Adipose mTORC1 Suppresses Prostaglandin Signaling and Beige Adipogenesis via the CRTC2-COX-2 Pathway

Highlights

- mTORC1 is a potent regulator of white to beige adipogenesis
- mTORC1 inhibition leads to WAT browning and occurs despite sympathetic denervation
- COX-2 and prostaglandins are essential for browning of WAT in Raptor-deficient mice
- mTORC1 suppresses COX-2/PG pathway via a CRTC2-dependent mechanism

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In Brief

Beige adipocytes, which develop in white adipose tissue (WAT), have become a promising avenue to counteract obesity. However, the repertoire of extracellular signals that control beige adipogenesis remains largely unknown. Here, Zhang et al. show that COX-2-mediated prostaglandins act as paracrine signals that orchestrate beige adipogenesis and are controlled by the mTORC1/CRTC2 pathway.

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SUMMARY

Beige adipocytes are present in white adipose tissue (WAT) and have thermogenic capacity to orchestrate substantial energy metabolism and counteract obesity. However, adipocyte-derived signals that act on progenitor cells to control beige adipogenesis remain poorly defined. Here, we show that adipose-specific depletion of Raptor, a key component of mTORC1, promoted beige adipogenesis through prostaglandins (PGs) synthesized by cyclooxygenase-2 (COX-2). Moreover, Raptor-deficient mice were resistant to diet-induced obesity and COX-2 downregulation. Mechanistically, mTORC1 suppressed COX-2 by phosphorylation of CREB-regulated transcription coactivator 2 (CRTC2) and subsequent dissociation of CREB to COX-2 promoter in adipocytes. PG treatment stimulated PKA and promoted differentiation of progenitor cells to beige adipocytes in culture. Ultimately, we show that pharmacological inhibition or suppression of COX-2 attenuated mTORC1 inhibition-induced thermogenic gene expression in inguinal WAT in vivo and in vitro. Our study identifies adipocyte-derived PGs as key regulators of white adipocyte browning, which occurs through mTORC1 and CRTC2.

INTRODUCTION

Recruitment and activation of beige adipocytes (the “browning” effect) may have potential therapeutic implications for the treatment of obesity and related disorders such as insulin resistance, type 2 diabetes, and cardiovascular diseases (Boström et al., 2012; Cohen et al., 2014; Harms and Seale, 2013; Seale et al., 2011; Vegiopoulos et al., 2010). However, while activation of the β3-adrenoreceptor signaling pathway promotes thermogenic gene expression in brown and beige adipocytes in rodents, this approach is clinically not feasible due to the low efficacy of the β3-adrenoreceptor signaling agonists in humans (Boström et al., 2012; Cristancho and Lazar, 2011; Farmer, 2008; Fisher et al., 2012; White and Stephens, 2010). Thus, identifying alternative drug targets that specifically and effectively promote beige adipogenesis is critical for the treatment of obesity and associated metabolic diseases.

Cyclooxygenase (COX), a rate-limiting enzyme responsible for the biosynthesis of prostaglandins (PGs), exists in two isoforms: COX-1, the constitutive form, and COX-2, the inducible form (Marnett et al., 1999). COX-2 oxygenates arachidonic acid and converts it into a number of PGs, including PGD2, PGE2, PGF2α, and prostacyclin (PGI2), all of which exert diverse hormone-like effects via autocrine or paracrine mechanisms (Marnett et al., 1999). In adipose tissue (AT), the COX-2/PG pathway is induced by cold exposure and plays a critical role in cold-induced beige/brite adipocyte formation and browning of white adipose tissue (WAT) (Bayindir et al., 2015; Vegiopoulos et al., 2010). In agreement with this, PGs shift the differentiation of progenitors toward a brown adipocyte phenotype (Bayindir et al., 2015; Vegiopoulos et al., 2010). The COX-2/PG axis also plays a critical role in regulating adipose tissue inflammation and obesity-induced insulin resistance (Chan et al., 2016; Gartung et al., 2016; Hsieh et al., 2010; Hu et al., 2016; Vegiopoulos et al., 2010). However, whether or not the COX-2/PG pathway is involved in the development of obesity remains an open question given that a 25-week high-fat diet (HFD) feeding had no significant effect on COX-2 expression in adipose tissue (Vegiopoulos et al., 2010). In addition, COX-2 is expressed constitutively in adipocytes as well as a variety of immune cells residing in
The mechanistic targets of rapamycin (mTOR), an intracellular energy sensor that integrates distinct signals such as hormones, nutrients, and stress, is a vital regulator of multiple cellular processes such as protein translation, lipid metabolism, cell growth, and survival (Wullschleger et al., 2006). mTOR exists in two distinct complexes, mTORC1 and mTORC2, which differ in subunit compositions and biological function (Loewith et al., 2002). mTORC1 contains the rapamycin-associated TOR protein (Raptor), while mTORC2 contains the rapamycin-insensitive companion of mTOR (Rictor) (Loewith et al., 2002). Raptor is also a regulatory-associated protein of mTOR that binds to and mediates mTOR action (Hara et al., 2002). Loss of Raptor eliminates mTORC1 activity irrespective of feeding status (Sen-gupta et al., 2010). mTORC1 is understood to regulate multiple metabolic processes including protein synthesis, lipogenesis, energy expenditure, and autophagy (Laplante and Sabatini, 2012; Wullschleger et al., 2006) and is highly active in the adipose tissue of obese and HFD-fed rodents (Khamzina et al., 2005; Tremblay et al., 2005). Furthermore, over-activation of mTORC1 results in the development of adiposity and obesity (Carnevalli et al., 2010; Laplante and Sabatini, 2012; Polak et al., 2008; Um et al., 2004; Zhang et al., 2009). However, accumulating evidence has raised questions regarding its role in adipose tissue thermogenesis (Labbé et al., 2016; Lee et al., 2016; Liu et al., 2014, 2016; Polak et al., 2008; Shan et al., 2004; Tran et al., 2016; Um et al., 2004; Wada et al., 2016). Several studies agree that adipose inhibition of mTORC1 leads to browning of WAT and that overactivation of mTORC1 in adipose tissue suppresses thermogenesis and exacerbates diet-induced obesity and insulin resistance in mice (Liu et al., 2014; Polak et al., 2008; Shan et al., 2004; Tran et al., 2016; Um et al., 2004; Wada et al., 2016). However, other studies report that inactivation of mTORC1 in adipose tissue results in impaired thermogenesis (Liu et al., 2016; Tran et al., 2016). While the work from Lee, Shan, and colleagues suggests an inhibitory effect of mTOR signaling on WAT browning, both studies argue that adipose-specific inhibition of mTOR or mTORC1 results in lipodystrophy and systemic insulin resistance during postnatal growth (Labbé et al., 2016; Lee et al., 2016; Shan et al., 2016). In comparison to the well-studied physiological role of mTORC1 in adipose tissue, the mechanisms by which mTORC1 regulates adipose thermogenesis remain largely unknown.

