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Supplemental Information

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

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INVENTORY OF SUPPLEMENTAL INFORMATION

Figure S1. Food intake and mRNA expression levels of the candidate genes in the congenic mouse lines. Relate to Figure 1.

Figure S2. Metabolic parameters of iDeptor mice. Relates to Figure 2.

Figure S3. Analysis of subcutaneous fat in humans. Relates to Figure 3.

Figure S4. Regulation of *Deptor* during adipogenesis *in vivo* and *in vitro.* Relates to Figure 4.

Figure S5. Impact of DEPTOR overexpression or knockdown *in vitro*. Relates to Figure 5.

Figure S6. DEPTOR positively regulates Akt/PKB activation and lipogenesis. Relates to Figure 6.

Table S1. Expression profile from the genome-wide Affymetrix array of the candidate genes in WAT of independent F2 congenic line cross.

Table S2. Bioinformatics analyses of positional candidate genes mapping to *Deptor*-containing *Fob3a* QTL region.

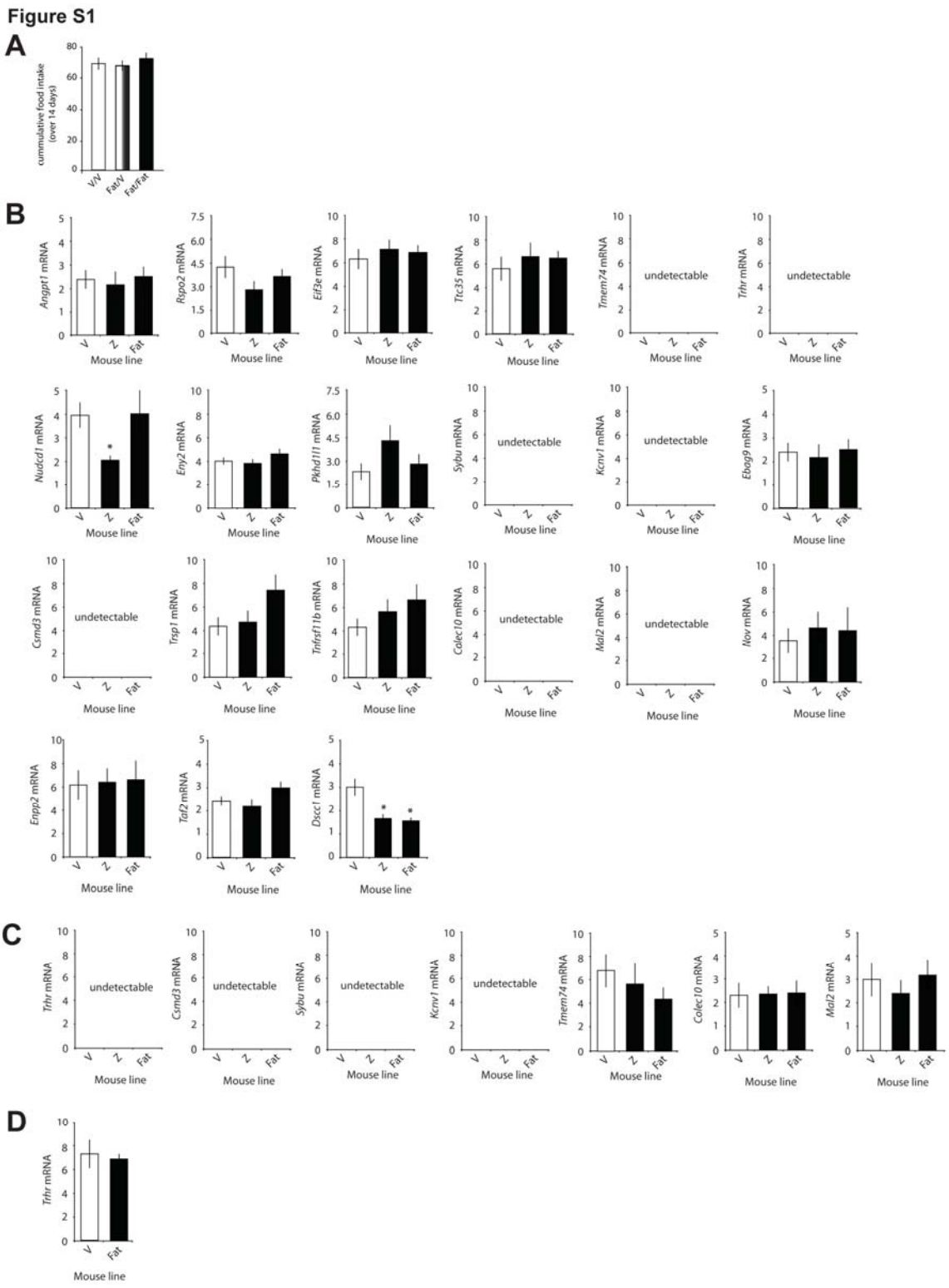


Figure S1. Food intake and mRNA expression levels of the candidate genes in the congenic mouse lines.

