DEPTOR Cell-Autonomously Promotes Adipogenesis, and Its Expression Is Associated with Obesity

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SUMMARY

DEP domain-containing mTOR-interacting protein (DEPTOR) inhibits the mechanistic target of rapamycin (mTOR), but its in vivo functions are unknown. Previous work indicates that *Deptor* is part of the *Fob3a* quantitative trait locus (QTL) linked to obesity/leanness in mice, with *Deptor* expression being elevated in white adipose tissue (WAT) of obese animals. This relation is unexpected, considering the positive role of mTOR in adipogenesis. Here, we dissected the *Fob3a* QTL and show that *Deptor* is the highest-priority candidate promoting WAT expansion in this model. Consistently, transgenic mice overexpressing DEPTOR accumulate more WAT. Furthermore, in humans, DEPTOR expression in WAT correlates with the degree of obesity. We show that DEPTOR is induced by glucocorticoids during adipogenesis and that its overexpression promotes, while its suppression blocks, adipogenesis. DEPTOR activates the proadipogenic Akt/PKB-PPAR-γ axis by dampening mTORC1-mediated feedback inhibition of insulin signaling. These results establish DEPTOR as a new regulator of adipogenesis.

INTRODUCTION

Obesity, which is defined by excessive white adipose tissue (WAT) accumulation, increases mortality and the risk of developing multiple disorders including insulin resistance, type 2 diabetes, cardiovascular diseases, and cancers (Flegal et al., 2003, 2007; Pischon et al., 2008; Wang et al., 2005). Although changes in lifestyle are responsible for the increase in incidence of obesity over the last decades, heritability studies provide evidence for a substantial genetic contribution to obesity risk (Bouchard et al., 1990; Maes et al., 1997). Because strategies aiming to reduce obesity have shown limited long-term success, an improvement in our understanding of the molecular mechanisms regulating WAT formation is important for the development of new tools to treat obesity and its related diseases.

The mTOR signaling pathway senses growth factors and nutrients to regulate many biological processes involved in the promotion of cell growth (Laplante and Sabatini, 2012). mTOR interacts with several proteins to form two distinct multiprotein complexes named mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). When active, mTORC1 promotes protein synthesis by phosphorylating the eukaryotic initiation factor 4E (eIF4E)-binding protein1 (4E-BP1) and the ribosomal S6 kinase 1 (S6K1), whereas mTORC2 regulates cell survival through the phosporylation of several AGC kinases. It is becoming increasingly clear that, in addition to protein synthesis, mTORC1 also regulates lipid metabolism and adipogenesis (Laplante and Sabatini, 2009). Overactivation of mTORC1 increases adipogenesis in vitro (Zhang et al., 2009), whereas the complete inhibition of mTORC1 by rapamycin or loss of RAPTOR, a key mTORC1 component, blocks this process (Cho et al., 2004; Gagnon et al., 2001; Kim and Chen, 2004; Polak et al., 2008; Yu et al., 2008; Zhang et al., 2009). For these reasons, targeting mTORC1 signaling represents a potential approach for reducing adiposity and improving obesity-related diseases.

Recently, we identified the DEP domain-containing mTOR-interacting protein (DEPTOR, also known as DEPDC6) as a protein that represses mTOR signaling (Peterson et al., 2009). DEPTOR interacts with mTORC1 and mTORC2, and its protein stability is reduced when these complexes are active. We observed that DEPTOR is low in most cancer cells but is surprisingly highly expressed in multiple myeloma (Peterson et al., 2009). In these cells, high DEPTOR expression reduces mTORC1 activity and S6K1-mediated feedback inhibition of phosphoinositide-3 kinase (PI3K), which contributes to the activation of protein kinase B (Akt/PKB) and cell survival.

The role of DEPTOR in the regulation of physiological processes in vivo is unknown. Before the identification of DEPTOR as an mTOR-interacting protein, a section on chromosome 15 that includes the *Deptor* gene was identified as part of the *Fob3a* quantitative trait locus (QTL) that is linked to obesity/leanness in mice (Stylianou et al., 2005). It was reported that *Deptor* expression is elevated in the adipose tissues of the mouse line

genetically prone to obesity. Because the attention was at that time focused on other candidate genes, the potential role of *Deptor* in regulating fat accumulation was not examined.

The possibility that *Deptor* could favor adiposity is counterintuitive, considering the well-established positive role of mTORC1 in regulating adipogenesis and adipose cell maintenance (Cho et al., 2004; Gagnon et al., 2001; Kim and Chen, 2004; Polak et al., 2008; Yu et al., 2008; Zhang et al., 2009). However, because DEPTOR impairs mTORC1 action to a much lower degree than rapamycin or RAPTOR loss, its impact on the regulation of adipogenesis and WAT accumulation may be different. Here, we used several congenic lines for dissecting the *Fob3a* QTL and confirmed that *Deptor* is a high-priority candidate regulating WAT accumulation in the polygenic mouse model of obesity/leanness. To demonstrate the positive role of *Deptor* in regulating adiposity, we generated a doxycycline-inducible transgenic mouse model for *Deptor* overexpression and found that *Deptor* promotes WAT expansion. Consistent with these observations, we noted that DEPTOR expression is elevated in WAT of obese humans and positively correlates with the degree of obesity. DEPTOR is induced during adipogenesis through a glucocorticoid receptor (GR)-dependent mechanism, and its expression facilitates adipocyte differentiation in a cell-autonomous fashion. We observe that DEPTOR positively regulates adipogenesis by promoting the activity of the proadipogenic factors Akt/PKB and peroxisome proliferator-activated receptor-γ (PPAR-γ). These results establish DEPTOR as a new physiological regulator of adipogenesis.

RESULTS

Deptor Is a Strong Candidate Gene for Causing WAT Accumulation in a Murine Polygenic Model of Obesity/Leanness

A polygenic mouse model of obesity was previously developed by divergent selective breeding and resulted in strains differing substantially in fat content (Horvat et al., 2000; Sharp et al., 1984). The mice were selected for high fat (Fat line) or low fat (Lean line) content from a genetically highly variable base population for more than 60 generations (Sharp et al., 1984), resulting in lines that differ in body fat content by more than 5-fold. The *Fob3* QTL is one of several chromosomal regions controlling obesity in the Fat and Lean mouse lines. The *Fob3* region has been dissected into distinct obesity QTLs called *Fob3a*, *Fob3b1*, and *Fob3b2* (Prevorsek et al., 2010; Stylianou et al., 2005). Genetic mapping and microarray studies showed that that *Deptor*, together with many other genes, is part of the *Fob3a* QTL and could be involved in regulating adiposity (Stylianou et al., 2004, 2005).

To examine the contribution of the *Deptor*-containing *Fob3a* segment in the regulation of fat accumulation in the Fat mouse strains, the *Fob3a* interval was genetically dissected by analyzing the phenotypes of several congenic lines (P, V, W, and Z) with overlapping *Fob3a* regions of various lengths (Figure 1A). Congenic lines P and Z carrying the Fat-line-derived *Fob3a-Deptor* region had significantly increased weights of WAT compared to lines V and W, which carry the *Fob3a-Deptor* region from the Lean line (Figure 1B, left panel). These results were confirmed in independent F_2 congenic intercrosses (Figure 1B, middle and

right panels) and were not dependent on changes in food intake (see Figure S1A online). A cross-section analysis of overlapping congenic segments fine mapped the *Fob3a* region down to an ∼15 Mbp region containing the *Deptor* gene (Figure 1C).

