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ARTICLE

Adipocyte-specific deletion of *mTOR* inhibits adipose tissue development and causes insulin resistance in mice

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Abstract
Aims/hypothesis The in vivo role of mechanistic target of rapamycin (mTOR) in the development and function of adipose tissue, especially brown adipose tissue (BAT), is not well understood. Here, we aimed to assess the effect of *mTOR* (also known as *Mtor*) knockout on adipose tissues and systemic energy metabolism.
Methods We generated adipocyte-specific *mTOR*-knockout mice (*Adipoq-mTOR*) by crossing adiponectin-*Cre* (*Adipoq-Cre*) mice with $mTOR^{flox/flox}$ mice. The mice were then subjected to morphological, physiological (indirect calorimetry, glucose and insulin tolerance tests) and gene expression analyses to determine the role of mTOR in adipose tissues.
Results We provide in vivo evidence that *mTOR* is essential for adipose tissue development and growth. Deletion of *mTOR* decreased the mass of both BAT and white adipose tissues (WAT) and induced browning of WAT. In addition, ablation of *mTOR* in adipose tissues caused insulin resistance and fatty liver in the *Adipoq-mTOR* mice. Furthermore, *mTOR* was required for adipocyte differentiation in vivo and activation of PPARγ ameliorated the differentiation deficiency of the *mTOR*-null adipocytes.
Conclusions/interpretation Our findings demonstrate that mTOR is a critical regulator of adipogenesis and systemic energy metabolism. Our study provides key insights into the role of mTOR in adipose tissues; such knowledge may facilitate the development of novel strategies with which to treat obesity and related metabolic diseases.

Keywords Adipose · Browning · Insulin resistance · mTOR · PPARγ

Tizhong Shan, Pengpeng Zhang, Qinyang Jiang and Yan Xiong contributed equally to this paper.

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Abbreviations

asWAT	Anterior subcutaneous WAT
BAT	Brown adipose tissue
C/EBPα	CCAAT/enhancer binding protein α
eWAT	Epididymal WAT
FABP4	Fatty acid binding protein 4
H&E	Haematoxylin and eosin
HFD	High-fat diet
ITT	Insulin tolerance test
iWAT	Inguinal WAT
KO	Knockout
mTOR	Mechanistic target of rapamycin
mTORC	mTOR complex

PPARγ	Peroxisome proliferator-activated receptor γ
RER	Respiratory exchange ratio
S6K1	Ribosomal S6 kinase 1
SVF	Stromal vascular fraction
TG	Triacylglycerol
UCP1	Uncoupling protein 1
WAT	White adipose tissue
WT	Wild-type

Introduction

Adipose tissue plays a critical role in regulating energy balance and metabolism and its dysfunction is closely associated with metabolic diseases such as obesity and insulin resistance. In mammals, there are two main types of adipose: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT is the major energy storage site that stores excess energy, whereas BAT contains numerous mitochondria with high levels of uncoupling protein 1 (UCP1) that dissipates energy as heat [1]. Adipocytes in the WAT and BAT are called white and brown adipocytes, respectively. Recently, a third type of adipocyte, called beige or brite [2], has been found in WAT. Beige adipocytes can be induced by chemical or hormonal stimulation, cold exposure or genetic manipulation [3–7]. Similar to classical brown adipocytes, beige adipocytes have high levels of UCP1 [3, 7, 8]. Both brown and beige adipocytes can increase energy expenditure and counteract obesity [7–9]. Thus, understanding the development and function of brown and beige adipocytes may provide novel strategies with which to counteract obesity.