(A) Food intake measured over a period of 14 days in F_2 congenic intercrosses (Lines V/V, VF, FF). Data are expressed as the mean ± SEM for n=11-21 per condition.

(B) Expression of candidate genes in WAT of various congenic mouse lines. mRNA was measured by qRT-PCR and normalized to *36B4* mRNA levels. Data are expressed as the mean ± SEM for n=6-10. * $p < 0.05$ versus control. The 'undetectable' indication means that the transcript was not detected using 2-6 independent sets of primers.

(C) Expression of candidate genes in the liver of various congenic mouse lines. mRNA for which no expression was detected in WAT was measured in the liver of the mouse lines. mRNA was measured by qRT-PCR and normalized to *36B4* mRNA levels. Data are expressed as the mean ± SEM for n=7-11. * $p < 0.05$ versus control. The 'undetectable' indication means that the transcript was not detected using 2-6 independent sets of primers.

(D) Expression of the high priority candidate gene *Trhr* in pituitary. mRNA was analyzed by northern blotting, *Trhr* levels quantified by Phosphor-image Scanner and expressed as values normalized to *18S* RNA levels (mean ± SEM for n=3 per line).

Figure S2

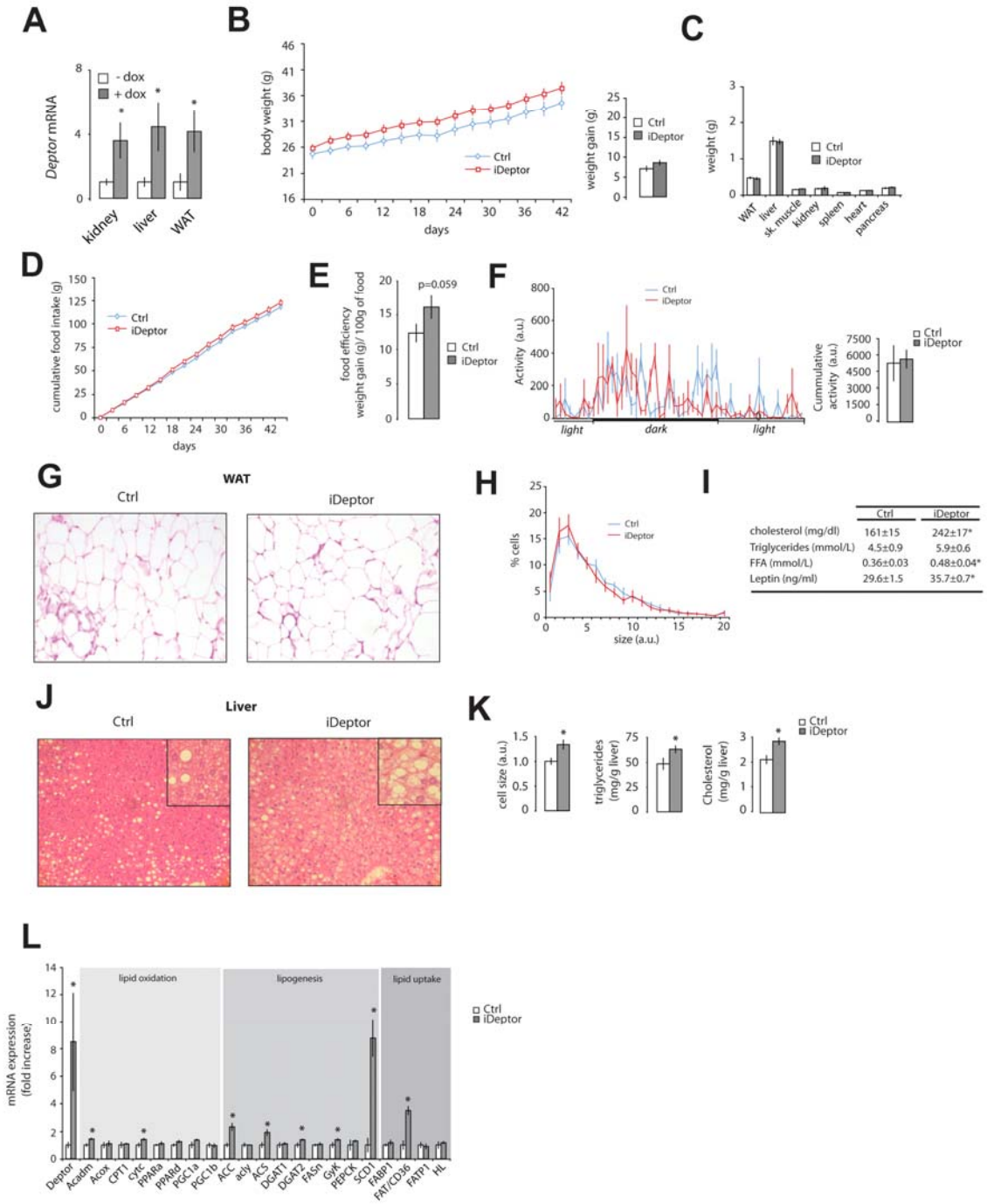

A
Deptor mRNA
- dox
+ dox
kidney
liver
WAT
B
body weight (g)
Ctrl
iDeptor
days
weight gain (g)
C
weight (g)
WAT
liver
sk. muscle
kidney
spleen
heart
pancreas
D
cumulative food intake (g)
E
p=0.059
food efficiency
weight gain (g)/ 100g of food
F
Activity (a.u.)
light
dark
light
Cummulative activity (a.u.)
G
WAT
Ctrl
iDeptor
H
% cells
size (a.u.)
I
Ctrl
iDeptor
cholesterol (mg/dl) 161±15 242±17*
Triglycerides (mmol/L) 4.5±0.9 5.9±0.6
FFA (mmol/L) 0.36±0.03 0.48±0.04*
Leptin (ng/ml) 29.6±1.5 35.7±0.7*
J
Liver
Ctrl
iDeptor
K
cell size (a.u.)
triglycerides (mg/g liver)
Cholesterol (mg/g liver)
L
mRNA expression (fold increase)
lipid oxidation
lipogenesis
lipid uptake
Deptor
Acadm
Acox
CPT1
cytc
PPARa
PPARd
PGC1a
PGC1b
ACC
acly
ACS
DGAT1
DGAT2
FASn
GyK
PEPCK
SCD1
FABP1
FAT/CD36
FATP1
HL

Figure S2. Metabolic parameters of iDeptor mice.

(A) *Deptor* mRNA expression in response to doxycycline in iDeptor mice *in vivo*. iDeptor mice were euthanized and tissues were collected 6 hours after PBS or doxycycline injection. *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.

