



Published in final edited form as:

Cell Rep. 2014 July 10; 8(1): 256–271. doi:10.1016/j.celrep.2014.06.007.

Rictor/mTORC2 Loss in the *Myf5*-lineage Reprograms Brown Fat Metabolism and Protects Mice against Obesity and Metabolic Disease

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Summary

The *in vivo* functions of mTORC2, and the signaling mechanisms that control brown adipose tissue (BAT) fuel utilization and activity, are not well understood. Here, by conditionally deleting Rictor in the *Myf5*-lineage, we provide *in vivo* evidence that mTORC2 is dispensable for skeletal muscle development and regeneration but essential for BAT growth. Furthermore, deleting Rictor in *Myf5* precursors shifts BAT metabolism to a more oxidative and less lipogenic state and protects mice from obesity and metabolic disease at thermoneutrality. We additionally find that Rictor is required for brown adipocyte differentiation *in vitro*, that the mechanism specifically requires AKT1 hydrophobic motif phosphorylation but is independent of pan-AKT signaling, and is rescued with BMP7. Our findings provide new insights into the signaling circuitry that regulates

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See also Supplemental Experimental Procedures

AUTHOR CONTRIBUTIONS

C-M.H. and D.A.G. designed the project and wrote and edited the manuscript. C-M.H. performed most experiments and D.A.G. assisted in analysis and interpretation. C.M.C., J.S-G., and H.L. assisted with rescue, lineage tracing, and mouse experiments. C.B.C. performed metabolite profiling. S.H. and A.J.W. assisted with muscle experiments.

The authors declare no conflict of interests.

brown adipocytes and could have important implications for developing therapies aimed at increasing energy expenditure as a means to combat human obesity.

Introduction

Adipose tissue is essential for many biological processes and its dysfunction, for example in obesity, is associated with a growing spectrum of human diseases. Thus, understanding the developmental and metabolic regulation of adipose tissue has broad clinical implications. There are two main classifications of adipose tissue: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT is the major energy storage site in the body and has critical endocrine functions (Gesta et al., 2007) while BAT dissipates energy as heat in a process called nonshivering thermogenesis (Cannon and Nedergaard, 2004). BAT is particularly important in small rodents and newborn humans to defend against cold exposure and its functional relevance in adult humans was only recently appreciated (Harms and Seale, 2013; Nedergaard and Cannon, 2010; Tseng et al., 2010). Brown adipocytes are thermogenic because they express Uncoupling Protein 1 (UCP1), which embeds in the inner mitochondrial membrane and produces heat by uncoupling oxidativemetabolism from ATP production. The energy expending properties of brown adipocytes coupled with the observation that human BAT amount inversely correlates with body fat mass is garnering interest in developing strategies to increase brown adipocyte number and/or activity to treat obesity (Harms and Seale, 2013; Nedergaard and Cannon, 2010; Tseng et al., 2010). However, the mechanisms—and in particular the signaling circuitry—by which BAT regulates its energy supply are poorly understood (Townsend and Tseng, 2014). With the obesity pandemic seemingly out of control, and with a desperate need for novel therapeutics, the importance of elucidating mechanisms controlling adipocyte growth and function cannot be overstated.

Studying the *in vivo* mechanisms of adipose tissue growth has been challenging because adipocyte origins are poorly understood and consequently few tools are available for genetically targeting adipocyte precursors *in vivo* (e.g. by Cre-Lox). Lineage tracing studies indicate early mesenchymal precursor cells expressing *Myf5* give rise to myocytes, brown adipocytes, and a subset of white adipocytes (Sanchez-Gurmaches and Guertin, 2014; Sanchez-Gurmaches et al., 2012; Seale et al., 2008) and several recent studies have used the *Myf5*-Cre knock-in allele (Tallquist et al., 2000) to study BAT development (Harms et al., 2014; Martinez-Lopez et al., 2013; Ohno et al., 2013; Sanchez-Gurmaches et al., 2012; Schulz et al., 2013). Thus, the multi-fate potential of *Myf5* precursors provides an opportunity to use genetics to distinguish between signaling mechanisms that are required *in vivo* for the growth of myocytes versus adipocytes.

The mechanistic target of rapamycin (mTOR) kinase is a master-regulator of growth that functions in two distinct complexes called mTORC1 (defined by the Raptor subunit) and mTORC2 (defined by the Rictor subunit) (Laplante and Sabatini, 2012). While much is known about the inputs, outputs, and regulatory features of mTORC1, mTORC2 regulation and function remains more enigmatic. The best-described biochemical function of mTORC2 is to phosphorylate the hydrophobic motif (HM) of AKT (S473 in AKT1) and the related

SGK (S422 in SGK1) kinases (Garcia-Martinez and Alessi, 2008; Sarbassov et al., 2005). AKT has many effectors including GSK3 β , FoxO1/3, and mTORC1 (through TSC2 and PRAS40) and most models indicate mTORC2 is an essential upstream regulator of pan-AKT activity (Laplante and Sabatini, 2012). However, the extent to which this is the case in vivo remains unclear because *Rictor* deficient mouse embryo fibroblasts, which lack mTORC2, have seemingly normal GSK3 β phosphorylation, mTORC1 activity, and only partially decreased FoxO1/3 phosphorylation (Guertin et al., 2006; Jacinto et al., 2006; Shiota et al., 2006).

Mice lacking *Rictor* die around E10.5 (Guertin et al., 2006; Jacinto et al., 2006; Shiota et al., 2006); therefore, mTORC2 function in vivo is mostly being investigated using floxed *Rictor* alleles. In adipose tissue, two studies using *aP2-cre* to delete *Rictor* reported no effect on individual adipocyte size or overall adipose tissue mass (Cybulski et al., 2009; Kumar et al., 2010). One of the studies finds *aP2-cre;Rictor^{fl/fl}* mice eventually develop mild glucose intolerance and ectopic lipid deposition although a mechanism was not elucidated (Kumar et al., 2010). Notably however, the utility of *aP2-cre* to target adipocytes has recently been questioned (Lee et al., 2013; Mullican et al., 2013; Wang et al., 2013); therefore, the exact function of mTORC2 in adipose tissue remains unclear. Deleting *Rictor* in skeletal muscle with *Hsa-cre* or *Mck-cre* also has no effect on muscle fiber size or overall muscle mass and only minor effects on insulin-mediated glucose metabolism (Bentzinger et al., 2008; Kumar et al., 2008). These relatively mild phenotypes are somewhat surprising considering the importance of AKT signaling in metabolism; however, in both cases (adipose tissue and muscle) the Cre drivers used target mature cells and thus the in vivo role of mTORC2 in adipose tissue and muscle precursors is unknown.

Here, we take advantage of the fact that *Myf5-Cre* expresses in precursors of muscle and brown adipocytes to investigate the role of Rictor (i.e. mTORC2) and for comparison Raptor (i.e. mTORC1) in muscle and BAT growth. We report that *Raptor* is essential in the *Myf5* lineage for myogenesis, establishing BAT precursors, and viability. In contrast, *Rictor* is dispensable for myogenesis and viability, but essential for normal BAT growth. Moreover, Rictor-deficient BAT is more metabolically active, having elevated mitochondrial activity and decreased lipogenesis. Importantly, deleting *Rictor* in the *Myf5*-lineage also augments diet-induced thermogenesis, which protects mice from an obesogenic diet at thermoneutrality. We additionally find that *Myf5*-lineage white adipocytes require Rictor for normal growth in vivo suggesting a broader role for mTORC2 in adipose tissue development. Finally, we show that Rictor is also required in vitro for brown adipocyte differentiation but not for pan-AKT activity, and that this differentiation defect is rescued with BMP7. Collectively, our results provide new insight into the regulation of brown adipocytes and implicate Rictor/mTORC2 as a critical signaling node that balances oxidative and lipogenic metabolic states.

Results

Rictor is dispensable in the *Myf5* lineage during embryogenesis

We investigated the role of mTORC1 versus mTORC2 in vivo in fat versus muscle development by generating *Myf5-Cre; Raptor^{fl/fl}* (*Raptor^{Myf5cKO}*) and *Myf5-Cre; Rictor^{fl/fl}*

(*Rictor^{Myf5cKO}*) conditional knockout mice. The *Rictor^{Myf5cKO}* mice are born at the expected Mendelian ratio and show no obvious motor or behavioral defects [not shown]. In contrast, *Raptor^{Myf5cKO}* mice die perinatally. E16.5 *Raptor^{Myf5cKO}* embryos are smaller due to a muscle development defect that is not apparent in control or *Rictor^{Myf5cKO}* embryos [Figure S1A-D]. Transverse sections through the head and neck of *Raptor^{Myf5cKO}* embryos reveal an underdeveloped tongue and the absence of the masseter, sternohyoid, hyoglossus, supraspinatus, prevertebral, and trapezius muscles, the later deficiency resulting in hind neck body wall fragility during specimen preparation [Figure S1A-D]. Thus, *Raptor* is essential in the *Myf5* lineage for viability and muscle development while *Rictor* is dispensable for both.

To confirm that *Rictor* is dispensable for myogenesis, we purified satellite cells (which express *Myf5*) from *Rictor^{Myf5cKO}* skeletal muscles, confirmed they are deleted for *Rictor* [Figure S1E], and show they differentiate ex vivo into myosin heavy chain-positive multinucleate myofibers [Figure S1F-G]. Moreover, deleting *Raptor* in satellite cells in vivo with *Pax7-CreER* blocks skeletal muscle repair, while deleting *Rictor* by the same approach does not prevent muscle regeneration following acute injury [Figure S1H-I]. Thus, *Rictor* is also dispensable for satellite cell differentiation ex vivo and for adult myogenesis induced by injury.