Mammalian target of rapamycin (mTOR) is a conserved serine/threonine protein kinase involved in regulating protein synthesis, cell growth and energy metabolism [10]. mTOR functions in two distinct complexes: mTOR complex 1 (mTORC1) and mTORC2 [11]. mTORC1, consists of mTOR, raptor and deptor, is sensitive to rapamycin [10]. mTORC2, containing mTOR, rictor and deptor, is insensitive to rapamycin [10]. Recent studies demonstrate that both complexes play a crucial role in adipogenesis [12, 13], lipid homeostasis [10, 14, 15], glucose metabolism and insulin actions [16, 17]. Inhibition of mTORC1 signalling by rapamycin or small interfering RNA against *Raptor* (also known as *Rptor*) decreases adipocyte proliferation and differentiation [18–21] and protects mice against high-fat diet (HFD)-induced obesity [22]. Adipose-specific ablation of *Raptor* also enhances energy expenditure and protects the mutant mice against HFD-induced obesity [23, 24]. Mice lacking ribosomal S6 kinase 1 (S6K1), a key downstream target of mTORC1, have relatively lower fat mass and higher energy expenditure [13, 25]. Moreover, deletion or overexpression of *Tsc1*/*Tsc2*, upstream regulators of mTORC1, further confirms a key role for mTORC1 in adipocyte differentiation and energy metabolism [26]. Compared with mTORC1, the function of mTORC2 in adipocytes is not well understood. *Rictor*-null mice die at around embryonic day 10.5 [27, 28]. In *Caenorhabditis elegans*, *Rictor*-null worms have comparatively more body fat [14, 29], indicating that mTORC2 may also play a role in regulating adipogenesis. In mice, adipose-specific deletion of *Rictor* decreases glucose uptake and glucose tolerance in vivo without affecting adipocyte size or fat mass [16, 17, 30]. These reports indicate that mTORC1 and mTORC2 play distinct roles in adipogenesis and lipid metabolism. However, most of the earlier studies involved perturbations of either mTORC1 or mTORC1. The direct effect of genetic deletion of *mTOR* (also known as *Mtor*), which has the effect of simultaneously suppressing mTORC1 and mTORC2, in adipose tissues has not been reported. Moreover, previous studies used *FABP4-Cre* to drive the adipose-specific deletion of *Raptor* or *Rictor* and this may have led to off-target deletion in non-adipose tissues [31, 32] and confound the interpretation of results. In this study, we directly and specifically deleted *mTOR* in adipocytes by crossing adipocyte-restricted adiponectin-*Cre* (*Adipoq-Cre*) mice with $mTOR^{flox/flox}$ mice.

Methods

Animals All procedures involving mice were guided by Purdue University Animal Care and Use Committee. Additionally, all experimental protocols were approved by Purdue University Animal Care and Use Committee. *Adipoq-Cre* (stock no. 010803) and $mTOR^{flox/flox}$ (stock no. 011009) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were of a C57BL/6 J background and housed in an animal facility with free access to water and standard rodent chow food or HFD (TD.06414; Harlan, Indianapolis, IN, USA). PCR genotyping (Sigma-Aldrich, St Louis, MO, USA) was carried out as described by the supplier.

Indirect calorimetry study Oxygen consumption ($\dot{V}O_2$), carbon dioxide production ($\dot{V}CO_2$), respiratory exchange ratio (RER) and heat production were measured by using an indirect calorimetry system (Oxymax, Columbus Instruments, Columbus, OH, USA) [7].

GTTs and insulin tolerance tests GTTs and insulin tolerance tests (ITT) were performed as described previously [7].

Haematoxylin–eosin staining For adipose tissues, section and haematoxylin and eosin (H&E) staining was carried out as described previously [7]. For liver tissues, frozen livers were cut into 10 μm thick cross sections using a Leica CM1850 cryostat (Leica Microsystems, Wetzlar, Germany) for H&E staining. Whole-slide digital images were collected at magnification × 20 with an Aperio Scan Scope slide scanner

(Aperio, Vista, CA, USA). Scanned images were analysed by Photoshop CS3 (San Jose, CA, USA) to calculate nuclei numbers.

Primary adipocyte isolation and culture

together, the reduced BAT mass in *Adipoq-mTOR* mice is a consequence of a decrease in cell size as well as cell numbers.