Here, we show that adipose-specific knockout of Raptor induces COX-2 expression and PG biosynthesis, leading to beige adipogenesis in WAT. In support of this, activation of the mTORC1 signaling pathway inhibits COX-2 expression and biosynthesis of PGs including PGI2, PGD2, and PGE2. In adipocytes, mTORC1 inhibits transcription of COX-2 through phosphorylating cyclic-AMP-responsive element-binding protein (CREB)-regulated transcription coactivator 2 (CRTC2) at Ser136. Moreover, inhibition of COX-2 blunts the effect of mTORC1 on beige adipocyte differentiation in vivo. Collectively, our study reveals that adipocyte mTORC1 is a key negative regulator of beige adipocyte development via a CRTC2/COX-2/PG-dependent paracrine mechanism and that activation of adipocyte COX-2/PG signaling protects against diet-induced obesity.
Raptor Deficiency-Induced Browning of WAT Does Not Require Sympathetic Tone

It has previously been shown that impaired BAT function promotes the compensatory browning of WAT by enhancing sympathetic input to WAT (Schulz et al., 2013; Shin et al., 2017). To determine whether Raptor deficiency-induced browning of iWAT is caused by cross talk between BAT and WAT, we examined the levels of tyrosine hydroxylase (TH), a key enzyme of catecholamine biosynthesis driven by sympathetic tone. The expression of TH was not significantly affected by Raptor deficiency in either BAT or iWAT (Figures 1A and 1B). Moreover, surgical denervation led to a marked reduction of TH but had no significant effect on Raptor deficiency-induced UCPI and C/EBPβ expression in iWAT (Figure 2A), suggesting that Raptor deficiency-induced beige adipocyte development in WAT is not due to altered sympathetic tone. Furthermore, the increased levels of UCPI and C/EBPβ in Raptor KO iWAT were also observed under thermoneutrality with lower sympathetic activity (Figure 2C). However, the inducing effect of Raptor deficiency on thermogenic gene expression in iWAT was blunted under cold stress, a condition associated with activation of sympathetic tone (Figure 2D).

In addition, Raptor-deficient mice displayed decreased levels of UCPI and C/EBPβ in BAT under cold stress but not thermoneutrality (Figures 2E and 2F). In accordance with this, Raptor KO mice displayed significantly lower O2 consumption under cold stress conditions with no significant effect on food intake and activity (Figures 2F, S2A, and S2B). Whereas there was no significant difference in systemic O2 consumption between Raptor KO and control mice at room temperature (Figure 2F), probably due to increased WAT browning but reduced BAT function under this condition (Figures 1A and 1B).

Raptor Deficiency in Adipocytes Induces COX-2 Expression and PG Production In Vivo and In Vitro

To elucidate the underlying mechanism by which mTORC1 signaling regulates beige adipocyte development, we performed
RNA-sequencing experiments on inguinal fat from 3-month-old male Raptor KO and control mice. Principal-component analysis (PCA) showed that three KO samples (red) were separated from two control samples (black) (Figure S2C). A total of 11,278 genes remained for analysis after filtering out genes with little or no expression, and a total of 1,812 genes was differentially expressed as shown in the Volcano plot (Figure S2D). The results of the differential expression analysis are summarized in the heatmap (Figure 3A), which shows the gene expression signatures of the most differentially expressed genes in these samples (a larger version with the genes labeled is given in Figure S2E). Among the differentially expressed genes, a group of inflammatory cytokines or immune cell markers including Cxcl2, Tnfsf4, Pdcd1, IL-5, IL1rl1, IL-7r, and Gata3 were elevated by Raptor deficiency (Figure 3B), suggesting the activation of type 2 inflammation, a pathway that has been well documented to be linked to WAT browning. Given the depletion of adipocyte-specific Raptor in these animals, our data also imply that adipocyte-derived paracrine signals may mediate the type 2 immunity and WAT browning in Raptor KO WAT. Notably, Raptor deficiency significantly upregulated PG-endoperoxide synthase 2 (ptgs2), known as COX-2, which is responsible for biosynthesis of PGs, a group of cyclic fatty acid compounds with varying hormone-like effects (Figure 3B). As the COX-2/PG pathway plays a key role in cold-induced browning of WAT as well as inflammation (Hu et al., 2016; Vegiopoulos et al., 2010), we asked whether COX-2 could mediate the promoting effect of mTORC1 deficiency on WAT browning. In support of this, the upregulation of ptgs2, cxcl2, pdc1, II-5, and gata3 by Raptor deficiency was validated by RT-PCR in inguinal fat (Figure S2D). Both protein and mRNA levels of COX-2 were higher in WAT compared to BAT (Figures 3F and 3G), suggesting that the COX-2 pathway is downstream of mTORC1 in WAT, whereas expression levels of COX-1 and COX-2 were only minimally affected by Raptor deficiency in BAT (Figure S2F). Consistent with this, levels of several PGs including PGD2, PGE2, and PI2 were increased in inguinal fat of Raptor KO mice, and the increase of PGI2 level by Raptor deficiency was greater than the increase of PGD2 and PGE2 in iWAT (Figure 3H). In support of our in vivo data, protein levels of COX-2 were upregulated in primary adipocytes from iWAT of Raptor KO compared to control mice (Figure 3I). COX-2 mRNA levels and production of PGD2,
PGE\(_2\) and PGI\(_2\) were also upregulated in Raptor KO adipocytes compared to control cells (Figures 3J and 3K). These results indicate that mTORC1 plays a critical role in regulating the COX-2/PG pathway in a cell-autonomous manner.

Inhibition or Suppression of COX-2 Diminishes Raptor Deficiency-Induced Development of Beige Adipocytes in iWAT

To gain insight into the role of COX-2 in Raptor deficiency-induced beige fat development, we administered 15 mg/kg/day celecoxib, a specific inhibitor of COX-2, to Raptor KO and control mice for 10 days. Inhibition of COX-2 reversed the Raptor deficiency-induced increase in protein (Figures 4A and S2G) and mRNA (Figure 4B) levels of UCP1 in iWAT but had little effect on thermogenic gene expression in BAT (Figure S2H), suggesting that COX-2 mediates the inducing effect of Raptor deficiency on the browning of WAT. In addition, COX-2 inhibition diminished Raptor deficiency-induced development of multilocular adipocytes as well as large adipocytes in both iWAT and eWAT (Figures 4C and 4D). The increased \(O_2\) consumption was reversed by COX-2 inhibition in iWAT but not BAT of Raptor KO mice (Figure 4E). In addition, factors from Raptor KO adipocytes significantly induced expression of UCP1 and C/EBP\(\beta\) during differentiation of primary preadipocytes from iWAT (Figure 4F). However, this inducing effect was suppressed by RNAi-mediated knockdown of COX-2 in Raptor KO adipocytes (Figures S2I and 4F). These data suggest that COX-2 mediates the inhibitory effect of mTORC1 on beige adipocyte differentiation.

Blocking PG Signaling Alleviates Raptor Deficiency-Induced Beige Adipogenesis in BAT

To delineate the role of PG signaling in Raptor deficiency-induced beige adipocyte development, we administered 100 \\(\mu g/kg\) PGD\(_2\), PGE\(_2\), or PGI\(_2\) to C57BL/6 mice through subcutaneous injection for 2 days. Administration of PGD\(_2\), PGE\(_2\), or PGI\(_2\) significantly upregulated protein levels of thermogenic genes including \(ucp1\) and C/EBP\(\beta\) in inguinal fat but not in BAT (Figures S3A and S3A). In addition, mRNA levels of UCP1 and
were upregulated by administration of PGD2, PGE2, or PGI2 (Figure 5B), indicating the promoting effects of PGs on beige adipocytes. The injection of PGI2 and PGE2 induced thermogenesis to a greater extent than PGD2 treatment (Figures 5A and 5B). Although treatment with PGD2, PGE2, or PGI2 had no significant effect on the expression of *ucp1* and C/EBPb in differentiated primary adipocytes (Figure S3B), treatment of these PGs upregulated protein and mRNA levels of UCP1 and C/EBPb during the differentiation of primary iWAT-derived preadipocytes (from day 1 to day 4) (Figures 5C, 5D, S3C, and S3D). In support of previous findings reporting that PGE2, PGD2, and PGI2 bind to their respective receptors and stimulate cyclic AMP (cAMP) signaling (Clasadonte et al., 2011; Mohan et al., 2012), we found that the receptors for PGE2 and PGI2 were enriched in the SVF, adipocytes and preadipocytes (Figure S3E), and that treatment of primary adipocytes with PGD2, PGE2, and PGI2 for 2 hr increased intracellular levels of cAMP along with PKA signaling (Figures 5E and 5F). Consistent with this, induction of UCP1 and C/EBPb by PGI2 was suppressed by PKA inhibition following treatment with 10 μM H89 during adipocyte differentiation (Figure 5G).