White adipose tissues develop postpartum in mice, but early brown adipocyte precursor cells (bAPCs) are detectable in E16.5 embryos by Hematoxylin and Eosin (H&E) stain. Qualitatively similar pools of cervical, interscapular, and subscapular bAPCs are detectable in control and *Rictor^{Myf5cKO}* E16.5 embryos [Figure S1A & S1D]. In contrast, interscapular and subscapular bAPCs are absent in E16.5 *Raptor^{Myf5cKO}* embryos [Figure S1A & S1D]. Notably, a diminished pool of cervical bAPCs is detectable in the *Raptor^{Myf5cKO}* embryos consistent with our lineage tracing data showing that only about half of the cervical brown adipocytes arise from *Myf5-Cre* expressing precursors [Figure 1SD] (Sanchez-Gurmaches and Guertin, 2014). Thus, *Raptor* but not *Rictor* is also essential in the *Myf5*-lineage for establishing bAPCs during embryogenesis.

Brown and white adipose tissue growth requires *Rictor*

Although *Rictor^{Myf5cKO}* mice show no obvious embryonic phenotypes, they tend to weigh less (not significantly) than controls at postnatal day 1 (P1) [Figure S1J], which reaches significance from 6-15 weeks of life [Figure 1A]. Individual tissue analysis indicates that the weight difference results from decreased adipose tissue mass. For example, the interscapular BAT (iBAT) in P1 *Rictor^{Myf5cKO}* neonates weighs about 30% less than normal [Figure S1K] and during the first weeks of life the mutant BAT grows but to a much smaller size, resulting in mutant iBAT and subscapular BAT (sBAT) depots at 6-weeks weighing about 50% less than controls and being darker [Figure 1B]. Adipocytes in the retroperitoneal and anterior subcutaneous WAT depots (rWAT, asWAT) are also derived largely from *Myf5-Cre* expressing precursors (Sanchez-Gurmaches et al., 2012) and both of these depots also decrease in mass by approximately 50% in the *Rictor^{Myf5cKO}* mice [Figure 1C]. In contrast, the posterior subcutaneous and peri-gonadal WAT depots (psWAT, pgWAT), which are composed of *Myf5*-negative lineage adipocytes, do not differ in weight [Figure 1C]. Skeletal muscles (e.g. triceps, quadriceps, and gastrocnemius) and all other lean tissues examined

except the kidneys (which are slightly larger) are normal size in the knockout [Figure 1D]. Western analysis for Rictor protein confirms Rictor deletion and reduced AKT-S473 phosphorylation in iBAT and muscle and to a lesser extent in rWAT and asWAT, but not in psWAT, pgWAT or liver [Figure 1E].

From 6 weeks to 6 months, the mutant iBAT and sBAT shows no additional growth increase while asWAT and rWAT grow to about half (asWAT) or one third (rWAT) the size of their anatomically matched control tissues [Figure 1F]. In contrast, *Rictor^{Myf5cKO}* psWAT, pgWAT, muscles, and liver grow to their normal size in the same time frame [Figure 1F]. Thus, *Rictor^{Myf5cKO}* mice can grow small BAT tissues in the first weeks of life; however, as *Rictor^{Myf5cKO}* mice age the iBAT and sBAT maintain their weight while asWAT and rWAT grow at a reduced rate. Collectively, these results indicate Rictor is essential in the *Myf5* lineage for adipose tissue growth but not for skeletal muscle growth.

Brown adipocytes lacking Rictor are smaller

To better define the BAT growth defect we histologically examined iBAT in control and *Rictor^{Myf5cKO}* mice. At E18.5 there is no qualitative difference between control and *Rictor^{Myf5cKO}* bAPCs pools [Figure 2A]. In P1 neonates however, lipids begin accumulating in control BAT but not in the *Rictor^{Myf5cKO}* BAT [Figure 2A]. From P1 to 6 months lipid droplets grow in size in control BAT but remain small in the *Rictor^{Myf5cKO}* BAT [Figure 2A] resulting in smaller cells measured by the increase in nuclei per mm² [Figure S2A]. Total genomic DNA content is also lower in the *Rictor^{Myf5cKO}* BAT indicating additional hypoplasia [Figure S2B]. In contrast, *Rictor^{Myf5cKO}* skeletal muscle fibers appear histologically identical to control fibers [Figure S2C].

Myf5-lineage white adipocytes lacking Rictor are also small and multilocular

Compared to controls, many adipocytes in the *Rictor^{Myf5cKO}* rWAT and as WAT are also smaller and multilocular [Figure 2B] but the pattern is heterogeneous in that several large unilocular white adipocytes are also detectable. The psWAT and pgWAT adipocytes appear unchanged in the knockout [Figure S2C]. The adipocyte precursor pools in rWAT and asWAT are a mix of *Myf5-Cre* lineage positive and negative precursors (Sanchez-Gurmaches et al., 2012). Therefore, we reasoned that the size heterogeneity in *Rictor^{Myf5cKO}* rWAT and asWAT could reflect a mosaic of *Myf5*-lineage negative (i.e. undeleted) and *Myf5*-lineage positive (i.e. *Rictor* knockout) cells. To test this we incorporated the *Rosa26-mTmG* reporter (Muzumdar et al., 2007) into control and *Rictor^{Myf5cKO}* mice to irreversibly label Cre-expressing cells and their lineages with membrane-targeted eGFP (mGFP); all other (*Cre^{neg}*) cells and their descendants are labeled with membrane targeted tdTomato fluorescent protein (mTFP). The result is unequivocal; only the small adipocytes are mGFP⁺ in *Rictor^{Myf5cKO}* rWAT and asWAT while all the large unilocular adipocytes are mTFP⁺ [Figure 2C]. As expected, in both the control and *Rictor^{Myf5cKO}* mice the iBAT adipocytes are mGFP⁺ and the psWAT and pgWAT adipocytes are mTFP⁺ [Figure S2D]. We also detect a slight increase in UCP1 staining in the *Rictor^{Myf5cKO}* adipocytes suggesting the cells might have brown-adipocyte like characteristics [Figure S2E] (not shown). These data confirm that the heterogeneous small cell phenotype results from cell-autonomous Rictor deletion in the *Myf5*-lineage white adipocytes.

Lipogenesis is decreased in Rictor-deficient BAT

We hypothesized that the paucity of lipid and marked color difference between control and *Rictor^{Myf5cKO}* BAT indicates a shift from a lipogenic to oxidative state. To test this we first examined AKT signaling in BAT, which positively regulates lipogenesis. In vivo AKT-T308 phosphorylation is intact in both fasted/re-fed and insulin-stimulated *Rictor^{Myf5cKO}* BAT despite ablation of pAKT S473 and pAKT T450 (which is also mTORC2 dependent) [Figure 3A & S3A], consistent with the ability of T-loop (T308) and hydrophobic motif (S473) phosphorylation to be regulated independently (Pearce et al., 2010). Surprisingly, phosphorylation of the AKT substrates FoxO1/3, GSK3 β , TSC2, PRAS40, and AS160 is normal in *Rictor^{Myf5cKO}* BAT [Figure 3A] indicating Rictor is not essential in BAT for pan-AKT signaling. Rictor loss in BAT also does not affect phosphorylation of the SGK substrate NDRG1 [Figure 3A] indicating mTORC2 is not essential for SGK signaling to NDRG1 in BAT or that a compensatory pathway exists.

Next we examined whether deleting *Rictor* in the *Myf5*-lineage affects BAT differentiation markers. In P1 neonates, *Prdm16*, *C/ebpa*, and *C/ebp β* expression do not differ between controls and knockouts while *Ppar γ* and *Ucp1* levels slightly decrease [Figure 3B] indicating a possible delay in BAT maturation in the knockouts. However, by 6 weeks *Ppar γ* , *Prdm16*, and *C/ebpa* express at control levels while *C/ebp β* , *Ucp1*, and *Dio2* express at significantly higher than control levels [Figure 3B]. The mature adipocyte markers *Cidea* and *aP2* are unchanged between control and knockout both at P1 and 6 weeks [Figure 3B]. Consistent with the gene expression data, PPAR γ , UCP1, and insulin receptor beta (IR β) proteins also express at near control levels in *Rictor^{Myf5cKO}* BAT [Figure 3A]. Thus, terminal differentiation per se (i.e. PPAR γ , UCP1, and IR β induction) occurs in vivo in *Rictor^{Myf5cKO}* brown adipocytes.

Next we examined lipogenesis genes. In P1 *Rictor^{Myf5cKO}* BAT, acetyl-coA carboxylase (*Acc*), fatty acid synthase (*Fasn*), and fatty acid elongase 6 (*Elovl6*) decrease expression by 40%, 40%, and 25% respectively [Figure 3C]. By 6 weeks, expression of ATP citrate lyase (*Acly*) in addition to *Acc*, *Fasn*, and *Elovl6* is reduced by 90%, 75%, 80%, and 40% respectively [Figure 3C], which we confirmed by Western blot for ACLY and ACC [Figure 3A]. In addition, stearoyl-CoA desaturase (*Scd1*) decreases expression by 45% in 6 week *Rictor^{Myf5cKO}* BAT [Figure 3C]. The SREBP1c and ChREBP transcription factors regulate lipogenesis gene expression (Czech et al., 2013; Filhoulaud et al., 2013). In both P1 and 6-week *Rictor^{Myf5cKO}* BAT, the mRNA expression of SREBP1c (*Srebf1c*), which is induced by insulin, and ChREBP (α and β isoforms), which is induced by glucose, is similar [Figure 3D]. However, there is a marked decrease in the amount nuclear SREBP1c (nSREBP1c), the transcriptionally active SREBP1c cleavage product, in Rictor-deficient BAT [Figure 3A] consistent with the decrease in lipogenic gene expression. The levels of *insig1*, another nSREBP1c target gene and negative regulator of SREBP1c processing, also decreases [Figure 3C]. The mRNA expression of SREBP2 (which regulates cholesterol biosynthesis) slightly decreases in *Rictor^{Myf5cKO}* BAT at 6 weeks, but the SREBP2 target genes HMG-CoA synthase (*Hmg-cs*) and HMG-CoA reductase (*Hmg-cr*) express at similar levels in control and knockout BAT [Figure 3D] and nuclear SREBP2 (nSREBP2) accumulates possibly to higher levels in the knockout BAT [Figure 3A]. We find no difference in AMPK

or hormone sensitive lipase (HSL) phosphorylation between control and *Rictor^{Myf5cKO}* BAT [Figure 3A & S3A]. Together, these results indicate that despite having seemingly normal AKT signaling, de novo lipogenesis is reduced in *Rictor^{Myf5cKO}* BAT.