We next analysed the molecular phenotype of BAT from WT and *Adipoq-mTOR* mice. Mitochondrial biogenesis and brown adipocyte marker proteins and their genes, such as *Ucp1*, *Prdm16*, *Cidea* and *Ppara*, were expressed at similar levels in WT and *Adipoq-mTOR* mice (Fig. 2c, d). However, the BAT from *Adipoq-mTOR* mice contained lower levels of fatty acid binding protein 4 (FABP4), a mature adipocyte marker (Fig. 2c). Consistently, the mRNA levels of mature adipocyte markers (*Fabp4*, *Adipoq*, *Lep*) were lower in the BAT of *Adipoq-mTOR* mice compared with WT mice (Fig. 2d). The expression levels of lipolysis-related genes *Pnpla2* and *Lipe* were unchanged (Fig. 2e) by *mTOR* deletion. These data strongly suggest that deletion of *mTOR* inhibits brown adipocyte adipogenesis in vivo.

To verify whether deletion of *mTOR* affects brown pre-adipocyte differentiation in culture, we isolated BAT SVF cells from the WT and *Adipoq-mTOR* mice. We observed that *mTOR* depletion had a more dramatic effect on BAT in vitro than in vivo (ESM Fig. 3a). *mTOR* deletion inhibited brown adipocyte differentiation and TG accumulation (ESM Fig. 3a). Consistent with the in vivo data, *mTOR*-deficient brown adipocytes expressed lower levels of FABP4 and *Adipoq* in culture (ESM Fig. 3b, c). In addition, the expression of *Ucp1* and *Cidea* was also decreased by *mTOR* knockout (ESM Fig. 3c). Together, these results indicate that deletion of *mTOR*