(B) Body weight and body weight gain of control or iDeptor mice fed chow diet supplemented with doxycycline (200mg/kg food) for 6-7 weeks. Data are expressed as the mean ± SEM for n=6-8 per condition.

(C) Tissue weight of control or iDeptor mice treated as described in (B). Data are expressed as the mean ± SEM for n=6-8 per condition.

(D) Cummulative food intake and (E) food efficiency (body weight gain/100g food) of iDeptor mice fed a high fat diet supplemented with doxycycline (200mg/kg food) for 6-7 weeks. Data are expressed as the mean ± SEM for n=6-8 per condition.

(F) Locomotor activity of iDeptor mice fed a high fat diet supplemented with doxycycline (200mg/kg food) for 6-7 weeks. Data are expressed as the mean ± SEM for n=4-8 per condition.

(G) Histological analysis of WAT of control and iDeptor mice treated or not with doxycycline and fed a high fat diet. Representative pictures of each group are presented.

(H) Quantification of adipocyte size in WAT of mice described in (G). Data are expressed as the mean ± SEM for n=6-7 per condition.

(I) Blood metabolites from mice treated or not with doxycycline and fed a high fat diet. Data are expressed as the mean ± SEM for n=6-8 per condition. * $p < 0.05$ versus control.

(J) Histological analysis of liver of control and iDeptor mice treated or not with doxycycline and fed a high fat diet. Representative pictures of each group are presented.

(K) Hepatocyte size was measured by counting the number of nuclei per field. Triglycerides and cholesterol were extracted and measured from the same samples. Data are expressed as the mean ± SEM for n=6-8 per condition. * $p < 0.05$ versus control.

(L) Gene expression analysis in the liver of control and iDeptor mice fed a high fat diet supplemented with doxycycline (200mg/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.

Figure S3

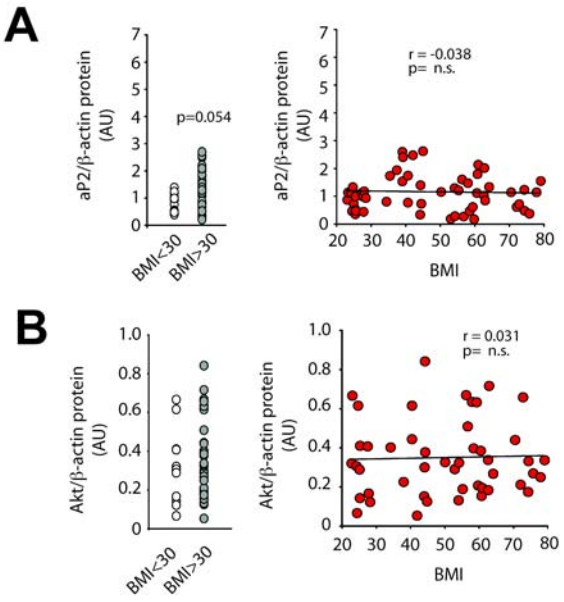


Figure S3. Analysis of subcutaneous fat in humans.

(A) aP2 and (B) Akt/PKB protein expression in human adipose tissue and its relation to BMI. Protein lysates were prepared from subcutaneous adipose tissue isolated from lean and obese humans. Lysates were then analyzed by immunoblotting for aP2 and β-ACTIN. aP2 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, n.s = non-significant.