Mitochondrial activity is elevated in Rictor-deficient BAT

To further examine the metabolic state of *Rictor^{Myf5cKO}* BAT we examined mitochondrial activity. In P1 neonate *Rictor^{Myf5cKO}* BAT, *Pgc1a* expresses normally, while expression of mitochondrial transcription factor A (*Tfam*), which regulates mtDNA replication, and carnitine palmitoyltransferase 1B (*Cpt1b*), which encodes the rate-limiting enzyme in beta-oxidation, slightly decreases [Figure 4A]. In contrast, *Pgc1a*, *Tfam*, and *Cpt1bin* addition to *Ucp1* express at higher levels in the BAT of 6-week old *Rictor^{Myf5cKO}* mice [Figure 4A & 3B] suggesting BAT mitochondrial activity progressively increases or is maintained at a higher level in *Rictor^{Myf5cKO}* mice as they age.

To explore this in more detail, we used qRT-PCR arrays to broadly measure mitochondrial gene expression in the 6-week old BAT. Using arrays for functional genes involved in mitochondrial molecular transport and biogenesis, we detect increases in several genes indicative of increased mitochondrial activity [Figure 4B]. Furthermore, the mitochondrial citrate and malate transporters *Slc25a1* and *Slc25a10* respectively—both of which function in fatty acid biosynthesis, the former also being an SREBP1c target gene (Infantino et al., 2007; Mizuarai et al., 2005)—significantly decrease expression in the mutant BAT. Using mitochondrial energy metabolism gene arrays, we found 58 additional genes involved in respiration (OXPHOS) are elevated in *Rictor^{Myf5cKO}* BAT [Figure S4A] suggesting an increase in mitochondrial mass, which we confirmed by CoxIV immunofluorescence [Figure 4C]. Transmission electron microscopy (TEM) reveals individual mitochondria in the mutant BAT are larger and have more disorganized cristae [Figure 4D]. To directly confirm elevated mitochondrial activity, we measured BAT oxygen consumption rate (OCR) in a Seahorse Flux Analyzer and determined that basal and pyruvate-stimulated OCR are elevated by around 18% in *Rictor^{Myf5cKO}* BAT [Figure 4E]. We did not detect a significant increase in overall oxygen consumption when *Rictor^{Myf5cKO}* mice were placed in metabolic cages at 22°C except when normalized for body weight [Figure S4B]. Notably however, mice are under thermal stress at this temperature, which can mask effects on BAT activity (Feldmann et al., 2009).

Interestingly, we also detect an approximate 2-fold increase in basal glucose uptake in *Rictor^{Myf5cKO}* BAT measured by ¹⁸F-DG-PET CT scanning [Figure S4C] and an increase in lipoprotein lipase (*Lpl*) expression [Figure 3C] suggesting that *Rictor^{Myf5cKO}* BAT may consume more nutrients than age matched control BAT. Small metabolite profiling reveals that *Rictor^{Myf5cKO}* BAT also has elevated levels of inosine monophosphate (IMP) [Figure S4D], a deamination product of adenosine monophosphate (AMP), the accumulation of which suggests increased uncoupling (Balcke et al., 2011). In an acute cold challenge *Rictor*-deficient BAT also induces *Ucp1* expression significantly more than control BAT and the mutants have no difficulty maintaining body temperature although body temperature regulation in an acute cold challenge is largely a function of muscle [Figure 4F]. Finally, we see no compensatory “browning” in the psWAT as would be expected if *Rictor^{Myf5cKO}* BAT

were dysfunctional [Figure S2C-D] (Schulz et al., 2013). These results are consistent with Rictor loss in BAT shifting metabolism to a more oxidative and less lipogenic state.

Brown preadipocytes require Rictor to differentiate in vitro

To examine if brown adipocyte differentiation also requires Rictor in vitro, we generated brown adipocyte precursor cells (bAPCs) harboring an inducible knockout system (i.e. *Rictor^{iKO}*) in which Rictor deletion is triggered by 4-hydroxytamoxifen (4-OHT) [Figure S5A]. Compared to isogenic controls, inducibly deleting *Rictor* rapidly and robustly depletes Rictor protein and AKT-S473 phosphorylation, and consistent with our in vivo data, leaves AKT-T308 phosphorylation intact [Figure S5B]. Also consistent with the in vivo results, both basal and insulin-stimulated phosphorylation of FoxO1/3, GSK3 β , TSC2, and PRAS40 are normal in *Rictor^{iKO}* bAPCs [Figure 5A]. S6K1 phosphorylation is also unaffected [Figure 5A]. Contrary to the in vivo results, acute *Rictor* loss in vitro decreases NDRG1 phosphorylation [Figure 5A]. This indicates Rictor is required in cultured bAPCs for SGK activity to NDRG1 but not for pan-AKT or mTORC1 activity.

To our surprise, *Rictor^{iKO}* bAPCs are completely incapable of synthesizing lipid droplets when induced to differentiate [Figure 5B]. This is surprising because *Rictor^{iKO}* cells maintain normal levels of pAKT-T308, pGSK3 β -S9, and pS6K1-T389 (i.e. PDK1, AKT, and mTORC1 activity respectively) throughout the differentiation protocol [Figure 5C]. The differentiation block occurs early as *Rictor^{iKO}* bAPCs fail to induce *C/ebp α* , *Ppar γ* , *Prdm16*, *Pgc1 α* , *Srebf1c*, *Ucp1*, and *Glut4* [Figure 5D & S5C]. The expression of *C/ebp δ* and *C/ebp β* on the other hand induces normally and slightly higher respectively in the *Rictor^{iKO}* bAPCs at differentiation day 6 [Figure 5D]. Consistent with the gene expression data, PPAR γ , IR β , UCP1, nSREBP1c, ACC, and ACLY levels fail to increase during differentiation in *Rictor^{iKO}* bAPCs [Figure 5Eh]. Notably, 4-OHT or CreER activation alone (i.e. in the absence of Rictor floxed alleles) has no effect on differentiation [not shown]. Moreover, bAPCs prepared from P1 *Rictor^{Myf5cKO}* neonates also fail to differentiate indicating that the ex vivo differentiation block is not unique to using the inducible knockout-system [Figure S5D]. Importantly, expressing recombinant PPAR γ in *Rictor^{iKO}* bAPCs rescues IR β , UCP1 and nSREBP1c expression [Figure 5F] and lipid droplet production [Figure 5G] indicating Rictor promotes differentiation at least in part by facilitating PPAR γ induction.

Insulin receptor substrate 1 (Irs1) and *Irs3* knockout bAPCs also fail to induce PPAR γ ex vivo (Fasshauer et al., 2001). It was later shown that *Irs1/3* KO bAPCs are unable to differentiate because they express high levels of *Pref-1*, *Wnt10a*, and *Necdin*, which encode adipogenesis inhibitors (Tseng et al., 2005). In contrast, *Rictor*-deficient bAPCs express normal levels of *Pref-1*, *Wnt10a*, and *Necdin* in culture, and during differentiation *Necdin* and *Pref-1* increase but only late in the differentiation protocol [Figure S5C]. Thus, the mechanism by which deleting *Rictor* inhibits brown adipocyte differentiation differs from that of deleting *Irs1/3*.

AKT1 functions downstream of Rictor in brown adipocyte differentiation

To further explore the mechanism by which Rictor regulates differentiation, we next asked whether an AKT or SGK pathway is required downstream of Rictor. To this end, we generated *Rictor^{iKO}* bAPCs that express HA-SGK1, HA-AKT1, HA-AKT2, or their phosphomimetic counterparts HA-SGK-S422D, HA-AKT1-S473D, HA-AKT2-S474D in which a phosphomimetic residue was placed at the mTORC2 hydrophobic motif site, confirmed they were functional [Figure S6A], and asked whether any of these constructs rescue differentiation. Only HA-AKT1-S473D efficiently rescues lipid biosynthesis [Figure 6A]. HA-AKT1-S473D-expressing *Rictor^{iKO}* bAPCs also induce PPAR γ and restore IR β , UCP1, nSREBP1c, ACLY, and ACC expression [Figure 6B]. Thus, Rictor promotes differentiation as part of mTORC2 through an AKT pathway.

Our rescue experiments point to AKT1 as the isoform driving bAPC differentiation in vitro. Consistently, AKT1 is highly expressed in undifferentiated precursors and decreases expression during differentiation while AKT2 expression increases during differentiation [Figure S6B]. To further examine the role of AKT1 and AKT2 in bAPC differentiation we generated bAPC lines that specifically lack either *Akt1* or *Akt2* and determined their in vitro differentiation capacity. Consistent with AKT1 but not AKT2 being required for differentiation, *Akt1*-deficient bAPCs cannot efficiently synthesize lipid droplets [Figure 6C] or up-regulate PPAR γ , IR β , or UCP1 when induced to differentiate [Figure 6D]. In contrast, *Akt2*-deficient bAPCs induce PPAR γ , IR β , and UCP1 normally [Figure 6C-D] indicating that AKT1 is indeed the isoform required downstream of Rictor/mTORC2 for brown adipocyte differentiation. Interestingly, we noticed in our in vitro differentiation assays that while the *Akt2*-deficient cells differentiate, they fail to induce nSREBP1C, that ACLY and ACC express at low levels and that lipid droplet content is reduced [Figure 6C-D]. This suggests that while AKT2 is not essential for differentiation it is important downstream of Rictor/mTORC2 for lipid metabolism. Indeed, when we immunoprecipitate AKT1 or AKT2 from undifferentiated bAPCs most of the AKT phosphorylation is on AKT1 while in vivo the bulk of AKT phosphorylation shifts to AKT2 [Figure S6C-D]. Thus, while the inability of *Rictor^{iKO}* bAPCs to differentiate in culture reflects an AKT1 deficiency, the in vivo metabolic phenotype appears to reflect an AKT2 deficiency.