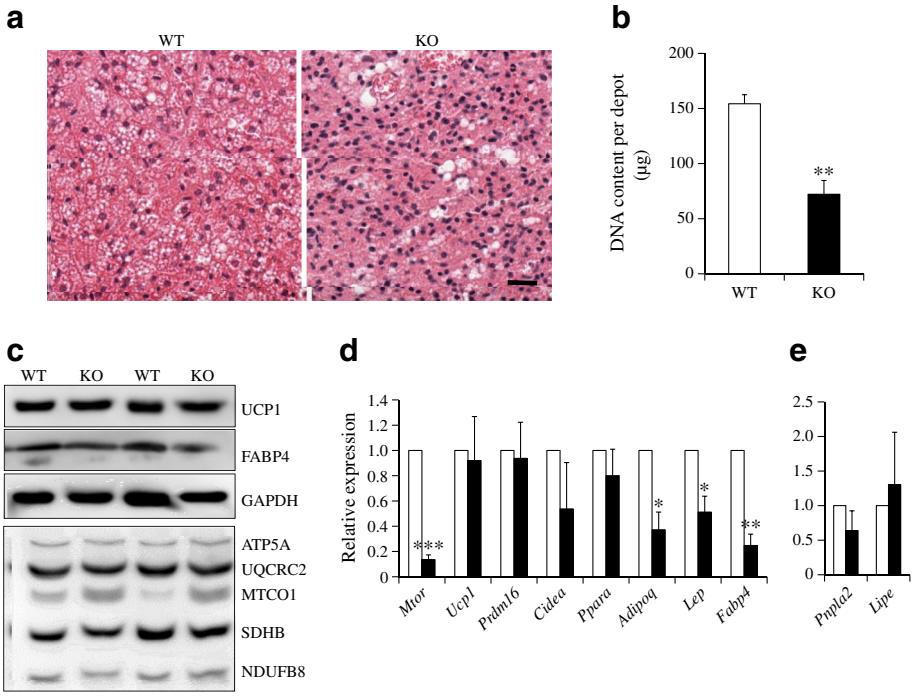

Fig. 2 mTOR deficiency inhibits BAT development. (**a**) H&E staining of BAT sections. Scale bar, 100 μm. (**b**) Genomic DNA content per BAT depot ($n = 4$). (**c**) Levels of UCP1, FABP4 and mitochondrial proteins in BAT. (**d**, **e**) mRNA levels of mature adipocyte marker genes and BAT-selective genes (**d**, $n = 4$ or 5) and lipolysis-related genes (**e**, $n = 3$) in BAT from WT and KO mice. White bars, WT mice; black bars, KO mice. Error bars represent SEM; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ for KO vs WT. ATP5A, ATP synthase, H^{+} transporting, mitochondrial F1 complex, alpha subunit 1; GAPDH, glyceraldehyde-3-phosphate; dehydrogenase; MTCO1, mitochondrially encoded cytochrome c oxidase I; NDUFB8, NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8; SDHB, succinate dehydrogenase complex, subunit B, iron sulfur (Ip); UQCRC2, ubiquinol cytochrome c reductase core protein 2

To examine whether the effects of *mTOR* deletion on browning of iWAT is cell autonomous, we isolated SVF cells from iWAT of WT and *Adipoq-mTOR* mice. Oil Red O staining indicated that deletion of *mTOR* inhibits adipocyte differentiation and TG accumulation (ESM Fig. 4a). Notably, the expression level of *Ucp1* and mature adipocyte markers was

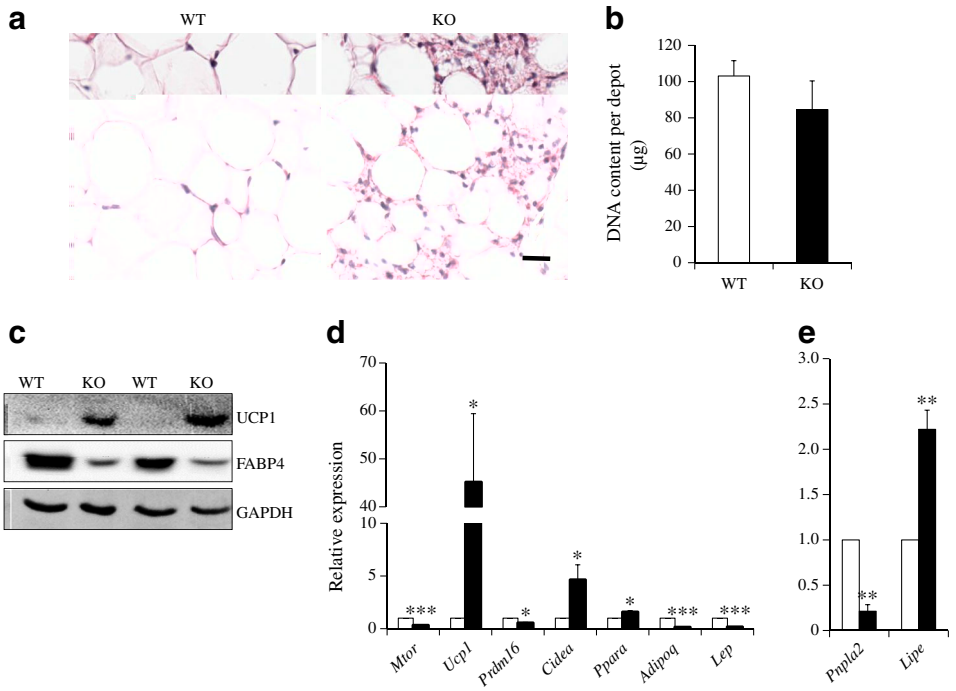

Fig. 3 mTOR deficiency inhibits WAT development and induces browning of WAT. (**a**) H&E staining of iWAT sections. Scale bar, 100 μm. (**b**) Genomic DNA content per iWAT depot of WT and KO mice ($n = 4$). (**c**) Protein levels of UCP1 and FABP4 in iWAT. (**d**, **e**) mRNA levels of mature adipocyte marker genes and BAT-selective genes (**d**) and lipolysis-related genes (**e**) in iWAT from WT and KO mice ($n = 5$). White bars, WT mice; black bars, KO mice. Error bars represent SEM; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ for KO vs WT. GAPDH, glyceraldehyde 3-phosphate dehydrogenase

significantly lower in *mTOR* KO white adipocytes (ESM Fig. 4b, c). These results indicated that deletion of *mTOR* induces browning of WAT in a non-cell autonomous manner.