To further narrow down the genetic interval likely to contain causal *Fob3a* QTL polymorphism(s), we performed *Fob3a*-interval-specific haplotype analysis that identified genomic blocks which are not identical by descent (non-IBD). Two non-IBD regions within the *Fob3a* segment were identified (Figure 1C). Of the 29 genes included in this region, 22 genes were considered as potential candidates (see the Experimental Procedures). Strikingly, *Deptor* was highly expressed in WAT of the Fat line (8.9 fold, p < 0.001) and in the congenic line Z (8.3 fold, p < 0.001) carrying the *Fob3a-Deptor* segment derived from the Fat line compared to the line V carrying the *Fob3a-Deptor* segment derived from the Lean line (Figure 1D). *Deptor* was similarly regulated in the liver (Figure 1E). Microarray analyses also confirmed the differential regulation of *Deptor* in WAT of an independent F_2 congenic intercross (Table S1). No other candidate gene showed such a consistent differential regulation between the lean and obese lines (Figures S1B–S1D and Table S1). In order to narrow down the list of potential candidate genes, we looked at the function (GO profile), the tissue distribution (BioGPS), and the available transgenic/knockout data for all the candidate genes included in the *Fob3a* region (see the Experimental Procedures). Together with the expression results, these analyses revealed that *Deptor* is the highest priority candidate gene regulating fat accumulation in this model (Figure 1F and Table S2).

Deptor Overexpression Promotes WAT Expansion in Mice

To test the hypothesis that upregulation of *Deptor* plays a causal role in obesity, we generated a doxycycline-inducible DEPTOR transgenic mouse model (Figure 2A). For ease, we refer to this model as iDeptor mice. This transgenic approach was selected because it allowed a modest overexpression of DEPTOR in many tissues, including WAT (Figure 2B, Figure S2A), thus grossly mimicking the differences observed between the Lean and Fat congenic mouse lines. iDeptor mice treated with doxycycline did not show changes in body weight, body weight gain, or tissue weight compared to control mice (Figures S2B and S2C). When fed a high-fat diet containing doxycycline, iDeptor mice gained more weight than controls (Figure 2C). The increase in body weight in iDeptor mice was not caused by changes in food intake or locomotion, but it was associated with an increase in food efficiency (Figures S2D–S2F). The weights of WAT and liver were significantly increased in iDeptor mice (Figure 2D). Histological analyses revealed that, despite the increase in WAT weight, DEPTOR expression did not change average adipocyte size (Figures S2G and S2H), indicating that DEPTOR affected WAT mass at least by increasing the number of fat cells. Consistent with the increase in WAT mass, plasma leptin was increased in iDeptor mice (Figure S2I). Lipid accumulation in the liver, a condition known as hepatosteatosis, was also increased by DEPTOR overexpression (Figures S2J and S2K).

In order to evaluate if DEPTOR modulates the ability of WAT to accumulate lipids, the expression of numerous genes encoding
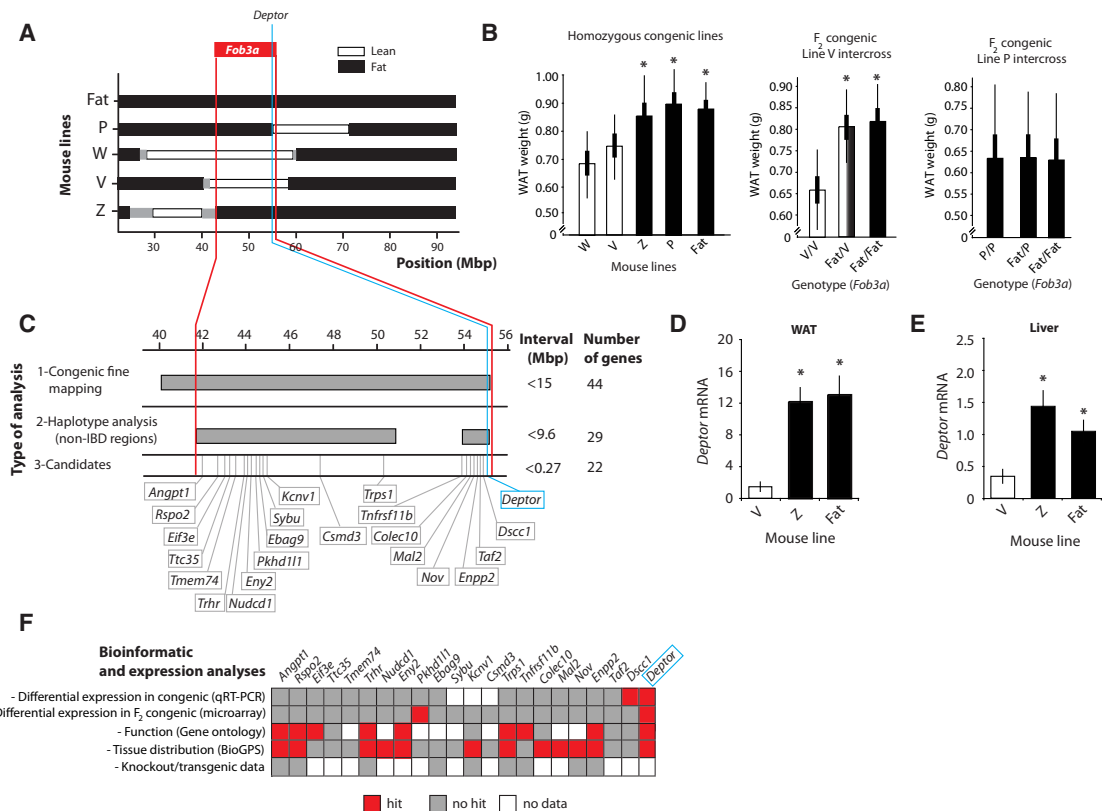
Figure 1. *Deptor* Gene Is Associated with Fat Accumulation in a Polygenic Model of Obesity/Leanness in Mice

(A) Genetic map of *Fob3a* QTL region on chromosome 15 based on congenic mapping analysis. Thick black box indicates the genome segment from the Fat line, the white box indicates the genome segment from the Lean line, and the gray box indicates the region with uncertain origin.

(B) Effect of *Deptor*-containing *Fob3a* QTL region on WAT accumulation in homozygous congenic lines (left) and F_2 congenic intercrosses (middle and right). Data are presented as the mean of WAT mass for each mouse line. Lines with thick and thin vertical lines represent genotype estimates and 50% and 95% confidence intervals. From the left panel, Fat line (n = 75) and lines P (n = 20), V (n = 32), W (n = 27), and Z (n = 37) were phenotyped. From the middle and right panels, the WAT phenotype was re-evaluated in independent F_2 populations. Mice were generated by crossing parental Fat line with congenic V (center panel) or P (right panel) line, intercrossing the F_1 generation and phenotyping the three F_2 genotypes (n = 79 for the line V intercross and n = 45 for the line P intercross). Asterisk denotes significance (p < 0.01) of the difference in WAT between the congenic line and the Fat line as well as the difference between genotypes in F_2 congenic intercrosses.

(C) High-resolution map of the reduced *Fob3a* interval. The position of potential candidates is shown on the map, and corresponding interval size and number of genes are indicated on the right side.

(D and E) *Deptor* mRNA was measured by qRT-PCR in (D) WAT or in the (E) liver of the mouse lines and normalized to *36B4* mRNA levels. Data are expressed as the mean ± SEM for n = 8–12. *p < 0.05.