To further investigate the role of PG signaling in mTORC1 inhibition-induced beige adipogenesis, we administered 2 mg/kg of PGI2 receptor antagonist CAY10441 through intraperitoneal (i.p.) injection in Raptor KO and control littermates for 10 days. Inhibiting PGI2 signaling suppressed Raptor deficiency-induced upregulation of UCP1 and C/EBPb in iWAT, suggesting that PGI2 mediates the browning effect induced by Raptor deficiency (Figure 5H). Moreover, the conditioned media of primary Raptor KO differentiated adipocytes induced the expression of UCP1 and C/EBPb during the differentiation of primary preadipocytes, an effect that was partially reversed by treatment with the PGI2 antagonist CAY10441 during the differentiation (Figure 5I). These findings suggest that PGI2 signaling is an important mediator of beige adipogenesis in response to mTORC1 inhibition.
C57BL/6 mice were fed with 45% HFD for 4, 16, or 24 weeks as well as phosphorylation of S6K at Thr389. In contrast, HFD feeding significantly downregulated COX-1 in iWAT (Figures 6A and 6B) and eWAT (Figure S4A), concurrent with increased phosphorylation of S6K at Thr389. In contrast, HFD feeding significantly upregulated COX-2 expression and suppressed phosphorylation of S6K in BAT (Figure S4B). While not statistically significant, trending COX-2 suppression in iWAT (Figures 6C, 6D, S4C, and S4D) and eWAT (Figures S4C–S4F) was also observed following 16 weeks of HFD feeding, with no effect seen at 24 weeks despite persistent activation of S6K. Along this line, mTORC1 was activated, and expression levels of COX-2 were suppressed in the iWAT and eWAT but not in the BAT of ob/ob mice compared to lean mice (Figures 6E and S4G). In addition, rapamycin treatment upregulated COX-2 in human adipocytes as we described previously (Liu et al., 2012, 2014). We found that 4-week HFD feeding significantly downregulated protein and mRNA levels of COX-2 but not COX-1 in iWAT (Figures 6A and 6B) and eWAT (Figure S4A), concurrent with increased phosphorylation of S6K at Thr389.
Figure 6. Adipocyte mTORC1 Inhibition Protects Mice against HFD-Induced Downregulation of COX-2 and Energy Expenditure

(A–D) 6-week-old male C57BL/6 mice were fed with normal chow diet (NCD) or HFD for 4, 16, or 24 weeks. Protein levels of COX-2 and COX-1 along with phosphorylation of S6K (A) and mRNA level of COX-2 and COX-1 (B) in iWAT after 4-week-HFD feeding. Phosphorylation of S6K at Thr389 in iWAT (C) were stimulated by HFD feeding for 4, 16, or 24 weeks. Conversely, the expression levels of COX-2 in iWAT (D) were suppressed by 4 or 16 weeks of HFD feeding, while not significantly altered by 24 weeks of HFD feeding. n = 4–8/group in (C) and (D).

(E) Phosphorylation of S6K at Thr389 was increased, and expression levels of COX-2 were suppressed in iWAT of ob/ob mice compared to lean mice. n = 5/group.

(F) Rapamycin treatment induced expression of COX-2 in human primary adipocytes from neck adipose tissue. n = 3/group.

(G) Raptor deficiency protected against the suppression of COX-2 induced by 4-week HFD and upregulated basal and HFD-induced UCP1 in iWAT in vivo. n = 5–7/group.

(H) O2 consumption in Raptor KO and control mice throughout light and dark cycles on the day before HFD feeding and day 7 and day 30 after HFD feeding. The average O2 consumption was normalized to whole-body mass and analyzed using Student’s t test. (F) was the representative data from three individual experiments. The data in (B)–(H) are presented as the mean ± SEM. *p < 0.05.
CRTC2 activity through phosphorylation rather than cellular well as food intake was stimulated by cold stress (Figure S6J), Phosphorylating CRTC2 at Ser136 in Adipocytes mTORC1 Suppresses COX-2 Expression by iWAT and BAT (Figure S6I). On the other hand, mTORC1 as HFD-induced decrease of O2 consumption with no change to 4 weeks of HFD feeding, Raptor deficiency protected against with this, despite no significant effect on body weight after 4 weeks of HFD feeding, Raptor deficiency protected against HFD-induced decrease of O2 consumption with no change to food intake or activity (Figure 6H and data not shown). Along with this, Raptor-deficient mice were protected against diet-induced obesity and glucose intolerance despite no significant changes in triglyceride (TG) content in liver, hepatic steatosis, or insulin tolerance (Figures S1A–S1F). These results together suggest that adipocyte mTORC1 inhibition protects mice against HFD-induced downregulation of COX-2, energy imbalance, and obesity. However, we did not observe the induction of COX-2 by 48-hr cold exposure (6°C) despite the upregulation of UCP1 in iWAT and BAT (Figure S6I). On the other hand, mTORC1 as well as food intake was stimulated by cold stress (Figure S6J), implying that cold stress activation of mTORC1 may be partially due to the increase of food intake, and that mTORC1/COX-2 pathway is dispensable for cold-induced thermogenesis.

mTORC1 Suppresses COX-2 Expression by Phosphorylating CRTC2 at Ser136 in Adipocytes

Given that β-adrenergic signaling drives lipolysis and COX-2 gene activation (Gartung et al., 2016; Klein et al., 2007; Vegiopoulos et al., 2010), we hypothesized that PKA signaling may mediate the inhibitory effect of mTORC1 on COX-2. We found that treatment of rapamycin or isoproterenol significantly induced COX-2 expression in primary adipocytes (Figures S5A and S5B). Moreover, inhibition of PKA by H-89 treatment diminished the stimulatory effect of Raptor deficiency or rapamycin treatment on COX-2 expression in primary adipocytes (Figures 7A and 7B), suggesting a mediatory role of PKA signaling in mTORC1 regulation of COX-2. However, rapamycin and Raptor deficiency had no significant effect on the phosphorylation of PKA substrates (Figure 7B). Because mTORC1 has been shown to interact with PKA signaling through phosphorylation of CRTC2 at Ser136 in hepatocytes (Han et al., 2015), we examined the effect of mTORC1 on the localization of CRTC2 and CRTC3 in primary adipocytes. We found that both CRTC2 and CRTC3 were expressed in adipocytes (Figures 7C and 7D). CRTC2 is localized in both the nucleus and the cytoplasm and CRTC3 is predominantly localized in the nucleus under growth medium-culturing conditions (Figures 7C and 7D). Treatment with a cAMP analog promoted CRTC2 translocation to the nucleus, while having little effect on CRTC3 localization (Figures 7C and 7D). In addition, inhibition of mTORC1 by rapamycin had no effect on the nuclear translocation of CRTC2 (Figure 7C). However, leucine treatment induced and rapamycin treatment suppressed phosphorylation of CRTC2 at Ser136 in adipocytes (Figures 7D and 7E). Moreover, Raptor deficiency suppressed leucine-induced P-CRTC2 at Ser136 in adipocytes (Figures 7F and 7G), suggesting that mTORC1 may modulate CRTC2 activity through phosphorylation rather than cellular localization.