***mTOR* deletion affects insulin sensitivity but not energy metabolism** Adipose tissues regulate systemic glucose metabolism and insulin sensitivity [7, 36]. To determine whether reduction of BAT and WAT mass in *Adipoq-mTOR* mice affects systemic glucose metabolism and insulin sensitivity, we conducted GTTs and ITTs. Compared with WT littermates, *Adipoq-mTOR* mice had higher blood glucose levels after glucose injection (Fig. 4a) and a slower rate of insulin-stimulated glucose clearance (Fig. 4b). However, compared with WT mice, the *Adipoq-mTOR* mice had similar values for $\dot{V}O_2$, $\dot{V}CO_2$, heat production and RER (Fig. 4c–f). Taken together, these results suggest that adipocyte-specific deletion of *mTOR* does not affect energy metabolism but results in insulin resistance.

To further confirm the effects of *mTOR* deficiency on energy metabolism and insulin sensitivity, we fed the WT and KO mice with HFD. Although the body weight of *Adipoq-mTOR* mice and WT mice was similar before and after HFD feeding, the fat mass of the KO mice was consistently less than that of the WT mice (ESM Fig. 5a, b). The liver mass in the *Adipoq-mTOR* mice was dramatically increased, whereas other tissues, including kidney, heart, lung, tibialis anterior muscle and gastrocnemius muscle, were unchanged (ESM Fig. 5c, d). Notably, the *Adipoq-mTOR* mice retained insulin resistance after HFD feeding (ESM Fig. 5e, f). These results together suggest that adipocyte-specific deletion of *mTOR* causes insulin resistance regardless of dietary conditions.

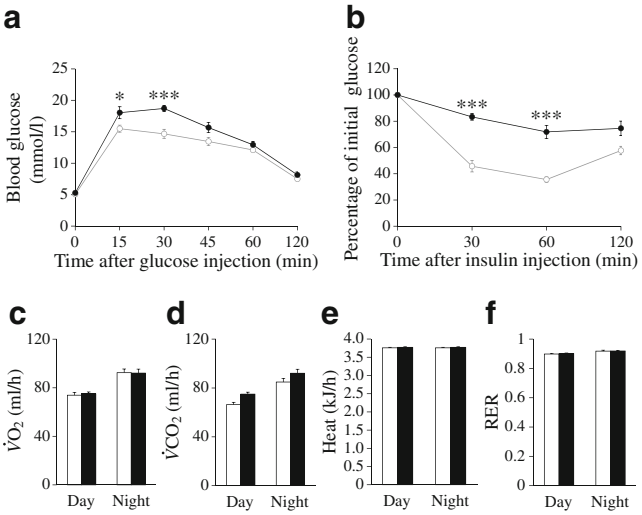

Fig. 4 Induced insulin resistance in *Adioq-mTOR* mice. (**a**, **b**) Blood glucose concentrations during IPGTT (**a**, $n = 8$) and ITT (**b**, $n = 7$) performed on WT and KO mice. (**c**–**f**) Average oxygen consumption (**c**), CO_2 production (**d**), heat production (**e**) and RER (**f**) of WT and KO mice ($n = 6$). White circles and bars, WT mice; black circles and bars, KO mice. Error bars represent SEM; $^*p < 0.05$ and $^{***}p < 0.001$ for KO vs WT

***mTOR* affects adipogenesis through PPARγ** To understand the molecular mechanism through which *mTOR* affects fat development and adipogenesis, we examined peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding protein α (C/EBPα), two key transcription factors that regulate adipogenesis [37]. Western blotting and real-time PCR results showed that *mTOR* deficiency dramatically decreased the expression of PPARγ and C/EBPα in BAT (Fig. 5a, b), as well as in brown adipocytes (Fig. 5c). Similar results were obtained using iWAT tissue and differentiated iWAT adipocytes from the KO and WT mice (ESM Fig. 6a–c). Rosiglitazone is a PPARγ agonist that induces PPARγ expression and increases its activity [6, 38]. Consistently, rosiglitazone treatment significantly enhanced expression of *Pparg*, *Cebpa*, *Fabp4*, *Adipoq* and *Ucp1* in brown adipocytes (Fig. 5d).