(F) Analysis of the bioinformatics and expression hits for the candidate genes included in the *Fob3a* interval. Hits were established as described in the Experimental Procedures. The red square indicates a positive/possible role of the gene in the fat phenotype (hit), the gray square indicates no apparent relation with the phenotype (no hit), and the white square indicates that data are not available.

proteins regulating lipogenesis and lipid uptake/retention was measured in WAT of iDeptor mice. DEPTOR overexpression significantly increased the mRNA levels of many of these genes (Figure 2E). Interestingly, most of the genes increased by DEPTOR have been previously identified as targets of the master regulator of adipogenesis, PPAR-γ (Berger and Moller, 2002; Guan et al., 2002; Nishino et al., 2008). These results indicate that DEPTOR promotes WAT expansion at least in part by improving the ability of this tissue to synthesize and accumulate lipids. Importantly, DEPTOR overexpression also induced the transcription of many lipogenic genes in the liver, which likely contributed to promote hepatic triglyceride accumulation and weight gain in iDeptor mice (Figure S2L).

DEPTOR Levels Are Elevated in WAT of Obese Humans

Because DEPTOR overexpression promotes adiposity and the expression of genes facilitating lipid accumulation in mice, we asked if DEPTOR expression might also be associated with obesity in humans. DEPTOR protein levels were measured in WAT of humans with body mass indexes (BMIs) ranging from 22 to 80. Strikingly, DEPTOR protein expression was strongly elevated in obese humans (3.47-fold in BMI >30, p = 0.0001) (Figures 3A and 3B). Additionally, we noted a significant correlation between DEPTOR protein levels and BMI (r = 0.42, p < 0.005). Importantly, no such correlation was observed with the expression of other proteins, such as aP2 or Akt/PKB (Figures S3A and S3B). Together with the results obtained in the mouse
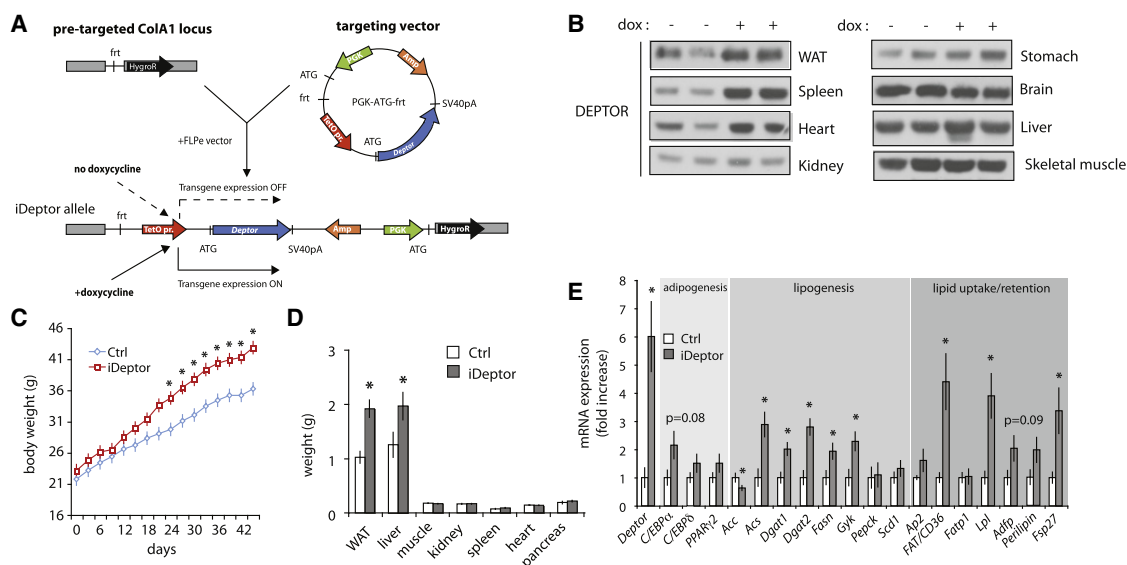
Figure 2. DEPTOR Overexpression Promotes Adiposity in Mice

(A) Schematic representation of the strategy used to produce the iDeptor mouse model.

(B) iDeptor mice were euthanized, and tissues were collected 6 hr after PBS or doxycycline injection (3.3 μg/g body weight). Protein lysates were analyzed by immunoblotting for indicated proteins.

(C and D) (C) Body and (D) tissue weight of control or iDeptor mice fed a high-fat diet supplemented with doxycycline (200 mg/kg food) for 6–7 weeks. Data are expressed as the mean ± SEM for n = 6–8 per condition. *p < 0.05 versus control.

(E) Gene expression analysis in WAT of control and iDeptor mice fed a high-fat diet supplemented with doxycycline (200 mg/kg food) for 6–7 weeks. mRNA expression was measured by qRT-PCR and normalized to *36B4* mRNA levels. Data are expressed as the mean ± SEM for n = 5–8 per condition. *p < 0.05 versus control.

models, these observations support the possibility that DEPTOR could play an important role in regulating WAT expansion in humans.

DEPTOR Is Induced during WAT Development and Adipocyte Differentiation

In order to better understand the biological function of DEPTOR in adipose tissue/adipocytes, we examined if DEPTOR is regulated during fat cell formation in vivo and in vitro. In WAT collected from murine embryos and pups at different stages of development, DEPTOR levels increased significantly during the WAT formation that occurs as mice begin to suckle and accumulate fat (Figure 4A and Figure S4A). Consistent with these results, DEPTOR levels also increased in mouse embryonic fibroblasts (MEFs) and 3T3-L1 cells treated with an adipogenic cocktail that promotes their differentiation into the mature adipocytes (Figures 4B and 4C and Figure S4B). Thus, in vivo and in two well-established in vitro models, DEPTOR expression increases during adipogenesis.

We next took advantage of the in vitro adipogenesis systems to investigate the mechanisms that regulate DEPTOR expression. Each component of the adipogenic cocktail was removed individually, and DEPTOR levels were measured after the induction of adipogenesis. Only the removal of dexamethasone, a synthetic glucocorticoid, blocked the induction of DEPTOR that occurs during differentiation (Figure 4D and Figure S4C). The addition of dexamethasone or corticosterone increased DEPTOR levels even in undifferentiated MEFs (Figures 4E–4G) or 3T3-L1 cells (Figure S4D). Analyses of publicly available tran-

scriptional profiling data showed that glucocorticoids induce *Deptor* mRNA levels in mouse primary chondrocytes in vitro and in the placenta in vivo, indicating that this mechanism of regulation is conserved in various conditions and cell types (Figures S4E and S4F). We observed that inhibition of transcription by actinomycin D or of the GR by RU-486 completely abrogated the dexamethasone-induced increases in *Deptor* mRNA and DEPTOR protein (Figure 4H). These results are consistent with glucocorticoids directly controlling *Deptor* expression through a transcriptional mechanism that depends on the GR. Bioinformatics analyses revealed the presence of a conserved glucocorticoid response element (GRE) in mouse and human *Deptor* promoter (Figure 4I). In order to demonstrate a physical interaction between the GR and the *Deptor* promoter, we treated MEFs with dexamethasone and performed chromatin immunoprecipitations (ChIPs) using an antibody against the GR. Using this approach, we observed that dexamethasone promotes GR binding to the *Deptor* promoter (Figure 4J). Importantly, binding of GR to the *Deptor* promoter was enriched when the PCR was performed close to the potential GRE, which supports the possibility that this specific site may play a role in the control of *Deptor* expression by glucocorticoids.