Next, we investigated the potential role of mTORC1-mediated CRTC2 phosphorylation in regulating COX-2 expression in preadipocytes given the promoting effect of CREB on cox-2 promoter activation (Reddy et al., 2000; Yang and Bleich, 2004). Suppressing CRTC2 by RNAi diminished rapamycin-induced COX-2 expression in preadipocytes (Figures 7G and S5E). In contrast, overexpression of CRTC2 markedly upregulated the expression levels of COX-2 and attenuated rapamycin-induced COX-2 expression in preadipocytes (Figure 7H). To dissect the mediatory role of CRTC2 in mTORC1 suppression of COX-2, we performed immunoprecipitation, chromatin immunoprecipitation (ChIP) analysis, and luciferase assays. We found that rapamycin treatment promoted the interaction between CRTC2 and CREB (Figure 7I), induced CREB binding to the cox-2 promoter (Figures 7J and S5F), and enhanced luciferase activity of the cox-2 promoter (Figure 7K) in preadipocytes. Moreover, overexpression of CRTC2 increased the association between CRTC2 and CREB as well as CREB binding to the cox-2 promoter, luciferase activity of the cox-2 promoter, and COX-2 expression, and diminished the inducing effect of rapamycin in preadipocytes (Figures 7H–7K). Furthermore, the ability of CRTC2 to interact with CREB, facilitate CREB binding to the cox-2 promoter, and activate the cox-2 gene to induce COX-2 expression was suppressed by a serine-to-aspartate mutation at 136 (S136D) but not by a serine-to-alanine mutation (S136A) (Figures 7H–7K). The results from this study indicate that mTORC1 suppresses COX-2 through phosphorylation of CRTC2 at Ser136 and subsequent inhibition of CREB binding to the cox-2 promoter in adipocytes.

DISCUSSION

Beige adipocytes, which develop in WAT, have become a promising avenue to counteract obesity (Harms and Seale, 2013). However, the adipocyte-derived signals that target progenitor cells and control beige adipogenesis in WAT have not yet been defined. Here, we show that COX-2-mediated PGs act as paracrine signals that target adipocyte progenitor cells and orchestrate beige adipogenesis. In addition, we propose that mTORC1 signaling is a key regulator of the COX-2/PG pathway through phosphorylation of CRTC2 in adipocytes and mediates obesity-induced suppression of COX-2 and beige adipogenesis in WAT. These results reveal that adipocyte mTORC1 controls beige adipogenesis via PG-mediated paracrine signaling, and establish a pathway underlying the suppression of COX-2 as well as energy imbalance during the development of obesity.

COX-2 plays a pivotal role in regulating adipose inflammation including macrophage recruitment and subsequent immune response and insulin resistance (Chan et al., 2016; Gartung et al., 2016; Hsieh et al., 2010; Hu et al., 2016). Additionally, COX-2 promotes energy metabolism such as beige adipocyte formation as well as browning of WAT (Davis et al., 2004; Fain et al., 2001; Lundholm et al., 2004; Vegiopoulos et al., 2010). However, whether COX-2 is critically involved in the development of obesity remains an open question. In addition, the physiological signals that control the COX-2 pathway during the development of obesity are unclear. We found that HFD feeding suppresses COX-2, which is accompanied by activation of
**Figure 7. mTORC1 Signaling Inhibits CRTC2 Phosphorylation at Ser$^{136}$ and Subsequently Suppresses COX-2 Transcription in Adipocytes**

(A) Raptor deficiency induced COX-2 expression, which was diminished by treatment of PKA inhibitor H89 in primary preadipocytes. $n = 3$/group.

(B) PKA inhibition attenuated the inducing effect of rapamycin treatment on COX-2 expression.

(C) Immunofluorescence staining of CRTC2 after treatment of 100 μM cAMP homolog dibutyryl cyclic AMP, 10 nM rapamycin, or co-treatment in primary preadipocytes.

(D) Leucine treatment stimulated the phosphorylation of CRTC2 at Ser$^{136}$ in primary preadipocytes.

(E) Rapamycin treatment inhibited CRTC2 phosphorylation at Ser$^{136}$ in primary preadipocytes.

(F) Raptor deficiency suppressed rapamycin-stimulated phosphorylation of CRTC2 at Ser$^{136}$ in primary preadipocytes.

(legend continued on next page)
mTORC1 in WAT, while BAT is protected from these alterations under HFD conditions (Figures 6A–6D, S4A, and S4B). In support of this, both protein and mRNA levels of COX-2 are more enriched in WAT compared to BAT (Figures 3D and 3E). Notably, mTORC1 activation and COX-2 suppression in iWAT by HFD feeding occurs within 4 weeks, even before UCP1 suppression (Figures 6A and 6B). In addition, inhibition of mTORC1 in adipocytes restores HFD-induced suppression of COX-2 (Figure 6G). Despite previous findings that 25 weeks of HFD (60% kcal from fat; Research Diet) feeding has no significant effect on COX-2 expression (Vegiopoulos et al., 2010), our data suggest that COX-2 expression is indeed suppressed in WAT in the earlier stage of obesity, as indicated by 4- to 16-week HFD feeding (Figures 6C, 6D, S4C, and S4D). However, diet-induced COX-2 suppression disappears at the later stage of obesity as indicated by 24-week HFD feeding. Given that COX-2 plays a critical role in obesity-induced adipose inflammation and insulin resistance, the mechanisms underlying the regulation of COX-2 expression at the later stage of obesity may be more complicated than in the earlier stage.

Previous studies have reported that β3-adrenergic receptors are required for the recruitment and activation of beige adipocytes in response to cold stress in WAT (Barbatelli et al., 2010; Cannon and Nedergaard, 2004; Jimenez et al., 2003; Namhias et al., 1991). However, these findings were challenged by evidence showing that, in the absence of β3-adrenergic receptors, cold-induced activation of thermogenic programming in both BAT and WAT remains fully intact (de Jong et al., 2017). These findings also indicate the possibility of an alternative β3-adrenergic receptor-independent signaling pathway that may control beige adipogenesis and activation. Adipocyte-derived PGs facilitates the activation of PKA-C/EBPβ pathway, which induces beige adipogenesis in adipocyte progenitor cells (Figures 5E–5G). Therefore, treatment with the PG receptor antagonist diminishes the ability of Raptor deficiency to induce progenitor cell differentiation into beige adipocytes (Figures 5H and 5I). The results of this study indicate that PG receptors in progenitor cells may be possible therapeutic targets for the treatment of obesity and its related disorders.

Despite accumulating evidence indicating that COX-2 is induced by lipolysis in adipocytes (Gartung et al., 2016; Klein et al., 2007; Vegiopoulos et al., 2010), the mechanisms underlying modulation of COX-2 transcription in adipocytes remain elusive. Our data suggest that overactivation of mTORC1 by obesity suppresses COX-2 expression and PG production through phosphorylation of CRTC2 at Ser136 and subsequent blocking of CREB binding to the cox-2 promoter (Figure 7). CRTC2s facilitate hepatic gluconeogenesis and lipid homeostasis at the transcriptional level (Han et al., 2015; Wang et al., 2009); however, the physiological role of CRTC2 in adipose tissue is incompletely understood. CRTC2 and CRTC3 are present in adipose tissue as well as in adipocytes and are involved in the regulation of energy balance and glucose uptake in adipose tissue (Henriksson et al., 2015; Park et al., 2014; Song et al., 2010). However, little is known about CRTC2s modulation and target genes in adipocytes. Our study established that mTORC1 inactivates the cox-2 gene by phosphorylating CRTC2 (Figure 7). Although mTORC1 signaling has little effect on cytoplasm-nucleus shuttling of CRTC2 in adipocytes, mTORC1-mediated phosphorylation of CRTC2 at Ser136 inhibits association between CREB and CRTC2, CREB binding to the cox-2 gene, and the promoter activity of the cox-2 gene (Figures 7I–7K). Our study establishes a pivotal role for mTORC1 in regulating COX-2 transcription and PG production/secretion in adipocytes through phosphorylation of CRTC2. Consistent with the findings of Paschos et al. (2018), mTORC1/COX-2 pathway is not required for cold-induced thermogenesis. Whereas adipocyte-derived PGs stimulate accumulation of cAMP in adipocyte progenitor cells and subsequently promote beige adipogenesis, favoring calorie restriction or weight loss induced WAT browning.