BMP7 rescues brown adipocyte differentiation in the absence of Rictor

In vitro *Rictor^{iKO}* bAPCs cannot differentiate (i.e. induce PPAR γ and UCP1), but in vivo PPAR γ and UCP1 positive Rictor-deficient BAT develops. One possible explanation for this paradox is that in vivo there are developmental signals present that are missing from the artificial in vitro differentiation assay. The signals that drive brown adipocyte differentiation in vivo are poorly understood. One proposed inducer of brown adipocyte differentiation is the transforming growth factor- β superfamily member BMP7 (Schulz and Tseng, 2013; Tseng et al., 2008). When given to control or *Rictor^{iKO}* bAPCs, BMP7 does not induce AKT phosphorylation [Figure S6E]. However, when supplemented into the differentiation cocktail BMP7 restores to *Rictor^{iKO}* bAPCs their ability to synthesize lipid droplets [Figure 6E] and express PPAR γ , IR β , UCP1 and to a lesser extent nSREBP1c, ACLY, and ACC [Figure 6F]. This is consistent with the in vitro differentiation assay lacking signaling molecules present in vivo and suggests BMP7 and mTORC2-AKT1 signaling converge

during brown adipocyte differentiation. A model depicting the role mTORC2-AKT signaling in vitro in brown adipocyte differentiation is shown in Figure 6G.

***Rictor*^{Myf5cKO} mice are less susceptible to obesity and metabolic disease at thermoneutrality**

The higher metabolic activity of *Rictor*-deficient BAT led us to wonder whether *Rictor*^{Myf5cKO} mice are resistant to obesity. Chronic consumption of high fat diet (HFD) triggers a phenomenon in mice called diet-induced thermogenesis, which requires UCP1, and counteracts obesity (Cannon and Nedergaard, 2010; Feldmann et al., 2009). Because BAT activity is masked by chronic thermal stress at 22°C we conducted the following studies at thermoneutrality (30°C for mice), which exempts mice from thermal stress (Feldmann et al., 2009). When eating a normal chow diet, control and *Rictor*^{Myf5cKO} mice gain equal weight [Figure 7A] and consume the same total energy [Figure 7B] over 12-weeks. In contrast, when eating a HFD control mice gain 14.67±1.05 grams while *Rictor*^{Myf5cKO} mice gain 10.57±1.18 grams [Figure 7A] despite both groups consuming the same energy [Figure 7B]. Thus, controls gain 64% more weight when eating HFD versus chow compared to *Rictor*^{Myf5cKO} mice. This suggests *Rictor*^{Myf5cKO} mice living at thermoneutrality and eating a HFD are less metabolically efficient than controls, which is indeed the case [Figure 7C].

The resistance to weight gain in the HFD-fed *Rictor*^{Myf5cKO} cohort is partly due to reduced growth of adipose tissue. For example, the pgWAT gains significantly less mass in the HFD-fed *Rictor*^{Myf5cKO} cohort than in HFD-fed controls [Figure 7D]. Liver and heart also grow larger in control eating HFD compared to chow, while liver and heart grow to the same mass in the *Rictor*^{Myf5cKO} cohorts regardless of diet [Figure 7D & S7A]. Diet has no effect on other lean tissues in either the controls or *Rictor*^{Myf5cKO} cohorts [Figure 7D & S7A]. That pgWAT grows less in HFD-fed *Rictor*^{Myf5cKO} mice compared to HFD-fed controls indicates systemic protection against obesity is occurring because *Myf5-Cre* does not target pgWAT [Figure 1F & S2C]. The reduction in pgWAT mass is due in part to smaller adipocyte size [Figure 7E]; the livers of *Rictor*^{Myf5cKO} mice also resist hepatic steatosis [Figure 7E]; and the HFD-fed *Rictor*^{Myf5cKO} mice perform better in a glucose tolerance test [Figure S7B].

In chow-fed cohort histology reveals that control BAT adopts a more “white adipocyte-like” appearance [Figure 7E]. In contrast, the BAT in chow-fed *Rictor*^{Myf5cKO} mice resists the whitening effects of living at thermoneutrality and maintains a more “brown-adipocyte-like” appearance [Figure 7E]. The resistance of *Rictor*^{Myf5cKO} BAT to whitening is reflected in the gene expression signature; for example, when normalized to BAT gene expression at 22°C, the shift to thermoneutrality decreases the expression of BAT-selective genes (*Prdm16*, *Sgk2*, *cideb*, *cyp2b10*) and increases the expression of WAT-selective genes (*Dpt1*, *Retn*, *Trim14*, *Nnmt*) to a greater extent in control BAT than in *Rictor*^{Myf5cKO} BAT, which maintains a more BAT-like identity. [Figure S7C].

In HFD-fed cohort histology reveals a large number of multi-locular adipocytes in control BAT [Figure 7F] that are not apparent in chow-fed controls [Figure 7E] suggesting diet-induced thermogenesis. This is reflected in the gene expression data as *Prdm16* increases in control BAT in HFD-fed mice compared to chow-fed [Figure 7G], while the WAT-specific

genes *Retn*, *Trim14*, and *Nnmt* decrease [Figure 7G]. Histology also reveals that *Rictor^{Myf5cKO}* BAT is even more “brown-like” in the HFD-fed cohort exhibiting a uniform abundance of small lipid droplets [Figure 7F] and a stronger BAT gene signature (i.e. elevated *Prdm16*, *Sgk2*, *Cideb*, *Cyp2b10*; decreased *Dpt1*, *Retn*, *Trim14*, *Nnmt*) [Figure 7G]. Consistently, BAT functional genes (*Ucp1*, *Pgc1 α* , *Cpt1 β* , *Dio2*) induce to a greater extent in HFD-fed *Rictor^{Myf5cKO}* mice [Figure 7H], which also maintain low *Acly*, *Acc*, and *Fasn* expression [Figure S7D]. Importantly, UCP1 protein level is higher in the BAT of *Rictor^{Myf5cKO}* mice eating HFD [Figure 7I]. Notably, after 20 weeks of eating a HFD the control BAT reverts to a more “white-adipocyte-like” histology; however, BAT character is preserved in *Rictor^{Myf5cKO}* mice [Figure S7E]. Collectively, these results suggest that inhibiting mTORC2 in BAT increases diet-induced thermogenesis and consequently, *Rictor^{Myf5cKO}* mice living without thermal stress and consuming an obesogenic diet are less susceptible to developing obesity and metabolic disease.

DISCUSSION

While transcriptional regulation of BAT development has been extensively described (Kajimura et al., 2010), less is known about the signaling mechanisms that regulate BAT. The control of brown fat fuel utilization is also incompletely understood (Townsend and Tseng, 2014). Previous studies reported that conditionally deleting *Rictor* in white and brown adipose tissue or skeletal muscle has no effect on WAT or BAT mass or individual adipocyte or myocyte size (Bentzinger et al., 2008; Cybulski et al., 2009; Kumar et al., 2008; Kumar et al., 2010). However, these studies used Cre drivers that reportedly delete *Rictor* in mature cells, which led us to hypothesize that *Rictor*/mTORC2 may be more important for brown/white adipose tissue and/or muscle development. By conditionally deleting *Rictor* in *Myf5* precursors we discovered that *Rictor* is not essential in vivo for muscle development or regeneration. In contrast, *Myf5*-lineage brown and white adipocytes lacking *Rictor* are reduced in size. Furthermore, *Rictor*-deficient BAT undergoes a metabolic shift to a more oxidative and less lipogenic metabolic despite having seemingly normal pan-AKT signaling. Importantly, at thermoneutrality this protects mice against an obesogenic diet. These findings implicate *Rictor*/mTORC2 as an essential signaling node in BAT that regulates the balance between fatty acid oxidation and storage. These findings could have important implications for understanding the signaling mechanisms that regulate fuel usage and metabolic activity in human BAT.

We also report that in vitro brown adipocyte differentiation requires *Rictor*/mTORC2. Mechanistically, *Rictor*/mTORC2 promotes *Ppar γ* induction through AKT1 independently of pan-AKT signaling and mTORC1 activity. In vivo however, brown adipocytes differentiate in *Rictor^{Myf5cKO}* mice despite lacking *Rictor* expression. We hypothesize that this paradox indicates that the artificial in vitro culture conditions lack important signals present in vivo that overcome this deficiency. Supporting this notion, supplementing the differentiation assay with BMP7, a proposed in vivo inducer of brown adipocyte differentiation and thermogenesis (Schulz and Tseng, 2013; Tseng et al., 2008), rescues differentiation in the absence of *Rictor*. Notably, we do detect low *Ppar γ* expression in *Rictor^{Myf5cKO}* P1 BAT, which may reflect the role of *Rictor*/mTORC2 in early brown adipocyte differentiation and explain the mutant BAT hypoplasia. Exactly how *Rictor*/

mTORC2 and BMP7 signaling might converge on PPAR γ is not yet clear. We also show that during brown adipocyte differentiation, the major AKT isoform switches from AKT1 to AKT2; thus, while Rictor/mTORC2 may regulate differentiation through an AKT1 pathway that can be bypassed in vivo, its role in BAT metabolism is likely mediated through an AKT2 pathway that cannot be compensated for. Consistent with this idea, whole body *Akt2* knockout mice among many other phenotypes have smaller BATs (Cho et al., 2001; Garofalo et al., 2003).

Why does deleting *Rictor* in BAT cause a metabolic shift? One possibility is that forkhead box O (FOXO) transcription factors are more active in Rictor-deficient brown adipocytes. FOXOs are regulated by multiple signals and function as cellular homeostasis regulators under stressful conditions (Eijkelenboom and Burgering, 2013). FoxO1 and FoxO3 are AKT substrates that are partially dephosphorylated in some Rictor-deficient cells (Guertin et al., 2009; Guertin et al., 2006; Hagiwara et al., 2012; Jacinto et al., 2006; Yuan et al., 2012). When dephosphorylated, FoxO1/3 translocate to the nucleus where they affect metabolism, survival, and cell cycle genes and the activity of transcriptional regulators (including PPAR γ and C/EBP α) (Eijkelenboom and Burgering, 2013). However, FoxO1/3 phosphorylation is not affected in Rictor-deficient BAT; thus, if the metabolic shift is driven by FoxO1/3, it may be through an alternative mechanism such as acetylation (Banks et al., 2011; Masui et al., 2013). Another possibility is that FoxC2 mediates the metabolic shift (Cederberg et al., 2001; Yao et al., 2013); however, we do not observe any change in FoxC2 expression in Rictor-deficient preadipocytes (not shown), nor do we see effects on the FoxC2 targets *C/ebp β* or *Wnt10b* during differentiation (Gerin et al., 2009). The shift could also be mediated through unidentified AKT substrates that uniquely require hydrophobic motif phosphorylation. This is an important ongoing area of investigation.