Figure S4

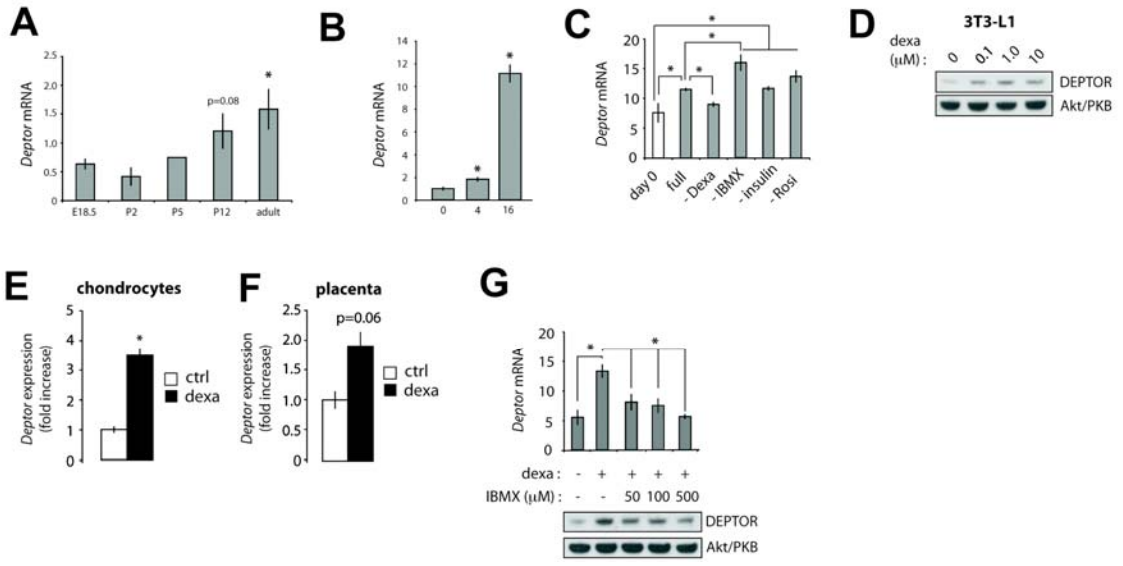


Figure S4. Regulation of *Deptor* during adipogenesis *in vivo* and *in vitro.*

(A) *Deptor* mRNA is induced during fat development *in vivo*. Subcutaneous adipose tissue from mice embryos and pups at various stages of development were collected and RNA was extracted. *Deptor* mRNA was measured by qRT-PCR and normalized to *36B4* mRNA levels. Data are expressed as the mean ± SEM for n=4-7 per condition, except P5 where only one sample was available. * $p < 0.05$ versus E18.5.

(B) *Deptor* RNA is induced during adipogenesis *in vitro*. MEFs were grown to confluence and differentiation was induced in post-confluent cells (2 days) in the presence of the adipogenic cocktail (insulin, IBMX, dexamethasone, rosiglitazone) for 2 days. The medium was then replaced by a medium containing serum and insulin for the rest of the differentiation process. *Deptor* mRNA was measured by qRT-PCR and normalized to *36B4* mRNA levels. Data are expressed as the mean ± SEM for n=3-9 per condition* $p < 0.05$ versus day 0.

(C) Dexamethasone controls *Deptor* expression during adipogenesis. MEFs were differentiated for 4 days using the adipogenic protocol (see Methods) but components of the adipogenic cocktail were removed as indicated (from day 0 to day 2). *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, n.s. : non-significant.

(D) Dexamethasone controls DEPTOR protein expression in 3T3-L1 cells *in vitro*. 3T3-L1 cells were seeded at equal density and grown in the presence of various doses of dexamethasone for 24 hours. Protein lysates were analyzed by immunoblotting for indicated proteins.

(E-F) Impact of dexamethasone on *Deptor* expression in other models. (E) Primary cultured fetal chondrocytes were treated with dexamethasone (0.1μM) or DMSO for 24 hours. Microarray data were collected from NCBI publicly available resources http://www.ncbi.nlm.nih.gov/sites/GDSbrowser?acc=GDS2802. Data are expressed as the mean ± SEM for n=2-3 per condition* p < 0.05. (F) Pregnant mice were treated with dexamethasone (0.5 mg/kg) or saline on gestational days 15, 16, and 17 and placenta was collected. Microarray data were collected from NCBI publicly available resources http://www.ncbi.nlm.nih.gov/sites/GDSbrowser?acc=GDS2314. Data are expressed as the mean ± SEM for n=2-3 per condition* p < 0.05.

(G) IBMX competes with dexamethasone for the control of DEPTOR expression. MEFs were treated with dexamethasone (0.1μM) and with various doses of IBMX for 10 hours. Protein lysates were analyzed by immunoblotting for indicated proteins. *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.