The role of adipocyte mTORC1 signaling in the regulation of thermogenesis remains controversial. Early studies show that inhibition of mTORC1 by S6K1 ablation as well as adipose-specific deficiency of Raptor enhances thermogenesis and energy expenditure and protects against diet-induced obesity and glucose intolerance (Polak et al., 2008; Um et al., 2004). In support of this, the basal expression levels of thermogenic genes are elevated in the white fat of Raptor- or mTOR-deficient mice (Lee et al., 2016; Meng et al., 2017; Shan et al., 2016; Tran et al., 2016). However, other studies show that adipose inactivation of mTORC1 by rapamycin or Raptor deficiency leads to lipodystrophy, fatty liver, and insulin resistance (Labbé et al., 2016; Lee et al., 2016; Shan et al., 2016; Tran et al., 2016). In addition, inhibition of mTORC1 results in impaired thermogenesis by interacting with PKA signaling in either BAT or both BAT and WAT (Liu et al., 2016; Tran et al., 2016). Therefore, addressing this controversy regarding mTORC1 in adipose tissue is urgently needed. Our present study shows that adipocyte mTORC1 differentially modulates thermogenic programing of BAT and WAT. Raptor deficiency promotes browning of white adipose tissue by regulating beige adipocyte differentiation in a PG-dependent manner, while suppressing thermogenesis in BAT through impairment of brown fat development. Interestingly, while increased thermogenesis and O2 consumption in iWAT occurs under thermoneutrality and at room temperature, experimental conditions in mice resemble human conditions, an effect that disappears under cold stress.
conditions. Our study demonstrates that, while mTORC1 inhibition does not intrinsically increase thermogenic capacity, it activates PKA signaling in adipocyte progenitor cells via CRTC2-COX-2 axis-dependent PG production. Our study uncovers PG as a paracrine signal that promotes beige adipogenesis and mediates adipocyte mTORC1 inhibition-induced browning of WAT. Given the activation of type 2 inflammation by Raptor deficiency in iWAT (Figures S2E, 3B, and 3C), further investigation is needed to address whether COX-2 mediates the promoting effect of mTORC1 inhibition on immune response in the context of thermogenic regulation. In addition, the type and function of immune cells controlled by adipose-specific mTORC1/COX-2 pathway remain to be clarified.

In summary, our data show that adipocyte mTORC1 signaling impacts adipocyte progenitor cells and controls beige adipocyte differentiation via a PG-dependent paracrine mechanism. Moreover, the mTORC1 pathway inhibits the production and secretion of PG through the phosphorylation of CRTC2 and the suppression of COX-2 in adipocytes. In addition, mTORC1-mediated suppression of COX-2 is critically involved in the development of obesity. Our study strongly suggests that adipocyte mTORC1 signaling plays a key role in regulating the development of beige adipocytes in WAT.

**EXPERIMENTAL PROCEDURES**

**ChIP Assay**

3T3-L1 preadipocytes or CRTC2-overexpressed preadipocytes were treated with or without 5 nM rapamycin for 10, 30, 60, or 120 min, and then fixed in 1% formaldehyde for 10 min at 37°C. Cells were washed twice with cold PBS containing protease inhibitor cocktail, and subsequently lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1). Extracts were sonicated until DNA fragments of 200–1,000 bp were achieved. Sonicated solution was diluted in ChIP dilution buffer (TE buffer, 1.1% Triton X-100, 167 mM NaCl, 1X cocktail). The immuno-complexes were precipitated using an anti-CREB (Millipore) antibody or IgG as the control. Precipitated complexes were reverse cross-linked at 65°C. The amount of precipitated DNA was detected by semiquantitative PCR using the primers 5'-CAGAGAGGGGGAAGAAAGTTGG-3' and 5'-GAGCAGAGTCCTGACTC-3'.

**Luciferase Assay**

The luciferase construct of the cox-2 promoter was generated by subcloning XhoI-HindIII fragment (~74 to ~727) of the cox-2 promoter into pGL3-basic with the primers 5'-CCCTCAAGAACCAGAGGTTAGTCAT-3' and 5'-CCAGCTGAGCAGTCTGACTGACTC-3'. 3T3-L1 preadipocytes were grown in a 24-well plate to 70% confluency. The cox-2 promoter luciferase construct was co-transfected with pSV-β-Galactosidase Control Vector (Promega, Madison, WI, USA) in combination with pcDNA 3.1, pcDNA/CRTC2, pcDNA/CRTC2-S136A, or pcDNA/CRTC2-S136D. 24 hr post-transfection, cells were treated with or without 5 nM rapamycin for 4 hr and then lysed for the measurement of the luciferase with the Luciferase Assay Kit (Promega) and β-galactosidase activities using a Turner TD20/20 luminometer.

**Human Study**

Patients were identified either before undergoing anterior cervical spine surgery, before undergoing parathyroidectomy or before undergoing thyroidectomy, and then were recruited in the ENT clinic at time of consent for primary surgery. This study has been reviewed and approved by Human Research Review Committee at UNMHSC. 5–10 mg neck fat was harvested during anterior cervical spine surgery, parathyroidectomy, or thyroidectomy. Primary adipocytes were isolated and treated with or without 5 nM Rapamycin for 20 hr.

**Animals**

The ob/ob mice were obtained from Jackson Laboratory (stock number 000632). The raptor floxed mice (raptorfl/f) (Jackson Laboratory, stock number 13188) were crossed with adiponectin cre mice (Jackson Laboratory, stock number 10803) to generate raptor fl/+ - adiponectin cre mice. The Raptor ad+/− mice were then crossed back with raptorfl/fl mice to generate adipose-specific raptor knockout (KO) and raptor-floxed control mice.

The knockout efficiency was confirmed in adipose tissue and other tissues by western blot analysis using anti-raptor antibodies. Unless otherwise noted, 10-week-old male mice were used for all experiments. Animals were housed in a specific pathogen-free barrier facility with a 12-hr light/12-hr dark cycle with free access to food and water. For cold stress study, animals were housed in the temperature-controlled chamber at 22°C for three days followed by either 30°C or 6°C for another 48 hr. For the high-fat diet challenge study, 6-week-old male mice were fed with 45% of normal chow diet provided by the animal facility at the University of New Mexico Health Sciences Center or high-fat diet (45% kcal from fat) from Research Diets (D12451; New Brunswick, NJ, USA) for 4, 16, or 24 weeks. For surgical denervation, an incision was made at the dorsal hindlimb of the animal and lateral to the spinal column that continued rostrally and then ventrally to the ventral hindlimb as described in our previous study (Luo et al., 2017). Two weeks post-surgery, mice were euthanized and inguinal fat was collected for western blot. All animal experimental protocols were reviewed and approved by the Animal Care Committee of the University of New Mexico Health Sciences Center.