Consistent with the *Myf5*-lineage giving rise to a subset of white adipocytes, we also uncovered an essential role for Rictor/mTORC2 in vivo in white adipocyte growth. This confirms our previous discovery that some white adipocytes arise from *Myf5-Cre* expressing precursors (Sanchez-Gurmaches and Guertin, 2014; Sanchez-Gurmaches et al., 2012). However, because in the *Rictor^{Myf5cKO}* mice the Rictor-deficient white adipocytes are interspersed heterogeneously with non-deleted adipocytes within the same depot, we could not perform the appropriate whole-tissue biochemical studies using Rictor-deficient WAT. We did however determine that *Rictor^{iKO}* white adipocyte precursors purified from the stromal vascular fraction of psWAT (which are not *Myf5*-lineage derived) are also defective at differentiating in vitro (not shown) indicating Rictor also has a cell autonomous role in white adipocyte differentiation that is not dependent upon being *Myf5*-lineage derived. To determine the in vivo relevance of these findings we will need to identify Cre drivers that express uniformly and specifically in white adipocyte precursors; however, the origins of adipocytes are just beginning to be revealed and appropriate tools are not yet available for this line of investigation.

Is Rictor/mTORC2 a master regulator of lipid metabolism? Recent studies of liver collectively report that deleting hepatic *Rictor* results in a complex phenotype including increased gluconeogenesis, decreased glycolysis, and impaired lipogenesis (Hagiwara et al., 2012; Lamming et al., 2012; Yuan et al., 2012). Two studies find that hepatic *Rictor* loss

also decreases SREBP1c activity; however, one study suggests AKT2 mediates this function (Hagiwara et al., 2012) while the other proposes an AKT-independent pathway (Yuan et al., 2012). These two studies are also inconsistent with respect to how *Rictor* loss affects AKT signaling and thus the role of hepatic Rictor/mTORC2 is currently controversial. Nevertheless, the glucose uptake and glycolysis defect is reportedly independent of the lipogenesis defect because restoring glucose flux in *Rictor*-KO hepatocytes did not rescue lipogenesis (Hagiwara et al., 2012). This study also reports that fatty acid oxidation genes are elevated in Rictor-deficient hepatocytes (Hagiwara et al., 2012). Thus, Rictor/mTORC2 may have a broad role in establishing a pro-lipogenic metabolic state. Going forward it is important to determine if Rictor/mTORC2 regulates de novo lipogenesis and beta-oxidation by a common or coordinate set of mechanisms, or whether one metabolic deficiency is indirectly driving the other. Notably, we detect a decrease in lipogenesis gene expression in P1 BAT lacking Rictor, but the increase in fatty acid oxidation gene expression we first detect in 6-week mutant BAT. Thus, mitochondrial activity may progressively increase in the Rictor-deficient BAT and be secondary to a lipogenesis defect. Regardless, our findings support the idea that targeting lipogenesis and/or beta-oxidation pathways in adipocytes could be one approach to treating obesity and diabetes.

One prediction is that increasing BAT energy expenditure could have anti-obesity therapeutic potential (Tseng et al., 2010). To achieve this goal, a deeper understanding of how BAT utilizes fuel is required (Townsend and Tseng, 2014). An important finding in our study is that *Rictor^{Myf5cKO}* mice living at thermoneutrality, when challenged with an obesogenic diet, induce higher levels of UCP1 and are more resistant to developing obesity and metabolic disease compared to HFD-fed controls. This suggests that inhibiting mTORC2 in BAT augments diet-induced thermogenesis (Cannon and Nedergaard, 2010; Feldmann et al., 2009) although we cannot yet rule out that Rictor loss in other *Myf5*-lineage tissues might also contribute to this phenotype. It is currently being debated as to whether humans have classic brown adipocytes or a potential third class of adipocyte called a brite/beige adipocyte (Nedergaard and Cannon, 2013). Recent work indicates that in the neck, deep fat is similar to rodent BAT and expresses high levels of UCP1 while more superficial fat expresses lower UCP1 levels and has more brite/beige characteristics (Cypess et al., 2013). Notably, humans typically adjust temperature to be around thermoneutrality (Cannon and Nedergaard, 2010) and the BAT of mice living at thermoneutrality appears more “white-fat like”, or perhaps more “brite/beige-fat” like [Figure 7]. Thus, it seems likely that humans possess classic brown fat and that studies of brown fat in mice will provide important insights into human BAT regulation. Continued elucidation of mTORC2 pathways in BAT bioenergetics could therefore lead to novel anti-obesity therapies that target cellular energy expenditure.

EXPERIMENTAL PROCEDURES

Gene Expression

Total RNA was isolated from using Qiazol (Invitrogen) and an RNeasy kit (Invitrogen). Equal amounts of RNA were retro-transcribed to cDNA using a High capacity cDNA reverse transcription kit (Applied Biosystems). *Tbp* expression was used as a

normalization gene. A different set of iBAT samples was used in RT-PCR arrays (Qiagen) according to manufacturer's instruction. See also Supplemental Experimental Procedures.

In vitro differentiation

Primary brown adipocyte precursors (bAPC) cells were isolated from P1 neonates and immortalized with pBabe-SV40 Large T antigen. To induce *Rictor* deletion *ubc-creERT2;Rictor^{fl/fl}* cells were treated on three consecutive days with 1 μ M 4-OHT. bAPCs were seeded at 4 \times 10⁴ cells/ml and allowed reach confluence over 3 days in medium containing 20nM insulin, 1nM T3 (differentiation medium). On day 4, cells were induced with 20nM insulin, 1nM T3, 0.125mM indomethacin, 2 μ g/mL dexamethasone and 0.5mM 3-isobutyl-1-methylxanthine. Two days later the induction medium was replaced with fresh differentiation medium and changed every two days until day 10. See also Supplemental Experimental Procedures.

Metabolic studies

For thermoneutrality studies, 6 week-old male mice were transferred to 30°C. At 7 weeks of age, mice were fed chow (Prolab Isopro RMH3000, LabDiet) or high fat diet (45% calories from fat; ResearchDiet # D12451). Body weight and food intake were accessed weekly for 12 weeks. Glucose tolerance tests were performed at the 11th week. Overnight fasted animals were subjected to GTT by i.p. injecting glucose at 2g/Kg of body weight and blood glucose levels were measured with a commercially available glucose meter. A small group (n=4) of mice were kept for 20 weeks on HFD for morphological studies.

Statistics

Unless otherwise stated, the results are described as mean \pm SEM. Two-way ANOVA was performed where indicated. For most experiments Student's t-test was used to determine statistical significance: *indicates P<0.05; **, P<0.01; ***, P<0.001.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH (R00CA129613 & R01DK094004), American Diabetes Association (ADA113BS-066), Pew Charitable Trusts and Charles Hood Foundation grants to D.A.G. We thank Yuefeng Tang, Xiaohao Yao, and Christine Powers for technical assistance, Morris Birnbaum for Aktflox mice, and Marcus Cooper for the PPAR γ 2 construct. Metabolic cage studies were performed in the UMass Mouse Phenotype Center (DK09300).

REFERENCES

- Balcke GU, Kollé SN, Kamp H, Bethan B, Looser R, Wagner S, Landsiedel R, van Ravenzwaay B. Linking energy metabolism to dysfunctions in mitochondrial respiration--a metabolomics in vitro approach. *Toxicology letters*. 2011; 203:200–209. [PubMed: 21402135]
- Banks AS, Kim-Muller JY, Mastracci TL, Kofler NM, Qiang L, Haeusler RA, Jurczak MJ, Laznik D, Heinrich G, Samuel VT, Shulman GI, Papaioannou VE, Accili D. Dissociation of the glucose and lipid regulatory functions of FoxO1 by targeted knockin of acetylation-defective alleles in mice. *Cell metabolism*. 2011; 14:587–597. [PubMed: 22055502]