An important question arising from this last set of results relates to the temporal regulation of DEPTOR by glucocorticoids during adipogenesis. If glucocorticoid signaling is an important regulator of DEPTOR expression during adipogenesis, why is the increase in DEPTOR levels relatively weak during the first 2 days of differentiation (Figure 4B)? Because in undifferentiated MEFs dexamethasone induced DEPTOR expression within 5 hr
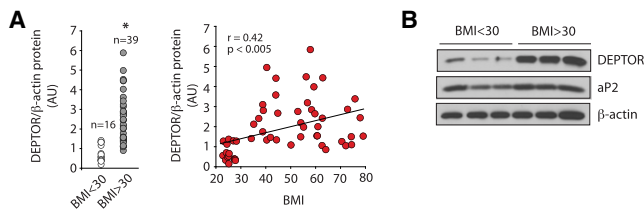
Figure 3. DEPTOR Expression Is Elevated in WAT of Obese Humans
(A) Impact of BMI on DEPTOR protein expression in WAT. Protein lysates were prepared from subcutaneous WAT isolated from lean and obese humans. Lysates were then analyzed by immunoblotting for DEPTOR and β-ACTIN. DEPTOR protein levels were quantified and normalized to β-ACTIN levels. n = 16 (BMI < 30) and n = 39 (BMI >30), *p < 0.05 versus BMI < 30.
(B) Representative samples from the western blots are shown.

of addition (Figures 4E and 4F), we hypothesized that a component of the adipogenic cocktail might counteract the positive effects of dexamethasone on DEPTOR expression at early time points. Indeed, we observed that IBMX dose-dependently blocked the stimulatory effect of dexamethasone on DEPTOR protein and *Deptor* mRNA levels (Figure S4G). Consistent with this observation, IBMX removal from the adipogenic cocktail promoted *Deptor* mRNA expression (Figure S4C). As a nonselective phosphodiesterase inhibitor, IBMX is thought to exert some of its effects by raising intracellular cAMP levels. Treating cells with various doses of the cAMP analog dibutyryl-cAMP or with the adenylate cyclase activator forskolin did not reduce *Deptor* expression in response to dexamethasone (data not shown), indicating that IBMX competes with dexamethasone for the control of *Deptor* expression by a mechanism that does not depend on variation in cAMP levels.

DEPTOR Promotes Adipogenesis in a Cell-Autonomous Fashion
To better understand the role of DEPTOR in adipogenesis, MEFs isolated from iDeptor mice were treated or not with doxycycline and were differentiated into mature adipocytes. Consistent with the in vivo findings, DEPTOR overexpression significantly increased triglyceride accumulation in MEFs (Figure 5A and Figure S5A). Moreover, we noted a dose-dependent effect of DEPTOR on the promotion of adipogenesis in this model (Figure S5B). Importantly, doxycycline per se had no effect on adipocyte differentiation in wild-type MEFs (Figure S5C). Similar to what was observed in WAT in vivo, DEPTOR overexpression induced the expression of many genes regulated by PPAR-γ (Figures 5B and 5C and Figures S5D–S5F). In order to determine which step in the adipogenic program is affected by DEPTOR overexpression (commitment versus terminal differentiation), DEPTOR was expressed in iDeptor MEFs during the early (day −2 to day +2) or the late (day +4 to day +12) stage of adipogenesis. Both treatments led to a significant increase in triglyceride accumulation, indicating that DEPTOR expression triggers fat cell development by promoting preadipocyte commitment as well as lipid synthesis in committed cells (Figures 5D and 5E).

We next determined if DEPTOR expression is necessary for adipocyte differentiation in vitro. Using 3T3-L1 cells, we observed that suppression of DEPTOR expression by short-hairpin RNA (shRNA) significantly reduced adipogenesis (Figure 5F and

Figure S5G). This was associated with a reduction in PPAR-γ protein levels and mRNA expression of genes controlled by PPAR-γ (Figures 5G–5I and Figure S5H). Together, these results indicate that DEPTOR controls adipogenesis in a cell-autonomous fashion by modulating the expression of PPAR-γ and its target genes.

DEPTOR Promotes Adipogenesis by Activating Akt/PKB and PPAR-γ
To better understand the mechanism by which DEPTOR promotes adipogenesis, we examined the effect of DEPTOR expression or depletion on mTOR signaling. Overexpression of DEPTOR in MEFs reduced the phosphorylation of insulin receptor substrate 1 (IRS1) at S636/639, a site directly targeted by mTORC1 that favors IRS1 degradation (Tzatsos, 2009; Tzatsos and Kandror, 2006), and promoted the phosphorylation/activation of Akt/PKB (Figure 6A). We also observed that dexamethasone and corticosterone, which both promote DEPTOR expression, also induced Akt/PKB phosphorylation in MEFs (Figures S6A and S6B). Moreover, the induction of DEPTOR expression during adipogenesis was associated with an increase in Akt/PKB activation (Figures S6C and S6D). Such an elevation in Akt/PKB phosphorylation was also observed in adipose tissue of mice overexpressing DEPTOR (Figure S6E). Conversely, suppression of DEPTOR expression in 3T3-L1 cells increased IRS1 phosphorylation at S636/639, reduced IRS1 levels, and blocked Akt/PKB activation (Figure 6B). DEPTOR depletion also reduced Akt/PKB-mediated Forkhead box O1/3a (FoxO1/3a) phosphorylation and nuclear exclusion (Figure S6F). Importantly, Akt/PKB activation was impaired in DEPTOR-depleted 3T3-L1 cells treated with insulin-like growth factor-1 (IGF-1), a key molecule promoting preadipocyte differentiation (Figure S6G) (Smith et al., 1988). We also noted a significant reduction in Akt/PKB activation over the differentiation process in DEPTOR-depleted 3T3-L1 cells (Figure S6H).

It was reported that the complete inhibition of mTORC1 blocks WAT accumulation in vivo and adipogenesis in vitro (Cho et al., 2004; Gagnon et al., 2001; Kim and Chen, 2004; Polak et al., 2008; Yu et al., 2008; Zhang et al., 2009). Because DEPTOR inhibits mTORC1 (Peterson et al., 2009), it might be expected that DEPTOR expression would repress lipid accumulation in adipocytes. Importantly, although DEPTOR had a significant impact on the phosphorylation of IRS1 S636/639, we noted that the activation state of other classic downstream effectors of mTORC1 (S6K1, S6, or 4E-BP1) was not severely affected by the overexpression/knockdown of DEPTOR (Figures 6A and 6B). This indicates that DEPTOR does not impair mTORC1 activity to the same extent as rapamycin or RAPTOR loss, which probably explains why DEPTOR does not similarly affect adipogenesis.