**RNA isolation and Sequencing**

Total RNA was isolated from single 10-μm slide-mounted FFPE sections using the RNeasy FFPE Kit from Qiagen. After isolation, total RNA was mixed with ERCC Spike-In Mix 1 control RNA (Life Technologies) and converted to cDNA using the SMARTer Universal Low Input RNA Kit for Sequencing (Clontech), which uses random primers for the conversion. The Ion Plus Fragment Library Kit (Life Technologies) was used to add barcodes, and amplify and size-select the final library. Four individually barcoded samples were sequenced together on Ion Proton P1v2 chips by the Analytical and Translational Genomics Shared Resource at the University of New Mexico Cancer Center (Brayer et al., 2016). RNA-sequencing data are available for download from the NCBI Sequence Read Archive using SRA study accession number SRP059557.

**Statistics**

Statistical analysis of the data was performed using a two-tailed Student’s t test between two groups or one-way ANOVA among three different groups. All of the results were presented as the mean ± SEM, and p value of <0.05 was considered to be statistically significant.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.08.055.

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AUTHOR CONTRIBUTIONS

M.L., X.O.Y., and M.B. designed the project. J.Z. provided technical support for the CHIP analysis. N.B. and M.S. completed the collection of human samples. X.Z., Y.L., C.W., X.Y., D.W., F.S., Z.Y., Q.Z., and M.L. conducted the experiments. M.L., X.Z., Y.L., C.W., X.D., and F.S. analyzed the results. M.L. wrote the manuscript. All authors reviewed and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES


is required for brown adipose tissue recruitment and metabolic adaptation to cold. Sci. Rep. 6, 37223.
Supplemental Information

Adipose mTORC1 Suppresses Prostaglandin Signaling and Beige Adipogenesis via the CRTC2-COX-2 Pathway

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Fig. S1. Adipose-specific raptor deficient mice are resistant to diet-induced obesity and glucose intolerance, Related to Fig. 1. 6 week-old raptor KO and control mice were fed with normal chow (NC) or high fat diet (HFD) for 14 weeks. The following studies were performed using these animals. A. The body weights were monitored weekly during feeding with NC or HFD. B. Raptor deficiency decreased fat mass and protected against diet-induced adiposity, while increasing the mass of liver and pancreas under NC but not under HFD feeding condition. C. The representative images of BAT, iWAT, eWAT and liver in raptor KO and control mice fed with NC or HFD. D. Raptor deficiency upregulated triglyceride levels in the liver under normal chow feeding condition, but diminished the diet-induced increase of triglyceride levels in liver. Raptor deficient mice were insulin intolerant under NC-feeding condition, but abrogated diet-induced glucose intolerance (E) and insulin intolerance (F) despite insulin resistance under normal chow condition compared to control mice. G. Raptor deficiency upregulated expression levels of UCP1 and C/EBPβ, while suppressed adipogenic markers PGC1α, PPARγ and adiponectin in eWAT at room temperature. H. Oil red O staining of primary differentiated adipocytes from preadipocytes from iWAT and BAT of raptor KO and control mice. I. Basal and CL316243-induced expression of UCP1 and adipogenic markers PPARγ and adiponectin in raptor KO and control primary differentiated adipocytes. The oxygen consumption rate (OCR) of raptor KO and control primary differentiated adipocytes from preadipocytes of iWAT (J) and BAT (K) determined by Seahorse XF analyzer. L. The data for Fig. 1H were quantified and analyzed. The data in Figs. S1H and S1I are representative from three individual experiments. The data in Figs. S1A-1B, S1D, and S1J-1L are presented as the mean ± S.E.M. *p<0.05, **p<0.01, comparison between two groups as indicated. For Fig. S1A, *p<0.05 control mice with NC vs control mice with HFD. For Figs. S1E and S1F, *p<0.05, **p<0.01, control mice with NC vs control mice with HFD, and *p<0.05, **p<0.01, control mice with HFD vs raptor KO with HFD.
Fig. S2. RNA sequencing of raptor KO and control iWAT, Related to Fig. 3 and Fig. 4. Food intake (A) and beam counts (B) of raptor KO and control mice at room temperature (22°C) and under cold stress condition (6°C). n=8/group in Figs. S2A and S2B. RNA seq Gene expression signatures of inguinal fat from 10 week-old male raptor KO and control mice. C. PCA of RNA-seq data from 20 ACC tumors (red) and 5 normal salivary gland samples (black). D. Volcano plot showing distribution of gene expression changes in ACC tumor samples, with genes showing >2-fold change and adjusted $P < 0.05$ shaded red. E. Heatmap summarizing differential gene expression in raptor KO iWAT versus control samples and illustrating gene expression signatures for raptor deficiency. Blue and red shading indicate downregulation and upregulation, respectively, as shown in the color key at upper right. n=2-3/group in Figs. S2C-S2E. F. The expression levels of COX-2 and COX-1 in raptor KO and control BAT. n=4/group. G. Quantification of UCP1 and C/EBPβ expression in Fig. 4A. H. The levels of UCP1 and C/EBPβ were not significantly affected by celecoxib treatment in BAT of raptor KO mice. n=8/group in Figs. S2G and S2H. I. Introduction of COX-2 siRNA suppressed basal and raptor deficiency-induced COX-2 expression in primary adipocytes. The data in Fig. S2I was representative from three individual experiments. Data in Figs. S2A, S2B and S2G are presented as the mean± S.E.M. *p<0.05.
**Fig. S3**

(A) Western blots of UCP1, C/EBPβ, PGC1α, and PPARγ in BAT and PGC1α in adipocytes treated with different concentrations of PGD2, PGE2, and PGI2.

(B) Western blots of UCP1, C/EBPβ, PGC1α, and PPARγ in adipocytes treated with different concentrations of PGD2, PGE2, and PGI2.

(C) Western blots of UCP1, C/EBPβ, PGC1α, and PPARγ in adipocytes treated with different concentrations of PGD2.

(D) Western blots of UCP1, C/EBPβ, PGC1α, and PPARγ in adipocytes treated with different concentrations of PGE2.

(E) Western blots of EP2, EP4, and IP in SVF, Pre-Adip, and Adipo treated with different concentrations of PGE2 and PGD2.
Fig. S3. Prostaglandin treatment induced beige adipogenesis, Related to Fig. 5. **A.** Administration of PGE2, PGD2 or PGI2 for 2 days had no significant effect on the expression levels of UCP1, C/EBPβ, PGC1α and PPARγ in BAT. **B.** Treatment of PGE2, PGD2 or PGI2 had no significant effect on the expression levels of UCP1, C/EBPβ, PGC1α and PPARγ in primary differentiated adipocytes. The expression levels of UCP1 and C/EBPβ but not PPARγ and PGC1α were induced by PGD2 (C) or PGE2 (D) treatment at the different dose during differentiation of primary preadipocytes. **E.** The expression of PGI2 receptor (IP) and PGE2 receptors EP2 and EP4 in primary preadipocyte, adipocyte and SVF.
Fig. S4. Activation of mTORC1 or obesity suppressed COX-2 expression in vivo and in vitro, Related to Fig. 6. Protein levels of COX-2 and COX-1 along with phosphorylation of S6K in eWAT (A) and BAT (B) after 4 week-HFD feeding. n=6-7/group. Protein levels of COX-2 and COX-1 along with phosphorylation of S6K in iWAT, eWAT and BAT after 6-week-old male C57BL/6 mice were fed with NC or HFD for 16 (C) or 24 weeks (D). n=4/group in Figs. S4C and S4D. The data in Figs. S4C (E) and S4D (F) were quantified and analyzed. G. Protein levels of COX-2 and COX-1 along with phosphorylation of S6K in eWAT and BAT of ob/ob and lean mice. n=5/group. H. Raptor deficiency had no significant effect on the expression of COX-2 and HFD-induced UCP1 in BAT in vivo. n=6-7/group. I. Cold exposure for 48 hours had no significant effect on the expression of COX-2 and COX-1 despite increased activity of mTORC1 in iWAT and BAT. n=6/group. J. Food intake was significantly increased by cold exposure for 48 hours. n=6/group. The data in Figs. S4E, S4F, S4H, and S4J are presented as the mean ± S.E.M. *p<0.05.
mTORC1 suppresses COX-2 in CRTC2-dependent manner, Related to Fig. 7. Expression of COX-2 was induced by the treatment of rapamycin (A) or the treatment of isoproterenol (B) in dose-dependent manner in preadipocytes. C. Immunofluorescence staining of CRTC3 after treatment of 100 µM cAMP homolog Dibutyryl cyclic AMP, 10 nM rapamycin or co-treatment in primary preadipocytes. D. Quantification of phosphorylation levels of CRTC2 at Ser136 and normalization with CRTC2 in Fig. 7F. E. Quantification of COX-2 levels in Fig. 7G. F. Treatment of rapamycin increased CREB binding to the cox-2 promoter in a time-dependent manner in 3T3-L1 preadipocytes. The binding of CREB to the cox-2 promoter was determined using CHIP assay. Figs. S5A-S5C are the representatives from three individual experiments. The data in Figs. S5D-S5F are presented as the mean ± S.E.M. *p<0.05.
Supplemental information