To confirm whether PPARγ mediates the effects of *mTOR* deficiency on adipogenesis, we treated the WT and KO brown adipocytes with rosiglitazone. Remarkably, rosiglitazone treatment rescued the differentiation of the *mTOR*-deficient brown pre-adipocytes (Fig. 5e). Consistently, the expression of *Fabp4*, *Adipoq* and *Ucp1* in brown adipocytes was significantly increased in both WT and KO brown adipocytes after rosiglitazone treatment (Fig. 5f). Taken together, we conclude that *mTOR* deletion affects adipogenesis, at least in part, through the PPARγ signalling pathway.

Discussion

Here, we generated the *Adipoq-mTOR* mouse model and directly identified a role for *mTOR* in BAT and WAT. We provided evidence that deletion of *mTOR* inhibits fat development and leads to insulin resistance. Consistently, we demonstrated that *mTOR* deletion decreases lipid accumulation and differentiation of brown and white adipocytes. We further elucidated the PPARγ signalling through which mTOR regulates the differentiation of pre-adipocytes. Our study reveals the critical role of the mTOR signalling pathway in regulating adipose tissues development, whole-body energy metabolism and insulin sensitivity.

The roles of mTORC1 and mTORC2 in adipose tissues have been studied separately by genetic perturbation of key components involved [15, 17, 24, 30], but the overall role of mTOR signalling has not been reported. Here, we used a highly adipocyte-specific *Adipoq-Cre* [31, 34, 35] to delete *mTOR* in mice and demonstrated that *mTOR* deletion inhibits BAT and WAT development. We found that adipose-specific deletion of *mTOR* dramatically decreased the levels of mTOR and phosphorylated S6 and 4EBP1 proteins but, surprisingly, increased phosphorylated AKT (S473) in BAT and iWAT. The increased levels of phosphorylated AKT might be due to the feedback activation of AKT signalling induced by mTORC1 deletion or a kinase that phosphorylates AKT, such as DNA-protein kinase