- Bentzinger CF, Romanino K, Cloetta D, Lin S, Mascarenhas JB, Oliveri F, Xia J, Casanova E, Costa CF, Brink M, Zorzato F, Hall MN, Ruegg MA. Skeletal muscle-specific ablation of raptor, but not of rictor, causes metabolic changes and results in muscle dystrophy. *Cell metabolism*. 2008; 8:411–424. [PubMed: 19046572]
- Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. *Physiological reviews*. 2004; 84:277–359. [PubMed: 14715917]
- Cannon B, Nedergaard J. Metabolic consequences of the presence or absence of the thermogenic capacity of brown adipose tissue in mice (and probably in humans). *Int J Obes (Lond)*. 2010; 34(Suppl 1):S7–16. [PubMed: 20935668]
- Cederberg A, Gronning LM, Ahren B, Tasken K, Carlsson P, Enerback S. FOXC2 is a winged helix gene that counteracts obesity, hypertriglyceridemia, and diet-induced insulin resistance. *Cell*. 2001; 106:563–573. [PubMed: 11551504]
- Cho H, Mu J, Kim JK, Thorvaldsen JL, Chu Q, Crenshaw EB 3rd, Kaestner KH, Bartolomei MS, Shulman GI, Birnbaum MJ. Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). *Science*. 2001; 292:1728–1731. [PubMed: 11387480]
- Cybulski N, Polak P, Auwerx J, Ruegg MA, Hall MN. mTOR complex 2 in adipose tissue negatively controls whole-body growth. *Proceedings of the National Academy of Sciences of the United States of America*. 2009; 106:9902–9907. [PubMed: 19497867]
- Cypess AM, White AP, Vernochet C, Schulz TJ, Xue R, Sass CA, Huang TL, Roberts-Toler C, Weiner LS, Sze C, Chacko AT, Deschamps LN, Herder LM, Truchan N, Glasgow AL, Holman AR, Gavrilu A, Hasselgren PO, Mori MA, Molla M, Tseng YH. Anatomical localization, gene expression profiling and functional characterization of adult human neck brown fat. *Nature medicine*. 2013; 19:635–639.
- Czech MP, Tencerova M, Pedersen DJ, Aouadi M. Insulin signalling mechanisms for triacylglycerol storage. *Diabetologia*. 2013
- Eijkelenboom A, Burgering BM. FOXOs: signalling integrators for homeostasis maintenance. *Nature reviews. Molecular cell biology*. 2013; 14:83–97. [PubMed: 23325358]
- Fasshauer M, Klein J, Kriauciunas KM, Ueki K, Benito M, Kahn CR. Essential role of insulin receptor substrate 1 in differentiation of brown adipocytes. *Molecular and cellular biology*. 2001; 21:319–329. [PubMed: 11113206]
- Feldmann HM, Golozoubova V, Cannon B, Nedergaard J. UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. *Cell metabolism*. 2009; 9:203–209. [PubMed: 19187776]
- Filhoulaud G, Guilmeau S, Dentin R, Girard J, Postic C. Novel insights into ChREBP regulation and function. *Trends in endocrinology and metabolism: TEM*. 2013; 24:257–268. [PubMed: 23597489]
- Garcia-Martinez JM, Alessi DR. mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). *The Biochemical journal*. 2008; 416:375–385. [PubMed: 18925875]
- Garofalo RS, Orena SJ, Rafidi K, Torchia AJ, Stock JL, Hildebrandt AL, Coskran T, Black SC, Brees DJ, Wicks JR, McNeish JD, Coleman KG. Severe diabetes, age-dependent loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/PKB beta. *J Clin Invest*. 2003; 112:197–208. [PubMed: 12843127]
- Gerin I, Bommer GT, Lidell ME, Cederberg A, Enerback S, Macdougald OA. On the role of FOX transcription factors in adipocyte differentiation and insulin-stimulated glucose uptake. *The Journal of biological chemistry*. 2009; 284:10755–10763. [PubMed: 19244248]
- Gesta S, Tseng YH, Kahn CR. Developmental origin of fat: tracking obesity to its source. *Cell*. 2007; 131:242–256. [PubMed: 17956727]
- Guertin DA, Stevens DM, Saitoh M, Kinkel S, Crosby K, Sheen JH, Mullholland DJ, Magnuson MA, Wu H, Sabatini DM. mTOR complex 2 is required for the development of prostate cancer induced by Pten loss in mice. *Cancer cell*. 2009; 15:148–159. [PubMed: 19185849]
- Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, Moffat J, Brown M, Fitzgerald KJ, Sabatini DM. Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that

- mTORC2 is required for signaling to Akt-FOXO and PKC α , but not S6K1. *Developmental cell*. 2006; 11:859–871. [PubMed: 17141160]
- Hagiwara A, Cornu M, Cybulski N, Polak P, Betz C, Trapani F, Terracciano L, Heim MH, Ruegg MA, Hall MN. Hepatic mTORC2 Activates Glycolysis and Lipogenesis through Akt, Glucokinase, and SREBP1c. *Cell metabolism*. 2012; 15:725–738. [PubMed: 22521878]
- Harms M, Seale P. Brown and beige fat: development, function and therapeutic potential. *Nature medicine*. 2013; 19:1252–1263.
- Harms MJ, Ishibashi J, Wang W, Lim HW, Goyama S, Sato T, Kurokawa M, Won KJ, Seale P. Prdm16 is required for the maintenance of brown adipocyte identity and function in adult mice. *Cell metabolism*. 2014; 19:593–604. [PubMed: 24703692]
- Infantino V, Iacobazzi V, De Santis F, Mastrapasqua M, Palmieri F. Transcription of the mitochondrial citrate carrier gene: role of SREBP-1, upregulation by insulin and downregulation by PUFA. *Biochemical and biophysical research communications*. 2007; 356:249–254. [PubMed: 17350599]
- Jacinto E, Facchinetti V, Liu D, Soto N, Wei S, Jung SY, Huang Q, Qin J, Su B. SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. *Cell*. 2006; 127:125–137. [PubMed: 16962653]
- Kajimura S, Seale P, Spiegelman BM. Transcriptional control of brown fat development. *Cell metabolism*. 2010; 11:257–262. [PubMed: 20374957]
- Kumar A, Harris TE, Keller SR, Choi KM, Magnuson MA, Lawrence JC Jr. Muscle-specific deletion of rictor impairs insulin-stimulated glucose transport and enhances Basal glycogen synthase activity. *Molecular and cellular biology*. 2008; 28:61–70. [PubMed: 17967879]
- Kumar A, Lawrence JC Jr, Jung DY, Ko HJ, Keller SR, Kim JK, Magnuson MA, Harris TE. Fat cell-specific ablation of rictor in mice impairs insulin-regulated fat cell and whole-body glucose and lipid metabolism. *Diabetes*. 2010; 59:1397–1406. [PubMed: 20332342]
- Lamming DW, Ye L, Katajisto P, Goncalves MD, Saitoh M, Stevens DM, Davis JG, Salmon AB, Richardson A, Ahima RS, Guertin DA, Sabatini DM, Baur JA. Rapamycin-induced insulin resistance is mediated by mTORC2 loss and uncoupled from longevity. *Science*. 2012; 335:1638–1643. [PubMed: 22461615]
- Laplante M, Sabatini DM. mTOR Signaling in Growth Control and Disease. *Cell*. 2012; 149:274–293. [PubMed: 22500797]
- Lee KY, Russell SJ, Ussar S, Boucher J, Vernochet C, Mori MA, Smyth G, Rourk M, Cederquist C, Rosen ED, Kahn BB, Kahn CR. Lessons on conditional gene targeting in mouse adipose tissue. *Diabetes*. 2013; 62:864–874. [PubMed: 23321074]
- Martinez-Lopez N, Athonvarangkul D, Sahu S, Coletto L, Zong H, Bastie CC, Pessin JE, Schwartz GJ, Singh R. Autophagy in *Myf5*⁺ progenitors regulates energy and glucose homeostasis through control of brown fat and skeletal muscle development. *EMBO reports*. 2013; 14:795–803. [PubMed: 23907538]
- Masui K, Tanaka K, Akhavan D, Babic I, Gini B, Matsutani T, Iwanami A, Liu F, Villa GR, Gu Y, Campos C, Zhu S, Yang H, Yong WH, Cloughesy TF, Mellinghoff IK, Cavenee WK, Shaw RJ, Mischel PS. mTOR Complex 2 Controls Glycolytic Metabolism in Glioblastoma through FoxO Acetylation and Upregulation of c-Myc. *Cell metabolism*. 2013
- Mizuarai S, Miki S, Araki H, Takahashi K, Kotani H. Identification of dicarboxylate carrier Slc25a10 as malate transporter in de novo fatty acid synthesis. *The Journal of biological chemistry*. 2005; 280:32434–32441. [PubMed: 16027120]
- Mullican SE, Tomaru T, Gaddis CA, Peed LC, Sundaram A, Lazar MA. A novel adipose-specific gene deletion model demonstrates potential pitfalls of existing methods. *Mol Endocrinol*. 2013; 27:127–134. [PubMed: 23192980]
- Muzumdar MD, Tasic B, Miyamichi K, Li L, Luo L. A global double-fluorescent Cre reporter mouse. *Genesis*. 2007; 45:593–605. [PubMed: 17868096]
- Nedergaard J, Cannon B. The changed metabolic world with human brown adipose tissue: therapeutic visions. *Cell metabolism*. 2010; 11:268–272. [PubMed: 20374959]
- Nedergaard J, Cannon B. How brown is brown fat? It depends where you look. *Nature medicine*. 2013; 19:540–541.