Methods

**Materials** Antibodies against PKA substrate phosphorylation, Flag (DYKDDDK tag), raptor, S6K, phospho-S6K at Thr389, COX-1, COX-2 (catalog #12282, anti-human) and PPARγ were from Cell Signaling Technology (Danvers, MA). Anti-PGC1α and tyrosine hydroxylase (TyrH) (catalog #2950738) were purchased from Millipore. Anti-C/EBPβ was from Santa Cruz. Anti-CRTC2 from Invitrogen was used for staining and anti-CRTC2 from Calbiochem was used for Western blot analysis. The phosphor-CRTC2 at S136 antibody as well as plasmids encoding wild-type and S136A/D mutant CRTC2 and the control vector were kindly provided by Dr. Yiguo Wang at Tsinghua University, China. PGD2, PGE2, PG12, Celecoxib, CAY10441 and antibodies to COX-2 (catalog #160106, anti-mouse), EP4, EP2, and IP were obtained from Cayman Chemical Company (Ann Arbor, MI). Polyclonal antibodies to adiponectin were a gift from Dr. Feng Liu at UTHSA as described previously (Liu et al., 2008). The anti-UCP1 and anti-CRTC3, and cAMP Assay Kit were purchased from Abcam. Dibutylryl cyclic AMP (dbcAMP), CL316,243, leucine, Rapamycin, H89, the medium without arginine, leucine and lysine and control medium were from Sigma-Aldrich.

**Administration of PGD2, PGE2, PG12, Celecoxib and CAY10441** PGE2, PGD2 and PG12 were dissolved in 100% dimethyl sulfoxide (DMSO) and diluted in vehicle solution containing 5% Tween-80 and 5% PEG-400 in PBS to reach the concentration of 100 µg/ml. 2 month-old male C57/BL/6 mice were administered with PGD2, PGE2, PG12 or vehicle through subcutaneous injection at 100 µg/kg once a day for 2 days as described previously (Madsen et al., 2010). The mice were then euthanized and fat tissues were collected for tissue respiration, Western blot analysis and RT-PCR. Celecoxib or CAY10441 was dissolved in 100% dimethyl sulfoxide (DMSO) and diluted in vehicle solution containing 5% Tween-80 and 5% PEG-400 in PBS. 2-month-old raptor KO or control mice were administered with celecoxib (15 mg/kg), CAY10441 (2 mg/kg) or vehicle by intraperitoneal (i.p.) injection once a day for 10 days as the previous studies described (Fan et al., 2013; Vegiopoulos et al., 2010). The mice were then euthanized and adipose tissue samples were collected for H&E staining, tissue respiration, RT-PCR and Western blot analyses.

**Food intake, body weight, and body composition** Mouse food intake and body weight were measured weekly for 14 weeks. To check body composition, mice were anesthetized by i.p. injection with avertin (120 mg/kg animal body weight). Bone mineral density, fat mass, lean mass, and percentage of fat were determined using dual-energy X-ray absorptiometry (DEXA) (GE Medical Systems, Madison, WI).

**Hematoxylin and eosin, Oil Red O staining and immunohistochemical analysis** For hematoxylin and eosin (H&E) staining, adipose tissues were fixed with 4% formalin for 48 h and embedded in paraffin. Tissue sections (6-µm thick) were stained with H&E according to standard protocols and analyzed using the NIH Image J software. Immunohistochemistry was performed as described in our previous study (Liu et al., 2014). For the analysis of adipocyte size in adipose tissue, stained tissues were visualized with NanoZoomer Slide Scanner. Five sections per animal were stained. Eight representative images per section were used to determine adipocyte size (Liu et al., 2014). Size of adipocytes were determined with ImageJ Analysis of H&E stained tissues (Zhao et al., 2018). For Oil Red O staining, differentiated primary adipocytes were fixed with
10% formalin for 5 min and stained with Oil Red O in 60% isopropanol for 20 min (Liu et al., 2012).

**Energy expenditure** The mice were individually housed in a Promethion Metabolic Phenotyping System (Sable Systems International) coupled with a temperature-control chamber. Oxygen consumption (VO2), carbon dioxide release (VCO2), food and water intake, and the activity of each animal (single-housed in 8 cages) were monitored at room temperature (22°C) for 72 hours followed with cold stress (6°C) for 48 hours. The data were analyzed using the software of ExpeData associated with the system and oxygen consumption was normalized by body weight as described in our previous study (Luo et al., 2017).

**Primary culture and differentiation of adipocytes** Primary stromal vascular fractions from interscapular brown and inguinal fat depots of 3-week-old raptor knockout, control or C57BL/6 wild type mice were isolated, cultured and differentiated into adipocytes according to the procedure as described previously (Liu et al., 2014). For prostaglandin treatment, primary preadipocytes from inguinal fat were differentiated and treated with PGD2, PDE2 or PGI2 for 4 days during the differentiation. On the day 5, cells were harvested for Western blot analysis or RT-PCR. To generate CRTC2-suppressed primary preadipocytes, one of two CRTC2 small interfering RNA (siRNA) constructs (ThermoFisher, catalog 4390771, ID s92672 and ID s92673) was transfected into preadipocytes using a NEON transfection system as described in our previous study (Liu et al., 2014). One of two control siRNA constructs, including Silencer® Select GAPDH Positive Control siRNA (ThermoFisher, 4390849) and Silencer™ Select Negative Control No. 1 siRNA (ThermoFisher, 4390843), was transfected as a control. A similar approach was used to generate COX-2-suppressed and control primary adipocytes using COX-2 small interfering RNA (siRNA) constructs (ThermoFisher, catalog AM16708, with ID 151322 and ID 151322) and control constructs respectively. On day 5 of adipocyte differentiation, COX-2 expression was suppressed using RNAi. The medium was changed to fresh differentiation medium and adipocytes were cultured for another 24 hours. The collected media was used for the differentiation of primary preadipocytes isolated from the iWAT of C57BL/6 animals. For treatment using the PGI2 antagonist, the media was collected from raptor KO and control differentiated primary adipocytes and used for the differentiation of primary adipocytes in the presence of 3 µM CAY10441, a PGI2 receptor antagonist. For the overexpression of CRTC2, a plasmid encoding wild-type, or mutant CRTC2 of S136A or S136D, or empty vector was transfected into 3T3-L1 preadipocytes using a NEON transfection system.