Figure S5

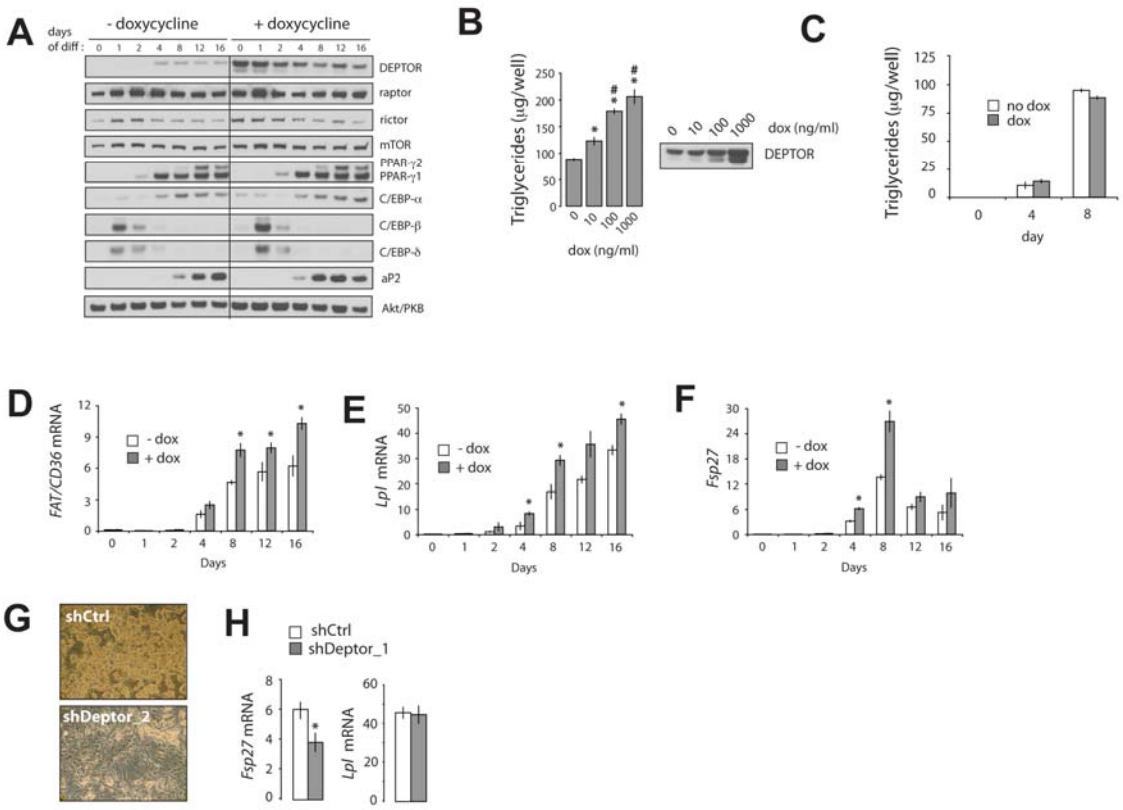


Figure S5. Impact of DEPTOR overexpression or knockdown in vitro.

(A) MEFs isolated from iDeptor mice were plated and grown to confluence. When confluent (day -2), doxycycline (100ng/ml) was added or not to the cells until the end of the experiment. Two days post-confluence (day 0), differentiation was induced using the classical adipogenic cocktail (insulin, IBMX, dexamethasone, rosiglitazone; see methods) for 2 days. The medium was replaced by a medium containing serum and insulin for the rest of the differentiation process. Protein lysates were prepared at day 0, 1, 2, 4, 8, 12, and 16 of the adipogenic protocol and were analyzed by immunoblotting for indicated proteins.

(B) iDeptor MEFs were treated with the indicated doses of doxycycline for the entire duration of the adipogenic protocol (day -2 to day 8). Cells were differentiated as described in A and triglycerides were extracted. Data are

expressed as the mean of ± SEM for n=4-5 per condition. * and # indicate $p < 0.05$ versus 0 or 10ng/ml respectively. Protein lysates were prepared as described above and analyzed by immunoblotting for indicated proteins.

(C) Wild-type MEFs were treated with the doxycycline (100ng/ml) for the entire duration of the adipogenic protocol (day -2 to day 8). Cells were differentiated until day 8 as describe in A and triglycerides were extracted. Data are expressed as the mean of ± SEM for n=3-4 per condition.

(D to F) MEFs were treated with doxycycline (100ng/ml) and differentiated using as described in A. 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.

(G-H) DEPTOR knock down decreases adipocyte differentiation of 3T3-L1 cells. 3T3-L1 cells were infected with control or *Deptor* shRNA lentiviruses and differentiated using the adipogenic protocol. (G) A representative picture of 3T3-L1 cells 8 days after the initiation of differentiation. (H) 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.