- Ohno H, Shinoda K, Ohyama K, Sharp LZ, Kajimura S. EHMT1 controls brown adipose cell fate and thermogenesis through the PRDM16 complex. *Nature*. 2013; 504:163–167. [PubMed: 24196706]
- Pearce LR, Komander D, Alessi DR. The nuts and bolts of AGC protein kinases. *Nature reviews. Molecular cell biology*. 2010; 11:9–22. [PubMed: 20027184]
- Sanchez-Gurmaches J, Guertin DA. Adipocytes arise from multiple lineages that are heterogeneously and dynamically distributed. *Nature communications*. 2014 in press.
- Sanchez-Gurmaches J, Hung CM, Sparks CA, Tang Y, Li H, Guertin DA. PTEN loss in the *Myf5* lineage redistributes body fat and reveals subsets of white adipocytes that arise from *Myf5* precursors. *Cell metabolism*. 2012; 16:348–362. [PubMed: 22940198]
- Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science*. 2005; 307:1098–1101. [PubMed: 15718470]
- Schulz TJ, Huang P, Huang TL, Xue R, McDougall LE, Townsend KL, Cypess AM, Mishina Y, Gussoni E, Tseng YH. Brown-fat paucity due to impaired BMP signalling induces compensatory browning of white fat. *Nature*. 2013; 495:379–383. [PubMed: 23485971]
- Schulz TJ, Tseng YH. Brown adipose tissue: development, metabolism and beyond. *The Biochemical journal*. 2013; 453:167–178. [PubMed: 23805974]
- Seale P, Bjork B, Yang W, Kajimura S, Chin S, Kuang S, Scime A, Devarakonda S, Conroe HM, Erdjument-Bromage H, Tempst P, Rudnicki MA, Beier DR, Spiegelman BM. PRDM16 controls a brown fat/skeletal muscle switch. *Nature*. 2008; 454:961–967. [PubMed: 18719582]
- Shiota C, Woo JT, Lindner J, Shelton KD, Magnuson MA. Multiallelic disruption of the rictor gene in mice reveals that mTOR complex 2 is essential for fetal growth and viability. *Developmental cell*. 2006; 11:583–589. [PubMed: 16962829]
- Tallquist MD, Weismann KE, Hellstrom M, Soriano P. Early myotome specification regulates PDGFA expression and axial skeleton development. *Development*. 2000; 127:5059–5070. [PubMed: 11060232]
- Townsend KL, Tseng YH. Brown fat fuel utilization and thermogenesis. *Trends in endocrinology and metabolism: TEM*. 2014; 25:168–177. [PubMed: 24389130]
- Tseng YH, Butte AJ, Kokkotou E, Yechoor VK, Taniguchi CM, Kriauciunas KM, Cypess AM, Niinobe M, Yoshikawa K, Patti ME, Kahn CR. Prediction of preadipocyte differentiation by gene expression reveals role of insulin receptor substrates and necdin. *Nature cell biology*. 2005; 7:601–611.
- Tseng YH, Cypess AM, Kahn CR. Cellular bioenergetics as a target for obesity therapy. *Nature reviews. Drug discovery*. 2010; 9:465–482. [PubMed: 20514071]
- Tseng YH, Kokkotou E, Schulz TJ, Huang TL, Winnay JN, Taniguchi CM, Tran TT, Suzuki R, Espinoza DO, Yamamoto Y, Ahrens MJ, Dudley AT, Norris AW, Kulkarni RN, Kahn CR. New role of bone morphogenetic protein 7 in brown adipogenesis and energy expenditure. *Nature*. 2008; 454:1000–1004. [PubMed: 18719589]
- Wang F, Mullican SE, DiSpirito JR, Peed LC, Lazar MA. Lipoatrophy and severe metabolic disturbance in mice with fat-specific deletion of PPARgamma. *Proceedings of the National Academy of Sciences of the United States of America*. 2013; 110:18656–18661. [PubMed: 24167256]
- Yao Y, Suraokar M, Darnay BG, Hollier BG, Shaiken TE, Asano T, Chen CH, Chang BH, Lu Y, Mills GB, Sarbassov D, Mani SA, Abbruzzese JL, Reddy SA. BSTA promotes mTORC2-mediated phosphorylation of Akt1 to suppress expression of FoxC2 and stimulate adipocyte differentiation. *Science signaling*. 2013; 6:ra2. [PubMed: 23300339]
- Yuan M, Pino E, Wu L, Kacergis M, Soukas AA. Identification of Akt-independent regulation of hepatic lipogenesis by mammalian target of rapamycin (mTOR) complex 2. *The Journal of biological chemistry*. 2012; 287:29579–29588. [PubMed: 22773877]

HIGHLIGHTS

- Brown and white adipocyte growth requires mTORC2
- mTORC2 promotes lipogenesis and suppresses β -oxidation in brown fat
- Brown preadipocytes also require mTORC2 to differentiate in vitro
- Inhibiting mTORC2 in BAT enhances diet-induced thermogenesis

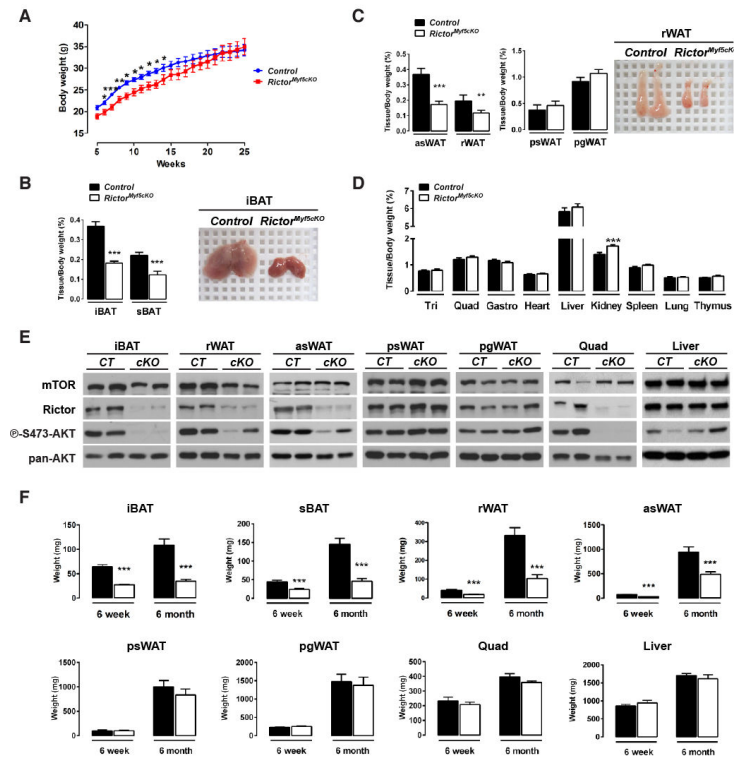


Figure 1. Post-natal brown and white adipose tissue growth requires Rictor

(A) Growth curves (n=13; bars represent mean \pm SEM; t-test; * p <0.05, ** p <0.01, *** p <0.001).

(B) (Left) BAT mass at 6-wks (n=19-21; mean \pm SEM; t-test; *** p <0.001) and (Right) representative image.

(C) (Left) Mass of WATs at 6-wks (n=14-16; mean \pm SEM; t-test; ** p <0.01, *** p <0.001) and (Right) representative image of control and mutant rWAT (6-wks).

(D) Lean tissue mass at 6-wks (n=15-19; mean \pm SEM; t-test; *** p <0.001).

(E) Westerns of tissue lysates (6-wks).

(F) Average tissue mass (mg) at 6-wks and 6-mos (n= 14-21 for 6-wks; n=7 for 6-mos; mean \pm SEM; t-test; *** p <0.001).

See also Figure S1.

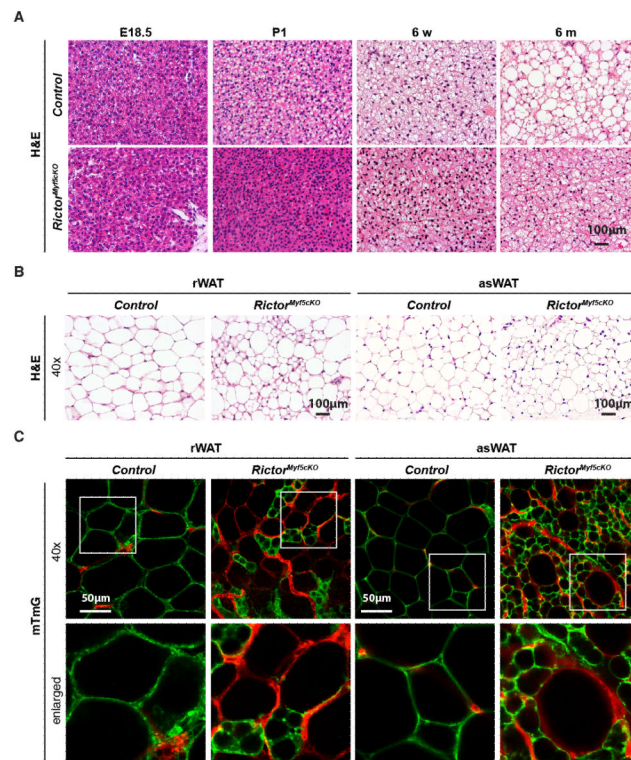


Figure 2. Brown and white adipocytes lacking *Rictor* are smaller and multilocular

(A) H&E stains of interscapular BAT (6 wks).

(B) H&E stains of retroperitoneal and anterior subcutaneous WAT.

(C) Representative images of mTFP and mGFP labeled adipocytes. Enlarged images indicated by white box.

See also Figure S2.

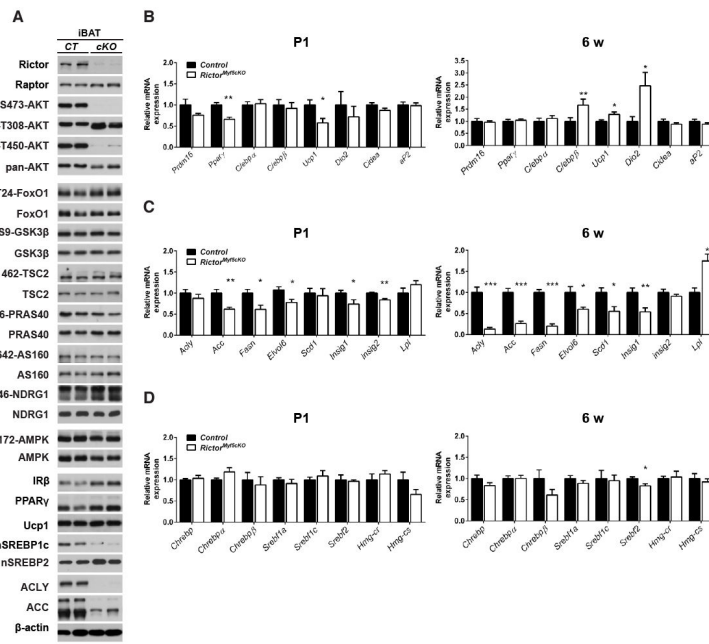


Figure 3. Rictor-deficient brown adipocytes have a lipid metabolism defect

(A) Western blots total and phospho-proteins using 6-wk iBAT lysates. Mice were fasted overnight and re-fed for 45mins prior to preparing lysates.

(B-D) qRT-PCR of the indicated genes in P1 (n=6) and 6wk (n=8) iBAT (mean ± SEM; t-test; *p<0.05, **p<0.01)

See also Figure S3.