Adipogenesis is a cellular process that requires the activation of Akt/PKB (Kohn et al., 1996; Magun et al., 1996; Menghini et al., 2005; Nakae et al., 2003; Peng et al., 2003). Akt/PKB induces glucose uptake, its metabolism, and its incorporation into lipids and promotes the activation of PPAR-γ through various mechanisms (Rosen and MacDougald, 2006). Because DEPTOR positively regulates Akt/PKB and the expression of many genes downstream of PPAR-γ, we tested the possibility that DEPTOR could affect adipogenesis by modulating the Akt/PKB-PPAR-γ

axis. Consistent with the role of Akt/PKB in regulating glucose uptake and lipogenesis, we observed that modulation in Akt/PKB activation by DEPTOR was associated with significant changes in glucose uptake and incorporation into lipids (Figure 6C and Figure S6I). Importantly, we also observed that expression of a constitutively active Akt/PKB corrected the adipogenic defect observed when DEPTOR was depleted, indicating that DEPTOR affects adipogenesis upstream of Akt/PKB (Figure 6D). Because Akt/PKB is a strong activator of PPAR-γ, we also looked at the impact of DEPTOR on the activity of this transcription factor. DEPTOR depletion in 3T3-L1 cells was associated with a significant reduction in the transactivation capability of PPAR-γ (Figure 6E). Interestingly, the inclusion of the potent PPAR-γ agonist rosiglitazone corrected adipogenesis and PPAR-γ expression in cells with suppressed DEPTOR levels (Figure 6F and Figure S6J). Together, these results indicate that DEPTOR controls adipogenesis by regulating the activation of the proadipogenic factors Akt/PKB and PPAR-γ. We propose a model in which the induction of DEPTOR during adipogenesis functions to reduce the feedback loop from mTORC1 to IRS1, which facilitates adipogenesis by upregulating the action of Akt/PKB and PPAR-γ.

DISCUSSION

Obesity increases mortality and the risk of developing multiple disorders, including insulin resistance, type 2 diabetes, cardiovascular diseases, and cancers (Flegal et al., 2003, 2007; Pischon et al., 2008; Wang et al., 2005). Here, we provide evidence that DEPTOR plays a role in regulating fat accumulation in vivo and in vitro. Observations made in two independent mouse models, in humans, and in cultured cells all indicate that DEPTOR expression positively associates with adiposity. These results establish DEPTOR as a new physiological regulator of adipogenesis.

The Fat and Lean mouse lines were previously selected based on their fat content from a genetically highly variable base population for more than 60 generations (Sharp et al., 1984), resulting in lines that differ in body fat content by more than 5-fold. The low-resolution mapping initially detected the obesity QTL Fob3 in a region on chromosome 15 (Horvat et al., 2000). Follow-up crosses of congenic line containing a large segment of chromosome 15 from the Lean line on otherwise Fat line background provided a medium-resolution genetic map and revealed that Deptor is located within the QTL (Stylianou et al., 2005). We provide a detailed fine genetic mapping with congenic strains carrying overlapping shorter Fob3a donor genomic segments that further narrowed down Fob3a candidate interval to below 15 Mbp. Expression and bioinformatics analyses confirmed that Deptor is a high-priority candidate gene for fat accumulation in this unique model of the common polygenic form of obesity.

Using a doxycycline-inducible mouse model, we observed that overexpression of Deptor promotes adiposity in vivo, thus confirming the analyses made in the congenic mouse lines. Interestingly, unlike the Fat-line-Deptor congenic mice that gain significantly more weight than the lines carrying Lean-line Deptor allele on a regular diet, iDeptor mice need a higher lipid flux to reproduce this phenotype. The exact reason for this phenomenon is unknown but may be related to differences in DEPTOR expression profile (duration and expression levels) or differences in the genetic background of these models. Nevertheless, these results indicate that DEPTOR overexpression promotes WAT expansion in mice.

In iDeptor mice, increased DEPTOR expression promotes the transcription of many genes regulated by PPAR-γ that favors lipid synthesis and uptake/esterification in WAT. Consistent with these results, we observed that DEPTOR modulates PPAR-γ activity and the expression of many lipogenic genes in vitro. The fact that DEPTOR cell-autonomously promotes adipogenesis in cultured cells strongly suggests that DEPTOR may play a direct role in promoting WAT expansion. In vitro, we noticed that DEPTOR expression triggers fat cell development by promoting preadipocyte commitment as well as lipid synthesis in committed cells. The increase in preadipocyte differentiation coupled to the increase in lipid synthesis in already committed cells may explain why we did not observe a major change in average adipocyte size in WAT of iDeptor mice.

The fact that DEPTOR expression is induced during adipocyte differentiation raises the possibility that elevated DEPTOR levels observed in WAT of the fat congenic mouse lines and in obese humans could be a consequence rather than a cause of the increased in adipogenesis. Although we cannot rule out the contribution of adipogenesis per se to the increase in DEPTOR levels in WAT of obese, a few facts indicate that adipogenesis may not be the only factor contributing to promote DEPTOR expression in obese. In the congenic mouse lines, elevated DEPTOR expression was observed not only in WAT but also in the liver of the obese congenic lines, indicating that high DEPTOR expression in WAT is unlikely to be a consequence of increased adipogenesis. Supporting this observation, preliminary analysis of the Deptor promoter revealed the presence of many polymorphisms between the Fat and the Lean lines that could contribute to the variation in Deptor expression levels between these lines (data not shown). Finally, in WAT of obese humans, we observed that the expression of the classic adipogenic marker aP2 was increased to a much lower extent than DEPTOR, suggesting that the process of adipogenesis is unlikely to be the only factor driving DEPTOR expression.