**Chromatin Immunoprecipitation (CHIP) assay** 3T3-L1 preadipocytes or CRTC2-overexpressed preadipocytes were treated with or without 5 nM Rapamycin for 10, 30, 60 or 120 min, and then fixed in 1% formaldehyde for 10 min at 37°C. Cells were washed twice with cold PBS containing protease inhibitor cocktail, and subsequently lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.1). Extracts were sonicated until DNA fragments of 200~1000 bp were achieved. Sonicated solution was diluted in ChIP dilution buffer (TE buffer, 1.1% Triton X-100, 167 mM NaCl, 1.1x cocktail). The immuno-complexes were precipitated using an anti-CREB (Millipore) antibody or IgG as the control. Precipitated complexes were reverse cross-linked at 65°C. The amount of precipitated DNA was detected by semi-quantitative PCR using the primers 5’ - CAGAGAGGGGAAAGTTGG - 3’ and 5’ - GAGCAGAGTCTGACTGACTTCTC - 3’.

**Luciferase assay** The luciferase construct of the cox-2 promoter was generated by subcloning Xhol-HindIII fragment (-74 to -727) of the cox-2 promoter into pGL3-basic with the primers 5’-
CCCTCGAGAACCCGGAGGGTAGTTCCAT-3' and 5'-CCAAGCTTGAGCAGA GTCCTGA-CTGACTC-3'.

3T3-L1 preadipocytes were grown in a 24-well plate to 70% confluency. The cox-2 promoter luciferase construct was co-transfected with pSV-β-Galactosidase Control Vector (PROMEGA, Madison, W1 USA) in combination with pcDNA 3.1, pcDNA/CRTC2, pcDNA/CRTC2-S136A, or pcDNA/CRTC2-S136D. 24 hours post transfection, cells were treated with or without 5 nM Rapamycin for 4 hours and then lysed for the measurement of the luciferase with the Luciferase Assay Kit (PROMEGA) and β-galactosidase activities using a Turner TD20/20 luminometer.

Seahorse assays and tissue respiration Primary preadipocytes from brown or beige fat were cultured and differentiated into adipocytes as described previously (Liu et al., 2014). The differentiated adipocytes were then cultured in a plate with XF Assay Media from SeaHorse Bioscience (Billerica, MA) containing 4500 mg/L glucose free of CO2 and incubated at 37°C overnight. SeaHorse XF24 Extracellular Analyzer injection ports were loaded with oligomycin at a final concentration of 1.0 μM for 24 minutes followed by the addition of carbonyl cyanide p-[trifluoromethoxy]-phenyl-hydrazone (FCCP) at a final concentration of 1.25 μM for 24 minutes. Post FCCP measurements, rotenone was added in for another 24 minutes to achieve a 1.0 μM final concentration. Oxygen consumption (OCR) was measured and normalized by the amount of protein in each well.

Analysis of adipose tissue respiration was performed using a Clark electrode (Strathkelvin Instruments) as previous studies described with minor modifications (Cohen et al., 2014; Kir et al., 2014). Tissues (10-15 mg) were minced and placed in respiration buffer (Dulbecco’s phosphate-buffered saline, 2% BSA, 25mM glucose and 1mM pyruvate). For each adipose depot, three separate pieces of tissue of equivalent size were analyzed, and O2 consumption was normalized to tissue weight.

Measurement of cAMP, PGD2, PGE2 and PGI2 The intracellular cAMP level in preadipocytes was determined by cAMP ELISA kit as described previously (Ohyama et al., 2015; Xu et al., 2013). For the measurement of PGD2, PGE2 and PGI2, the sample preparation for PGs release was performed following the previous study (Vegiopoulos et al., 2010). In brief, 3-month old mice were sacrificed and WAT was dissected. Approximately 50 mg pieces of tissue were plated in duplicate in 0.5 ml medium (Hank’s basic plus CaCl2 1.28 mM, MgSO4 1.2 mM, NaHCO3 25 mM, HEPES 15 mM, BSA 0.5 %, D-glucose 5 mM, Penicillin/Streptomycin 1 %) and incubated at 37°C, 5% CO2 for 8 hours. Conditioned media was centrifuged at 5 krpm, for 5 minutes at 4°C and the supernatants were snap-frozen. To measure secreted PGs in primary adipocytes, preadipocytes from iWAT were cultured and differentiated into adipocytes. Differentiated adipocytes were starved for 4 h in 0.4% BSA/DMEM, washed with PBS, and incubated with fresh DMEM containing 1% BSA overnight. Culture media was centrifuged at 10,000 g for 10 min, and the supernatants were collected for the assays of PGs. The levels of PGD2, PGE2 or PGI2 were determined using their enzyme immunoassay kits according to the manufacturer’s protocol (Caymann, Ann Arbor, MI, USA). Values were normalized to wet tissue weight or total protein level in the cells.

Real-time PCR Mouse tissue samples were homogenized and the total RNA was isolated with the RNeasy Lipid Tissue Mini Kit (Qiagen). RNA (1 μg) was reverse-transcribed using cDNA kit (Qiagen). PCR amplification was detected using SYBR Green PCR master mixture on a Roche 480 Real-time PCR system (Roche). Primer sequences are listed in Table 1. Each primer pair amplifies products spanning several exons, distinguishing spliced mRNA from genomic...
DNA contamination. The reaction yielded about 200 bp of PCR products for specified genes and internal control genes, including β-tubulin and β-actin. The relative expression of target genes was normalized to GAPDH.

**Immunoprecipitation** For overexpression of CRTC2, a plasmid encoding wild-type, empty vector or mutant CRTC2 of S136A or S136D was transfected into 3T3-L1 preadipocytes using a NEON transfection system. Cells were treated with or without 5 nM Rapamycin for 30 minutes. Protein A beads (GE Healthcare) were pre-incubated with 1.5 μg IgG or anti-CREB for 2 hours at room temperature, and then washed with WGB buffer before incubation with cell lysate at 4ºC for overnight followed by Western blot as described in our previous study (Liu et al., 2008).

**Western blot** The general procedures were used for Western blot as described in our previous study (Liu et al., 2014). Quantification of protein levels was performed by analyzing Western blots using the Scion Image Alpha 4.0.3.2 program (Scion Corp.). The protein level was normalized for the amount of protein loaded in each experiment and the relative change in protein level was expressed as percentage of control protein levels. The relative level of each control sample was arbitrarily set at 1.0 in the cell and in vivo studies.

**Immunofluorescence staining** Treated preadipocytes in 96 well plates were fixed in 4% paraformaldehyde for 5 min, and then permeabilized with 0.1% saponin in 3% bovine serum albumin (BSA) for 30 min followed by incubation with anti-CRTC2 or CRTC3 for 2 hours and secondary antibodies for 1 hour. Cell Nuclei were stained by DAPI (blue). Confocal images were taken using EVOS FL Cell Imaging System in the Department of Biochemistry and Molecular Biology at UNMHSC.

**Leucine treatment** 3T3-L1 preadipocytes were maintained with DMEM-high glucose (ATCC) containing 10% bovine serum. The medium was changed into DMEM-low glucose without arginine, leucine, and lysine (D9443, Sigma). 4 hours post medium change, starved cells were treated with and without 100 mM leucine at different time points as indicated in the figures.

**Adipocyte size analysis** H&E staining was performed on tissues. Stained tissues were visualized with NanoZoomer Slide Scanner. Five sections per animal were stained. Eight representative images per section were used to determine adipocyte size. Size of adipocytes were determined with ImageJ Analysis of H&E stained tissues (Zhao et al., 2018).

**Supplemental References**