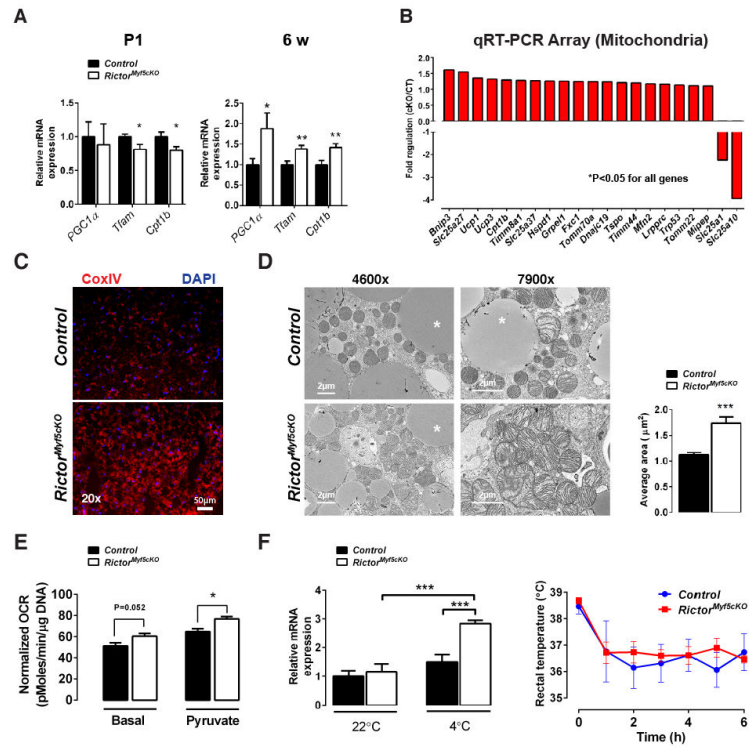


Figure 4. Mitochondrial activity is elevated in Rictor-deficient BAT

(A) qRT-PCR of mitochondrial genes in P1 (n=6) and 6wk iBAT (n=8) (mean ± SEM; t-test; *p<0.05, **p<0.01)

(B) Differentially expressed genes using mitochondrial qRT-PCR arrays(n=4; t-test; p<0.05)

(C) Representative immunofluorescence images of Cox IV staining in 6wk iBAT (n=3).

(D) (Left) Representative TEM images of 6wk iBAT and (Right) mitochondria size (n=3;mean ± SEM; t-test; ***p<0.001)

(E) Oxygen consumption of iBAT using a Seahorse Flux Analyzer (12 wks, n=5; normalized to DNA content;mean ± SEM; t-test; *p<0.05)

(F) (Left) qRT-PCR of *Ucp1* mRNA in iBAT with or without cold exposure (n=3 for 22°C; n=4 for 4°C; mean ± SEM; two-way ANOVA; ***P<0.001) and (Right) rectal temperature in acute cold challenge (n=4; mean ± SEM; t-test; no significant difference).

See also Figure S4.

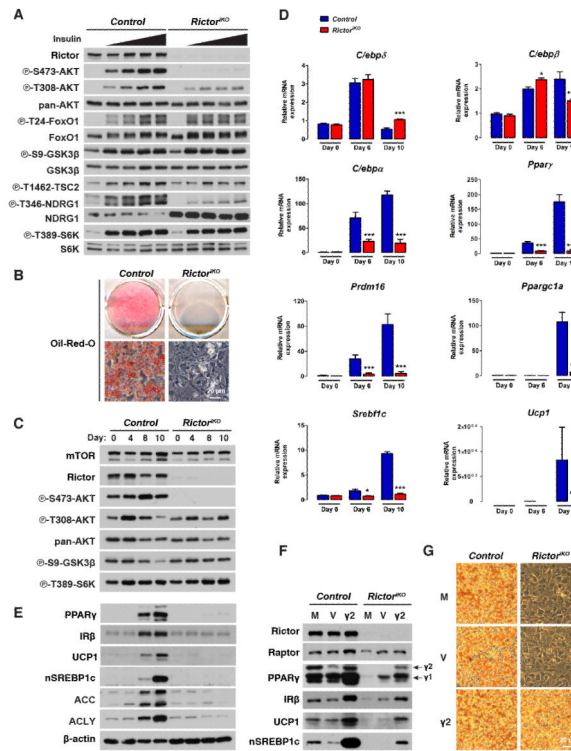


Figure 5. Rictor is required for brown adipocyte differentiation in vitro

(A) Western immunoblots using control and *Rictor^{IKO}* brown preadipocyte lysates. Cells were serum deprived 3 hours then stimulated with 0, 5, 25, 120, or 600nM insulin for 15 minutes prior to lysis.

(B) Oil Red O staining after differentiation.

(C) Western immunoblots using lysates from the indicated days of differentiation.

(D) qRT-PCR for differentiation-related genes (n=3; mean ± SEM; t-test; *p<0.05, ***p<0.001).

(E) Same as (C).

(F) Western immunoblots of cell lysates collected at day 10 of differentiation. M=mock; V=empty vector; γ2=recombinant PPARγ2. The γ1 and γ2 isoforms are indicated.

(G) Oil Red O staining of cells in (F).

See also Figure S5.

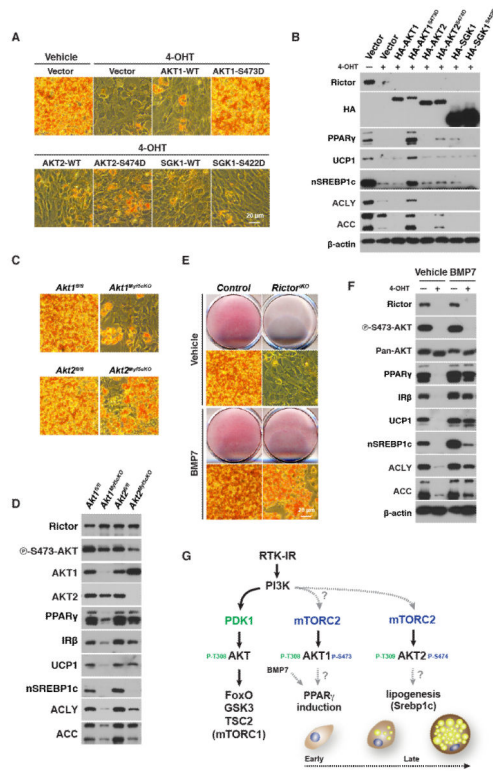


Figure 6. Recombinant AKT1-S473D or BMP7 supplementation rescue differentiation in the absence of Rictor

(A) Oil Red O staining of differentiated control (vehicle) and *Rictor^{iKO}* cells (4-OHT) cells stably expressing the indicated constructs.

(B) Western immunoblots corresponding to (A).

(C) Oil Red O staining of differentiated *Akt1* and *Akt2* conditional knockout and control bAPCs.

(D) Western immunoblots corresponding to (C).

(E) Oil Red O staining of differentiated control and *Rictor^{iKO}* cells in the presence or absence of BMP7 (3.2nM added day 1 during differentiation).

(F) Western immunoblots of corresponding to (E).

(G) Model summarizing the role of mTORC2 in vitro in brown adipocyte differentiation.

See also Figure S6.

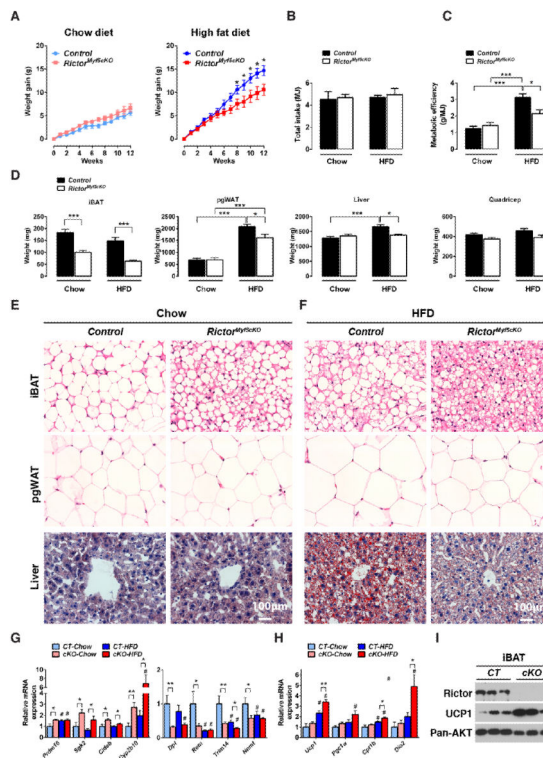


Figure 7. *Rictor^{Myf5cKO}* mice exempt from thermal stress and consuming a high fat diet are resistant to obesity and metabolic disease

(A) Weight gain of control and *Rictor^{Myf5cKO}* mice during 12-weeks of normal chow (chow) or high fat diet (HFD) (n=8 control and n=12 for KO in chow; n=10 for both genotypes on HFD; mean \pm SEM; t-test; *p<0.05). Control mice initially weighed 21.63 \pm 0.812g in the chow group and 21.24 \pm 0.621 in the HFD group; The *Rictor^{Myf5cKO}* mice initially weighed 19.42 \pm 0.305g in the chow group and 19.32 \pm 0.348 in the HFD group.

(B) Total energy intake (MJ) during the feeding regimen in (A). Control mice consumed 3.75 \pm 0.56g of chow and 2.81 \pm 0.12g of HFD; *Rictor^{Myf5cKO}* mice consumed 3.85 \pm 0.24g of chow and 2.95 \pm 0.35g of HFD.

(C) Metabolic efficiency determined as the amount of body weight increase (g) per MJ food consumed (n=8 control and n=12 KO on chow; n=10 for both genotypes on HFD; mean \pm SEM; two-way ANOVA; *p<0.05, ***p<0.001).

(D) Mass (mg) of the indicated tissues collected from control and KO mice after 12 weeks on chow or HFD. (n=8 control and n=12 KO on chow; n=10 for both genotypes on HFD; mean \pm SEM; two-way ANOVA; *p<0.05, ***p<0.001).

(E-F) H&E staining of iBAT and pgWAT and Oil red O staining of livers after 12-weeks of eating chow (E) or high fat diet (F).

(G) qRT-PCR of the indicated brown and white fat genes in iBAT from chow or HFD mice (n=8 control and n=12 KO on chow; n=10 for both genotypes on HFD; mean \pm SEM; two-way ANOVA; *p<0.05, **p<0.01, ***p<0.001; # indicates significant difference over the control chow group).

(H) qRT-PCR of the indicated metabolic genes in iBAT from chow or HFD mice (n=8 control and n=12 KO in chow; n=10 for both genotypes in HFD; mean \pm SEM; two-way

ANOVA; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; # indicates significant difference over the control chow group).

(I) Western immunoblots of BAT lysates.

See also Figure S7.