Figure S6

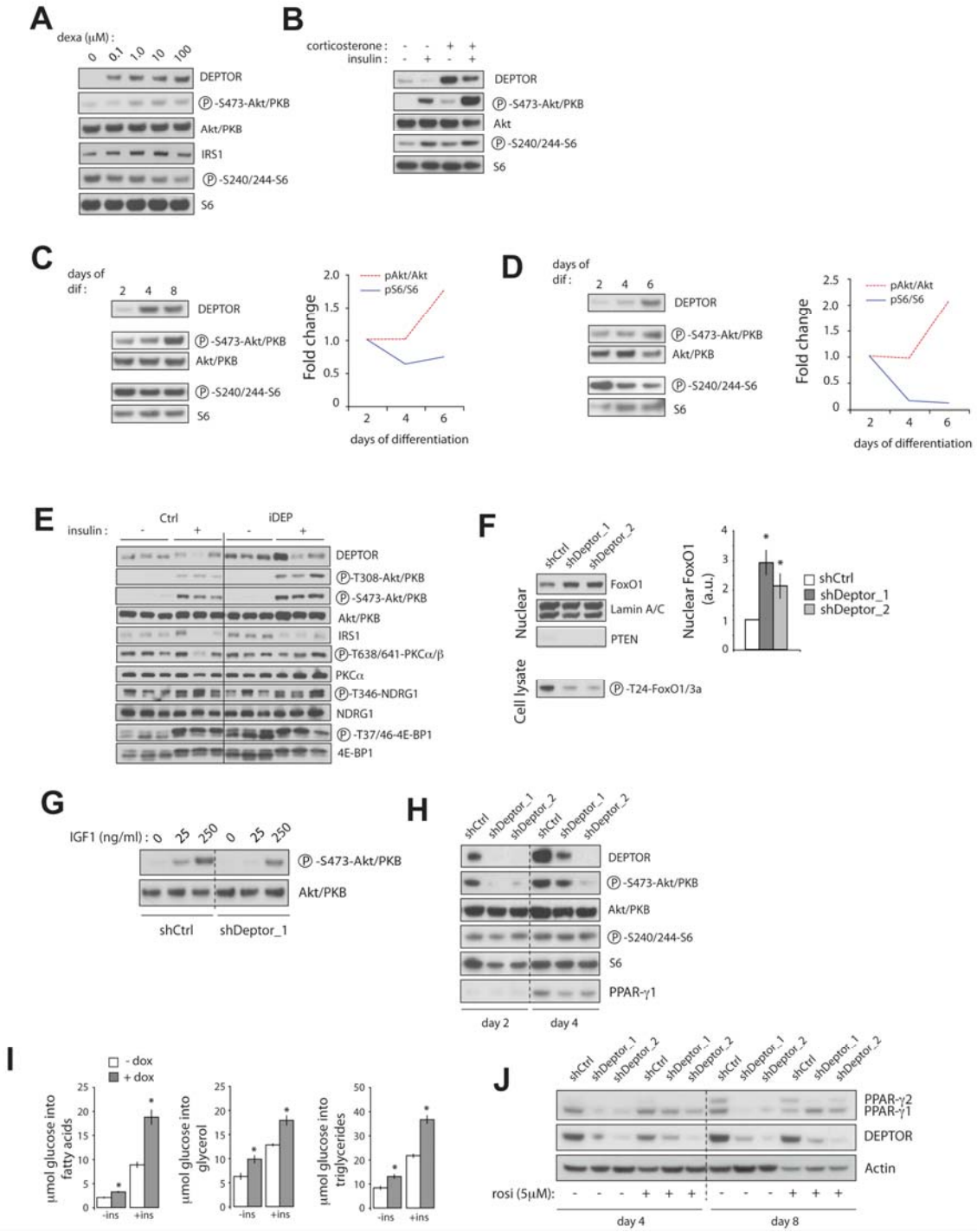

A
dexa (μM) :
0 0.1 1.0 10 100
DEPTOR
Ⓟ -S473-Akt/PKB
Akt/PKB
IRS1
Ⓟ -S240/244-S6
S6
B
corticosterone :
insulin :
DEPTOR
Ⓟ -S473-Akt/PKB
Akt
Ⓟ -S240/244-S6
S6
C
days of dif :
2 4 8
DEPTOR
Ⓟ -S473-Akt/PKB
Akt/PKB
Ⓟ -S240/244-S6
S6
pAkt/Akt
pS6/S6
Fold charge
days of differentiation
D
days of dif :
2 4 6
DEPTOR
Ⓟ -S473-Akt/PKB
Akt/PKB
Ⓟ -S240/244-S6
S6
pAkt/Akt
pS6/S6
Fold change
days of differentiation
E
insulin :
Ctrl
iDEP
DEPTOR
Ⓟ-T308-Akt/PKB
Ⓟ -S473-Akt/PKB
Akt/PKB
IRS1
Ⓟ-T638/641-PKCα/β
PKCα
Ⓟ-T346-NDRG1
NDRG1
Ⓟ -T37/46-4E-BP1
4E-BP1
F
shCtrl
shDeptor_1
shDeptor_2
Nuclear
FoxO1
Lamin A/C
PTEN
Cell lysate
Ⓟ -T24-FoxO1/3a
Nuclear FoxO1 (a.u.)
shCtrl
shDeptor_1
shDeptor_2
G
IGF1 (ng/ml) : 0 25 250 0 25 250
Ⓟ -S473-Akt/PKB
Akt/PKB
shCtrl
shDeptor_1
H
shCtrl
shDeptor_1
shDeptor_2
DEPTOR
Ⓟ -S473-Akt/PKB
Akt/PKB
Ⓟ -S240/244-S6
S6
PPAR-γ1
day 2
day 4
I
- dox
+ dox
μmol glucose into fatty acids
μmol glucose into glycerol
μmol glucose into triglycerides
-ins
+ins
J
shCtrl
shDeptor_1
shDeptor_2
PPAR-γ2
PPAR-γ1
DEPTOR
Actin
rosi (5μM):
day 4
day 8