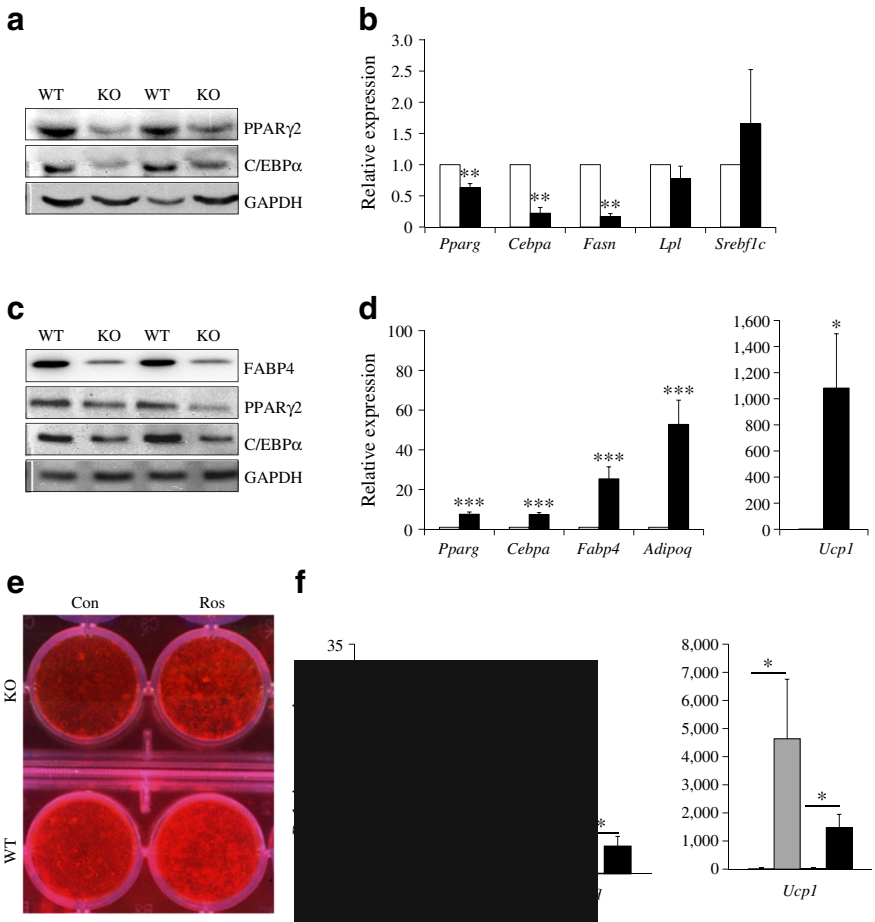

Fig. 5 *mTOR* deletion affects brown pre-adipocyte d
through PPARγ. (**a**) Protein levels of PPARγ and C/EBI
Adipoq-mTOR (KfesœvT

[39] and integrin-linked kinase [40]. In this regard, the increased phosphorylated AKT level cannot improve insulin sensitivity due to blockage of downstream mTOR.

We found that both the number and size of adipocytes were decreased in KO mice. Consistent with our findings, adipose-specific deletion of *Raptor* also results in leaner mice with lighter fat pads due to smaller and fewer WAT cells [24]. Inhibition of mTORC1 with rapamycin or by knockout of *S6k1* (also known as *Rps6kb1*) also reduced fat mass and cell size [25, 41]. These results together suggest that mTORC1 plays a positive role in regulating the development of adipose. The role of mTORC2 in adipose tissue has been studied recently [15, 17, 30], but the results are controversial. Two studies using *FABP4-Cre* to drive the deletion of *Rictor* reported no effect on fat mass or individual adipocyte size [17, 30]. However, these results are inconsistent with the phenotypes observed in *Myf5-Cre* driven *Rictor*-knockout mice, in which the mass of *Myf5*-lineage adipocytes was decreased [15]. The different phenotypes from these studies may be due to the *Cre* mice that were used. Although BAT and some white adipocytes are from *Myf5* lineage, the *Myf5-Cre* also drives gene deletion in skeletal muscles [42, 43], which can secrete myokines that regulate adipose development and function [3, 33]. Moreover, leaky expression of *FABP4-Cre* was found in adipocyte progenitors and non-adipose tissues [31, 32, 44]. Therefore, the leaky expression of *FABP4-Cre* or *Myf5-Cre* makes it difficult to interpret the data from these studies. Our *Adipoq-mTOR* mouse model drives specific deletion of *mTOR* in adipocytes and provides an excellent model with which to examine the role of mTOR in adipocytes and adipose tissues.

We found that adipocyte-specific deletion of *mTOR* drives browning of WAT. The KO iWAT has numerous small adipocytes with higher levels of *Ucp1* and *Lipe* expression. Likewise, the browning of WAT is also commonly seen in lipodystrophic

models, such as *Bscl2* knockout mice [45], in which fatty acids produced from unbridled lipolysis induce UCP1 and browning of WAT [46]. Moreover, previous studies have also reported that adipose-specific deletion of *Raptor* upregulated the expression of genes related to mitochondrial biogenesis in WAT [24]. *S6k1* deletion also induces formation of multilocular adipocytes and increases expression of *Ucp1* and *Pgc1a* [25]. In contrast, deletion of *Tsc1* induced the brown-to-white adipocyte phenotypic switch [26]. These results indicate that inhibition of mTORC1 signalling contributes to the white-to-brown adipocyte switching. In addition, a recent study reported that adipocytes lacking *Rictor* have stronger UCP1 staining and brown-adipocyte-like characteristics [15], suggesting that mTORC2 may also regulate white-to-brown switching.