What promotes DEPTOR expression in WAT of humans is an interesting question. To our knowledge, the Deptor locus has not been linked to human obesity in any genome-wide association studies published so far. Although we do not exclude the possibility that polymorphisms in DEPTOR locus or in other elements regulating DEPTOR expression/stability may be found in obese humans, the striking elevation in DEPTOR levels observed here among a population of unrelated individuals suggests that a common mechanism taking place in WAT of obese could promote DEPTOR expression. Interestingly, we show that DEPTOR is highly induced by glucocorticoids, steroid hormones that are secreted by the adrenal cortex in response to stress (Morton, 2010). Excess of glucocorticoids, as seen in Cushing's syndrome or in humans chronically treated with exogenous glucocorticoids, causes many adverse effects, including obesity (Stanbury and Graham, 1998). Many reports indicate that the local conversion/activation of glucocorticoids is increased in WAT of obese humans (Morton, 2010). In this context, it is tempting to speculate that the increase in the local
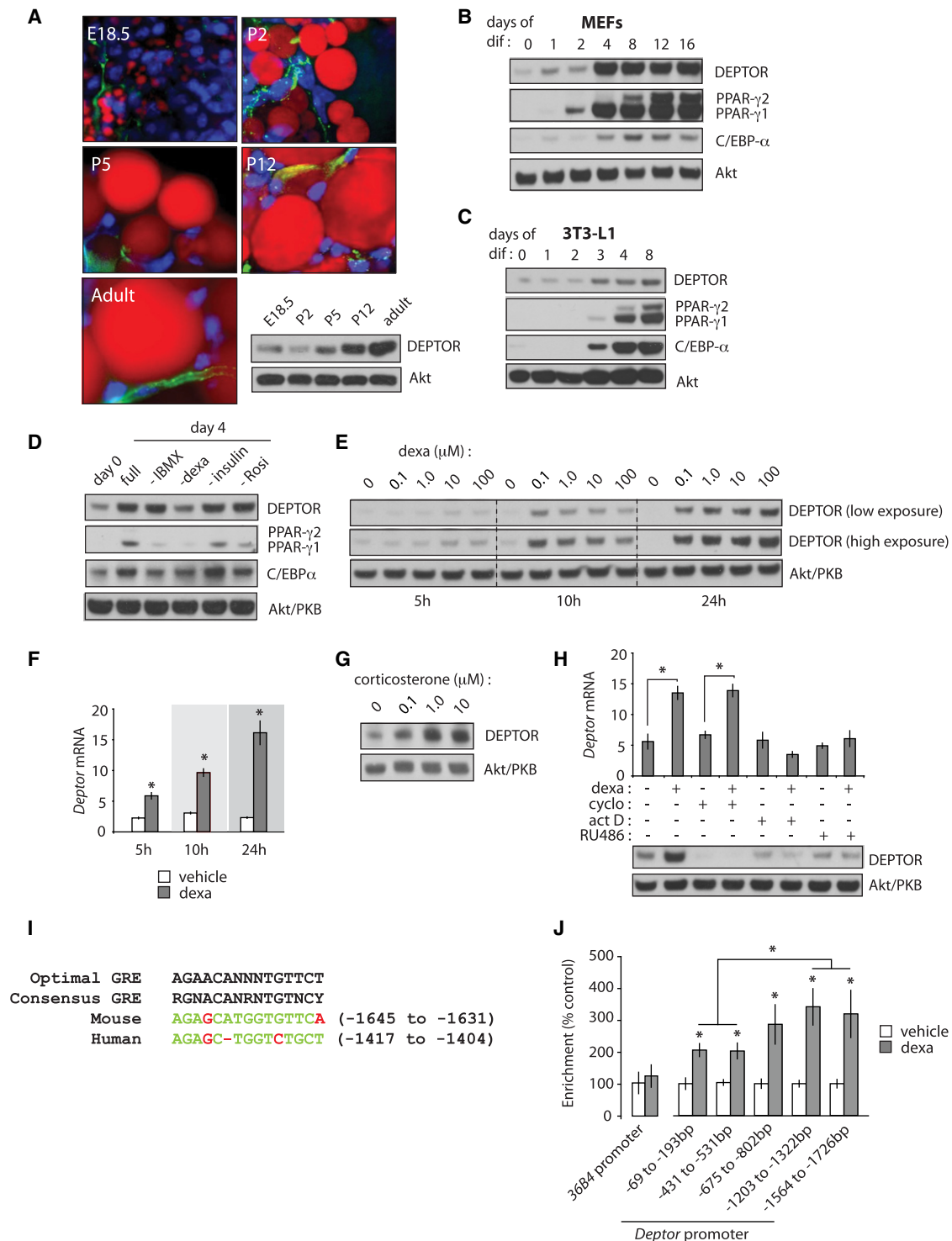
Figure 4. DEPTOR Expression Is Induced during Adipogenesis through a Transcriptional Mechanism Dependent on the Glucocorticoid Receptor

(A) Subcutaneous adipose tissue from mouse embryos and pups at various stages of development were collected and stained with lipidTOX deep (neutral lipids/red), isolectin GS IB$_4$ (endothelial cells/green), and Hoechst (nucleus/blue) and then analyzed by microscopy. Representative pictures are shown for each developmental stage. Protein lysates were prepared from tissues and analyzed by immunoblotting for indicated proteins.

(B and C) (B) MEFs or (C) 3T3-L1 were grown to confluence, and differentiation was induced in postconfluent cells (2 days) in the presence of the adipogenic cocktail (insulin, IBMX, dexamethasone [and rosiglitazone in the case of MEFs] [see the Experimental Procedures]) for 2 days. The medium was replaced by a medium containing serum and insulin for the rest of the differentiation process. Protein lysates were analyzed by immunoblotting for indicated proteins.

conversion of glucocorticoids in WAT of obese humans may contribute to increase DEPTOR expression, which in turn could facilitate WAT expansion by promoting its ability to store lipids. The increase in fat accumulation observed in response to DEP-TOR overexpression in the iDeptor mouse and in iDeptor MEFs in vitro supports this possibility.

The positive role of DEPTOR in regulating adipogenesis, lipogenesis, and fat accumulation is counterintuitive, considering that DEPTOR was reported to inhibit mTORC1 (Peterson et al., 2009), a protein complex known to promote adipogenesis and adipose cell maintenance (Cho et al., 2004; Gagnon et al., 2001; Kim and Chen, 2004; Polak et al., 2008; Yu et al., 2008; Zhang et al., 2009). We showed in the first DEPTOR report that this protein inhibits S6K1 activation, which reduces the negative feedback loop on IRS1/PI3K and promotes Akt/PKB activity (Peterson et al., 2009). Here, we confirm that DEPTOR promotes Akt/PKB action but, unexpectedly, found that this was not associated with severe inhibition of the classical downstream effectors of mTORC1 (S6K1/S6, 4E-BP1), which have been implicated in the regulation of adipogenesis downstream of mTORC1 (Carnevalli et al., 2010; Le Bacquer et al., 2007). Instead, we observed that DEPTOR reduces the phosphorylation of IRS1 on S636/639, a site directly targeted by mTORC1 that promotes IRS1 degradation (Tzatsos, 2009; Tzatsos and Kandror, 2006). These results indicate that DEPTOR selectively relieves the negative feedback loop on IRS1, which promotes the action of the proadipogenic Akt/PKB, while preserving the function of mTORC1 toward other substrates. From that perspective, it is clear that DEPTOR does not block mTORC1 to nearly the same degree as rapamycin or RAPTOR loss, which have both been shown to block adipogenesis. The incomplete inhibition of mTORC1 action probably explains why DEPTOR does not block adipogenesis. How DEPTOR selectively regulates IRS1 without impairing S6K1/S6 or 4E-BP1 is unclear. It is possible that chronic modulation of DEPTOR expression could rewire the mTOR signaling pathway by modulating the relation between mTORC1 and the numerous feedback loops (Laplante and Sabatini, 2012). Such reorganization in the pathway could lead to a new signaling equilibrium in which the elevation in Akt/PKB could reactivate the action of mTORC1 toward some substrates.

To dissect the respective contribution of mTORC1 and Akt/PKB in the control of adipogenesis, Zhang et al. used *Tuberous sclerosis 2* (*Tsc2*) null MEFs (Zhang et al., 2009). Loss of TSC2 activates mTORC1 and, through mTORC1-dependent feedback mechanism, completely inhibits Akt/PKB. Using this model, Zhang et al. showed that adipogenesis is enhanced when mTORC1 is constitutively activated. These results differ from our findings by suggesting that adipogenesis depends on high mTORC1 activation and that the mTORC1-dependent negative feedback loop on PI3K-Akt/PKB axis is not playing a significant inhibitory role on this process. Importantly, the supraphysiological activation of mTORC1 creates a signaling context that might override regulatory processes that normally take place during adipogenesis. For instance, basal PPAR-γ expression is induced by more than 30-fold in *Tsc2* null cells (Zhang et al., 2009). Overexpression of PPAR-γ is sufficient to induce adipogenesis and can partially correct the adipogenic defect caused by the loss of Akt/PKB (Peng et al., 2003; Tontonoz et al., 1994; Yun et al., 2009). The difference in PPAR-γ expression levels likely account for the different results observed between the present study and the one from Zhang et al.