Figure S6. DEPTOR positively regulates Akt/PKB activation and lipogenesis.

(A) MEFs were seeded at equal density and grown in the presence of various doses of dexamethasone for 24 hours. Protein were extracted and lysates were analyzed by immunoblotting for indicated proteins.

(B) MEFs were seeded at equal density and grown in the presence of 10μM of corticosterone for 24 hours. Cells were washed and incubated in serum free media with or without corticosterone (10μM) for 150 minutes and then stimulated with insulin (100ng/ml) for 15 minutes. Proteins were extracted and lysates were analyzed by immunoblotting for indicated proteins.

(C) MEFs were plated and grown to confluence. Two days post-confluence, differentiation was induced using the classical adipogenic cocktail (insulin, IBMX, dexamethasone, rosiglitazone; see methods) for 2 days. The medium was replaced by a medium containing serum and insulin for the rest of the differentiation process. Protein lysates were prepared at day 2, 4 and 8 of the adipogenic protocol. Fresh media was added to the cells 30 minutes prior lysis and cell lysates were analyzed by immunoblotting for indicated proteins. Akt/PKB and S6 phosphorylation was quantified using ImageJ and normalized to the total level of Akt/PKB and S6 protein respectively.

(D) 3T3-L1 were plated and grown to confluence. Two days post-confluence, differentiation was induced using the classical adipogenic cocktail (insulin, IBMX, dexamethasone; see methods) for 2 days. The medium was replaced by a medium containing serum and insulin for the rest of the differentiation process. Protein lysates were prepared at day 2, 4 and 6 of the adipogenic protocol. Fresh media was added to the cells 30 minutes prior lysis and cell lysates were analyzed by immunoblotting for indicated proteins. Akt/PKB and S6 phosphorylation was quantified using ImageJ and normalized to the total level of Akt/PKB and S6 protein respectively.

(E) DEPTOR expression induces insulin-stimulated Akt/PKB phosphorylation in the WAT of iDeptor mice. Following 1 week of treatment with doxycycline (200mg/kg food), mice were fasted for 15 hours, injected IP with PBS or insulin (0.75U/kg mouse), and euthanized 30 minutes after. During fasting, doxycycline (2g/L) was added to water to insure a continuous expression of the transgene. Protein lysates were analyzed by immunoblotting for indicated proteins.

(F) Control and DEPTOR knock down 3T3-L1 produced as described in Figure 5F were seeded at equal density, grown to confluence, and lysed. Cell lysates were analyzed by immunoblotting to measure the total amount of phospho-Foxo1/3a. Nuclei were extracted from control and DEPTOR depleted cells and protein lysates were analyzed by immunoblotting for indicated proteins. Data are expressed as mean ± SEM of three independent experiments. n=3, * $p < 0.05$ versus control.

(G) 3T3-L1 cells were infected with control or *Deptor* shRNA lentivirus as described previously and were seeded at equal density and grown to confluence. Cells were serum starved for 90 minutes and treated with the indicated doses of IGF1 for 15 minutes. Protein lysates were analyzed by immunoblotting for indicated proteins.

(H) 3T3-L1 cells were infected with control or *Deptor* shRNA lentivirus as described previously and were seeded at equal density and grown to confluence. Two days post-confluence, differentiation was induced using the classical adipogenic cocktail (insulin, IBMX, dexamethasone; see methods) for 2 days. The medium was replaced by a medium containing serum and insulin for the rest of the differentiation process. Protein lysates were prepared at day 2 and 4 of the adipogenic protocol. Fresh media was added to the cells 30 minutes prior lysis and cell lysates were analyzed by immunoblotting for indicated proteins.

(I) DEPTOR overexpression promotes glucose incorporation in the fatty acid and glycerol fractions of lipids. Cells were differentiated for 8 days as described in the methods and then incubated with tritiated water with or without insulin (100nM) overnight. Cells were washed and triglycerides were extracted and counted as

described in the Methods. Data are expressed as the mean ± SEM for n=4 per condition. * $p < 0.05$ versus control.