Adipose tissues participate in regulating whole-body energy metabolism, glucose homeostasis and insulin sensitivity [17]. Notably, we found that adipocyte-specific deletion of *mTOR* has no effect on whole-body energy metabolism but causes insulin resistance. It has been reported that mTORC1 is a regulator of adipose metabolism and controls whole-body energy homeostasis [24]. Adipose-specific deletion of *Raptor* improves energy expenditure and insulin sensitivity and protects mice against HFD-induced obesity [24]. Consistently, *S6k1*-deficient mice have enhanced β-oxidation, faster glucose clearance and insulin sensitivity and are resistant to HFD-induced obesity [25]. However, administration of rapamycin prevents HFD-induced obesity and exacerbates glucose intolerance [41]. Distinct from mTORC1, loss of *Rictor* reduces glucose transport in fat cells [16, 17] and adipose-specific knockout of *Rictor* in mice leads to mild glucose intolerance and severe insulin resistance [17]. These reports suggest that the insulin resistance in *Adipoq-mTOR* mice might mainly result from the absence of mTOC2 in adipose tissues. However, we cannot exclude the effects of other factors, such as adipose tissue inflammation (data not shown), on insulin resistance in the KO mice.

Numerous studies have shown that hepatic steatosis is strongly associated with insulin resistance [47]. Notably, higher serum NEFA and increased hepatic steatosis were found in the *Adipoq-mTOR* mice. Consistent with our results, adipose-specific deletion of *Rictor* increases hepatic steatosis [17, 30]. However, the weight of liver was not affected in the *S6k1*-deletion mice [25] and even less lipid accumulation was found after rapamycin treatment [41]. Taken together, the insulin resistance in *Adipoq-mTOR* mice may be mainly due to hepatic steatosis induced by mTOC2 deficiency.

We found that deletion of *mTOR* inhibits pre-adipocyte differentiation through PPARγ, a master regulator of adipogenesis [37]. Treatment with the PPAR agonist rosiglitazone rescued the differentiation and lipid accumulation in *mTOR*-knockout adipocytes. Previous studies have demonstrated that mTORC1 affects adipocyte differentiation and lipid accumulation by regulating lipogenesis and lipolysis [48]. In 3 T3-L1 adipocytes, activation of mTORC1 signalling suppresses lipolysis and promotes lipid accumulation [48]. Basal rates of lipolysis were increased in *S6k1*-knockout mice [25]. Inhibition of mTORC1 signalling by knockdown of raptor or by rapamycin stimulates lipolysis by activation of *Pnpla2* [48]. In addition, S6K1 depletion or rapamycin treatment inhibits adipocyte differentiation [13, 19, 20]. *mTOR* regulates pre-adipocyte differentiation and PPARγ activity probably not through the production of a ligand but rather through its direct impact on PPARγ transactivation activity [49] or through upregulation of AKT, a regulator of PPAR [12]. However, absence of *Raptor* in *Myf5*-lineage cells promotes osteogenesis and inhibits adipogenesis, while deletion of *Rictor* promotes adipogenesis and inhibits osteogenesis [50]. Therefore, mTORC1 and mTORC2 appear to counteract each other during adipocyte differentiation.

In conclusion, we demonstrate that mTOR plays several important roles in adipose development and adipocyte differentiation. These results provide a novel insight into the important role of mTOR in systemic metabolism. Such knowledge may be useful for developing strategies for treating obesity and related metabolic diseases.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement TZS, PPZ and SHK designed and conceived the research. TZS, PPZ, QYJ and YX performed the experiments, analysed and interpreted the data and revised the manuscript. TZS and SHK wrote the manuscript. YZW assisted with data analysis and interpretation and with revision of the article. All authors approved the final version of the manuscript. TZS and SHK are responsible for the integrity of the work as a whole.

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