DEPTOR overexpression increased glucose uptake, lipogenesis, and PPAR-γ activation, which all represent key Akt/PKB-controlled processes contributing to adipogenesis. Interestingly, examples of proteins affecting adipogenesis through the modulation of Akt/PKB and PPAR-γ exist in the literature. The Tribbles homolog 2 (TRB2) and TRB3 are pseudokinases acting as dominant-negative regulators of several kinases, including Akt/PKB (Du et al., 2003). TRB2/3 expression is reduced during adipogenesis, and their overexpression blocks Akt/PKB and PPAR-γ activation and adipogenesis (Bezy et al., 2007; Naiki et al., 2007; Takahashi et al., 2008). The coordinated increase in DEPTOR levels and the decrease in TRB2/3 during adipogenesis indicate that preadipocytes trigger various signaling events to insure high activation of proadipogenic signals.

In conclusion, we show that DEPTOR cell-autonomously promotes adipogenesis and that elevated expression of DEPTOR associates with obesity in mice and humans. These findings improve our understanding of the molecular mechanisms regulating WAT formation and may ultimately contribute to the development of new tools to treat obesity and its related diseases.

EXPERIMENTAL PROCEDURES

Antibodies and Cell Lines

Antibodies were obtained from the following sources: antibody to DEPTOR (09-463) from Upstate/Millipore; antibodies to phospho-S473 Akt/PKB(4058), phospho-T308-Akt/PKB (2965), Akt/PKB (4691), ap2 (2120), C/EBP-α (2295),

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(D) MEFs were differentiated for 4 days as described, but components of the adipogenic cocktail were removed as indicated (from day 0 to day 2). Protein lysates were analyzed by immunoblotting for indicated proteins.

(E and F) MEFs were seeded at equal density and grown in the presence of various doses of dexamethasone for the indicated time, (E) protein lysates were analyzed by immunoblotting for indicated proteins, and (F) *Deptor* mRNA was measured by qRT-PCR and normalized to *36B4* mRNA levels. Data are expressed as the mean ± SEM for n = 4 per condition. *p < 0.05 versus control.

(G) MEFs were seeded at equal density and grown in the presence of various doses of corticosterone for 24 hr. Protein lysates were analyzed by immunoblotting for indicated proteins.

(H) MEFs were treated with dexamethasone (0.1 μM), cycloheximide (10 μg/ml), actinomycin D (2.5 μg/ml), or RU486 (10 μM) for 10 hr. DEPTOR protein and *Deptor* RNA expression were quantified and analyzed as described in (E) and (F).

(I) Promoter sequences were analyzed for the presence of glucocorticoid response elements (GREs). Letters in green refer to conserved nucleotides (compared to the optimal and the consensus GRE). Letters in red refer to nucleotides that are not conserved. N, any nucleotide; R, purine; Y, pyrimidine.

(J) MEFs were treated with dexamethasone (1 μM) or vehicle for 16 hr. ChIP assays were performed using an antibody raised against the glucocorticoid receptor or IgGs (see the Supplemental Experimental Procedures). qPCR was performed on various sections of *Deptor* promoter and in the promoter of a control gene (*36B4*). Data are expressed as the mean of two experiments ± SEM (n = 3–6 per condition). *p < 0.05 versus control.
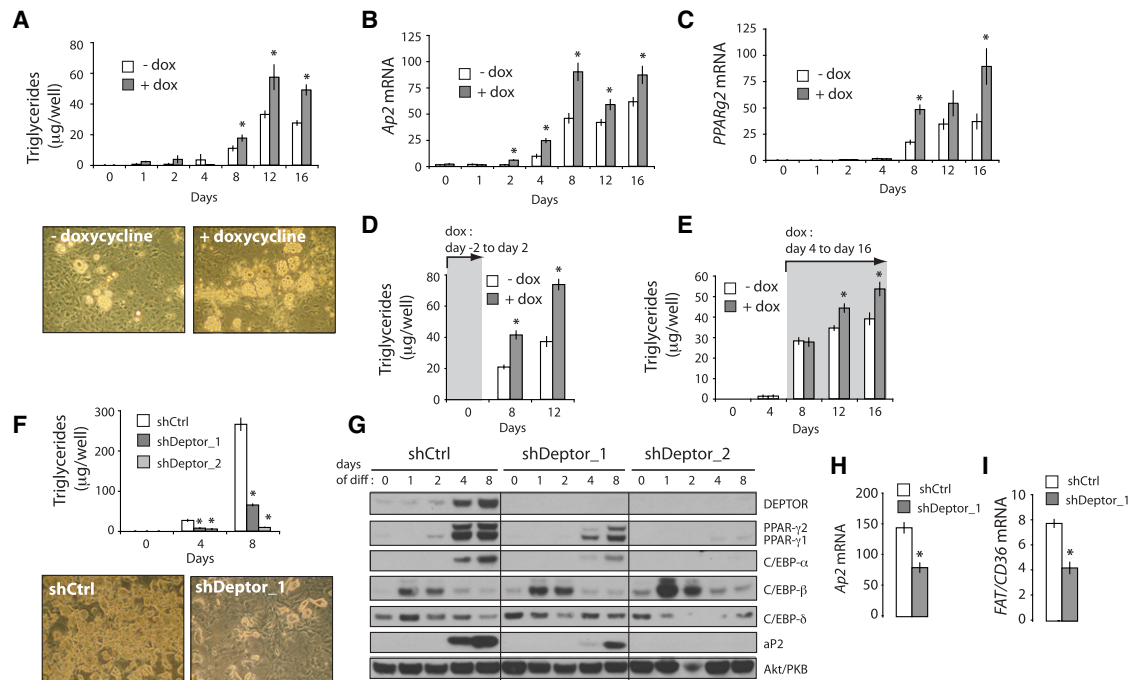
Figure 5. DEPTOR Regulates Adipogenesis in a Cell-Autonomous Fashion

(A–C) MEFs isolated from iDeptor mice were plated and grown to confluence. When confluent (day −2), doxycycline (100 ng/ml) was added or not to the cells until the end of the experiment. Two days after confluence (day 0), differentiation was induced using insulin, IBMX, dexamethasone, and rosiglitazone (see the Experimental Procedures) for 2 days. The medium was replaced by a medium containing serum and insulin for the rest of the differentiation process. (A) Triglycerides were extracted over the course of differentiation and then quantified. Data are expressed as mean ± SEM for n = 2–3 per condition. The experiment was repeated using another iDeptor MEF line. *p < 0.05 versus control. A representative picture of MEFs 12 days after the initiation of differentiation is shown. (B and C) mRNA expression of adipogenic genes was measured by qRT-PCR and normalized to *36B4* mRNA levels. Data are expressed as the mean ± SEM for n = 3–6 per condition. *p < 0.05 versus control.

(D) DEPTOR induces the commitment of preadipocyte to adipocyte. iDeptor MEFs were grown to confluence, and adipocyte differentiation was induced as described in (A). iDeptor MEFs were treated or not with doxycycline (100 ng/ml) from day −2 to day 2 of the adipogenic protocol. Cells were then washed, and the differentiation was completed without doxycycline. Triglycerides were extracted over the course of differentiation and then quantified. Data are expressed as mean ± SEM for n = 5–6 per condition. *p < 0.05 versus control.

(E) DEPTOR induces lipogenesis in already committed cells. iDeptor MEFs were grown to confluence, and adipocyte differentiation was induced exactly as described in (A). Differentiating cells were treated or not with doxycycline (100 ng/ml) from day 4 to day 16 of the adipogenic protocol, and triglycerides were extracted. Data are expressed as the mean of three independent experiments ± SEM for n = 5–6 per condition. *p < 0.05 versus control.

(F–I) 3T3-L1 cells were infected with control or *Deptor* shRNA lentivirus. After selection with puromycine, cells were differentiated using the classical adipogenic protocol. (F) Triglycerides were extracted over the course of differentiation and then quantified. Data are expressed as the mean ± SEM for n = 6 per condition. *p < 0.05 versus control. A representative picture of 3T3-L1 cells 8 days after the initiation of differentiation. (G) Protein lysates were prepared at the indicated days and analyzed by immunoblotting for indicated proteins. (H and I) mRNA expression of adipogenic genes was measured 4 days after the initiation of adipogenesis by qRT-PCR and normalized to *36B4* mRNA levels. Data are expressed as the mean of two independent experiments ± SEM for n = 4 per condition. *p < 0.05 versus control.

C/EBP-δ (2318), PPAR-γ (2443), mTOR (2983), Rictor (2140), Raptor (2280), phospho-S636/639-IRS1 (2388), IRS1 (2382), 4E-BP1 (9452), phospho-T37/46-4E-BP1 (2855), S6K1 (9202), phospho-T389 S6K1 (9205), S6 (2212), phospho-S240/24-S6 (2215), NDRG1 (5196), phospho-T346-NDRG1 (3317), phospho-PKCαβ-T636/641 (9375), FoxO1 (9462), phospho-T24 FoxO1/3a (9464), and PTEN (9559), from Cell Signaling Technology; antibodies to C/EBP-β (sc-150), Actin (sc-1616), PKCα (SC-208), GR (SC-1004), IgGs (sc-2027), and Lamin A/C (sc-6215) from Santa Cruz Biotechnology. Secondary antibodies were all purchased from Santa Cruz Biotechnology. 3T3-L1 cells were purchased from ATCC and cultured as indicated. Primary mouse fibroblasts were established from E13.5 embryos.

Differentiation of MEFs and 3T3-L1 into Adipocytes

For MEF differentiation, at 2 days postconfluence (day 0), cells were treated with the adipogenic cocktail containing containing DMEM, FBS (10%), insulin (830 nM), dexamethasone (1 μM), 3-isobutyl-1-methylxanthine (IBMX, 0.5 mM), and rosiglitazone (5 μM). Two days later, cells were switched to main-

tenance medium containing DMEM, 10% FBS, and 830 nM insulin for the remaining duration of differentiation. The maintenance medium was changed every 48 hr. 3T3-L1 cells were differentiated the same way, but rosiglitazone was not included in the adipogenic cocktail.

Analysis of Adipose Tissue Development In Vivo

C57BL6/J females mice were mated with C57BL6/J males, and plugs were checked to identify potential pregnant mice. Embryos were collected 18.5 days postimpregantion (E18.5), and pups were sacrificed 2, 5, and 12 days after birth. Subcutaneous adipose tissue was collected from the inguinal region using microdissection tools, and samples were frozen in liquid nitrogen for later analysis (western blotting and qRT-PCR) or directly used for imaging. Small pieces of tissue were incubated in PBS containing Hoechst (1:1000) (33342, Invitrogen), HCS LipidTOX (1:2000) (Invitrogen, H34476), and isolectin GS-IB4M (1:5000) (I21411, Invitrogen) for 20 min. Tissues were then washed twice with PBS, mounted, and analyzed by confocal microscopy.
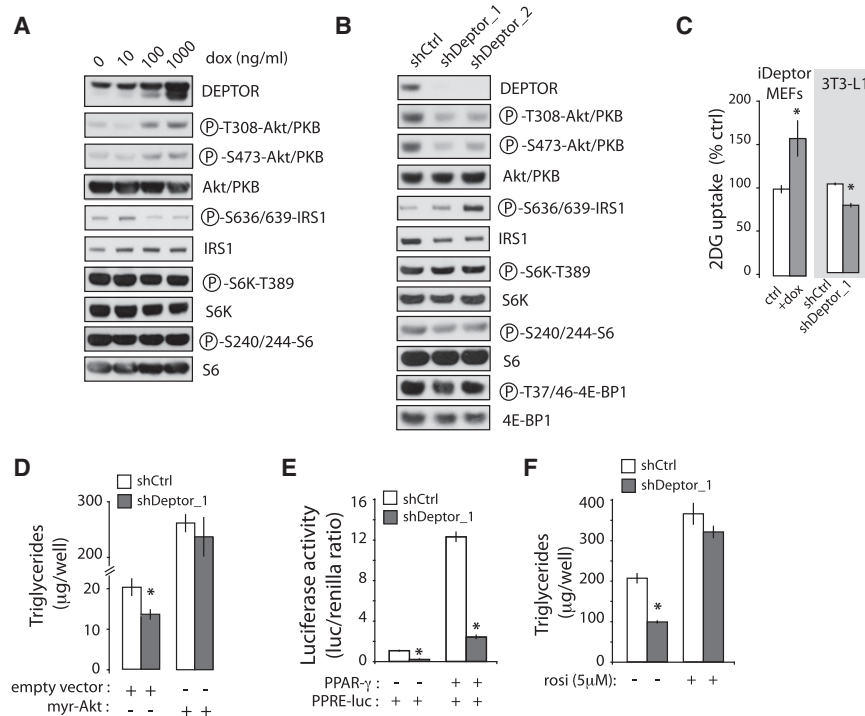
Figure 6. DEPTOR Modulates Adipogenesis by Regulating Akt/PKB and PPAR-γ Activation

(A) MEFs isolated from iDeptor mice were plated and grown to confluence. When confluent (day −2), cells were treated with the indicated doses of doxycycline and differentiated using media supplemented with insulin, IBMX, dexamethasone, and rosiglitazone (day 0 to day 2) and with media supplemented with insulin only (day 2 to day 8). Protein lysates were prepared as described above and analyzed by immunoblotting for indicated proteins.

(B) Control and DEPTOR knockdown 3T3-L1 produced as described in Figure 5F were seeded at equal density, grown to confluence, and lysed. Protein lysates were analyzed by immunoblotting for indicated proteins.

(C) Confluent iDeptor MEFs were treated or not with doxycycline (100 ng/ml) for 2 days. Confluent shCtrl or shDeptor_1 3T3-L1 cells were plated and used when confluent. Cells were treated with insulin (MEFs) or 10% serum (3T3-L1) and incubated in the presence of ^{3}H-2 deoxy-D-glucose (2DG). Cells were then lysed, and radioactivity was measured and corrected to protein content. Results are expressed as the mean ± SEM for n = 4–6 per condition. *p < 0.05 versus control.

(D) Control and DEPTOR knockdown 3T3-L1 cells were infected with pBABE-MyR-Akt or pBABE-empty lentiviruses and were differentiated for 7 days. Results are expressed as the mean ± SEM for n = 6 per condition. *p < 0.05 versus control.

(E) Luciferase assay measuring the transactivation potential of PPAR-γ toward a reporter containing several peroxisome proliferators response elements (PPRE) transfected in 3T3-L1 cells with suppressed expression of *Deptor* by shRNA. Data are expressed as mean ± SEM for n = 6–8 per condition. This experiment was repeated four times. *p < 0.05 versus control.

(F) Control and DEPTOR knockdown 3T3-L1 cells were differentiated with or without rosiglitazone (5 μM) for 8 days, and triglycerides were extracted and quantified. Data are expressed as mean ± SEM for n = 3–5 per condition. *p < 0.05 versus control.

Statistical Analysis

The mean values for biochemical data from each group were compared by Student's t test where p < 0.05 was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, two tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2012.07.008.

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