(J) Rosiglitazone corrects PPAR-γ protein levels in cells DEPTOR knockdown cells. 3T3-L1 cells were infected with control or *Deptor* shRNA and selected with puromycin. Then, cells were differentiated with the classic adipogenic cocktail supplemented with DMSO or rosiglitazone (5 μM) for 4 or 8 days. Protein lysates were prepared at the indicated days and analyzed by immunoblotting for indicated proteins.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Generation of the iDeptor mouse model

The iDeptor mouse model was produced as described before (Beard et al., 2006). Briefly, embryonic stem (ES) cells (KH2) have been engineered to contain, at the ColA1 locus, a frt-flanked neomycin resistance gene and a hygromycin resistance gene that lacks a promoter and an ATG initiation codon. The presence of the frt site in the ES cells allows the unique integration of the transgene at this locus. These cells also contain a M2rtTA transactivator at the endogenous Rosa26 promoter to allow the transactivation of the TetO promoter and the expression of the transgene in response to doxycycline. The Deptor coding sequence was cloned in a vector containing a tetO minimal promoter and the PGK-ATG-Frt cassette necessary for the integration of the transgene and the selection of the positive ES cells. This vector was then electroporated in the KH2 ES cells concomitantly with a vector containing a FLPe recombinase (pCAAGS FLPe-puro). In the presence of the FLPe recombinase, the transgene is inserted in genomic DNA at the frt site along with a PGK promoter and the ATG initiation codon necessary for the expression of the hygromycin resistance gene, thereby conferring hygromycin resistance to the correctly targeted cell (Figure 3A). Cells were selected and injected into blastocysts. Chimeric mice were then crossed to C57BL/6J until germline transmission was achieved. Genotyping of iDeptor mice was performed by PCR with the following primers:

	Sense primer	Antisense primer
iDeptor allele	GCCTCTTTTACCCTTTTCCTCTTCC	CCCTCCATGTGTGACCAAGG CTAGAGTCGCAGATCCAGACATGA
rtTA allele	AAAGTCGCTCTGAGTTGTTAT	GCGAAGAGTTTGTCCTCAACC GGAGCGGGAGAAATGGATATG

This PCR reaction produces fragments of 800bp (iDeptor allele) and 300bp (wild-type allele). For the rtTA genotyping reaction, the PCR leads to fragments of 300bp (rtTA allele) and 500bp (wild-type allele).

Animal experiments

All procedures were performed in accordance with the guidelines of the Massachusetts Institute of Technology Committee on Animal Care (CAC). All mice were housed in a temperature-controlled environment with 12 hr light/dark cycles. iDeptor mice were maintained in a mixed strain background C57BL/6J:129S6/SvEv (75:25) and ~7-9 week-old male mice were used for all experiments. For the duration of the studies, control ($rtTA^{?/?}$, $iDeptor^{-/-}$) or iDeptor ($rtTA^{+/?}$, $IDeptor^{+/-}$) mice had free access to water and were fed *ad libitum* with either a chow diet (Bio-Serv, S3888) or a high fat diet (60%kcal fat, Research Diets, D09050201) supplemented with doxycycline (200mg/kg of diet). The animals were treated for 8-9 weeks. The day before the sacrifice, the animals were fasted overnight with access to water supplemented with doxycycline (2g/L). The next morning, mice were fed for 2 hours before being euthanized with CO_2. A few animals where similarly treated with doxycycline in chow for 1 week and injected with saline or with insulin (0.75U/kg) (Lilly,HumulinR, HI-210) following an overnight fast. Mice were euthanized 30 minutes post-injection.

Experiments using the congenic mouse lines

From a previous F_2 cross (Stylianou et al., 2005), a recombinant individual within *Fob3a* QTL region was used as a founder for development of subsequent congenic lines P, W, V, and Z used here. A total of 11-12 backcrosses to the Fat line with additional marker-assisted elimination of other QTL regions was performed. Positions of Lean donor segments in congenic lines were defined based on high density SNP genotyping with the exception of the Z line, where intervals were defined based on microsatellite markers (SNPs were not available for this line). Food chow (1324 maintenance diet, Altromin, Germany) and acidified water were offered *ad libitum*. Mice were weaned at 3 weeks of age and housed in pairs. All the procedures involving animals were performed according to local ethical and regulatory guidelines, which are all in compliance with the EU regulations regarding research on experimental animals (project license number

34401-3/2007/4). Genetic screening of congenic lines and crosses was performed by microsatellite or SNP analysis. Mice were weighed at 14 weeks and adipose tissues (inguinal-femoral fat pad) were dissected and weighed. For the experiment comparing homozygous congenic lines the Fat line (n=75) ,P (n=20), V (n=32), W (n=27), and Z (n=37) were phenotyped. Additionally, F_2 mice from the crosses between the Fat line and the congenic lines V (n=79) and P (n=45) were phenotyped. Empirical distributions of collected phenotypic data suggested a normal distribution for each analyzed variable. Therefore, a statistical model (Eq [1]) with multivariate normal distribution was fitted:

[1] $\mathbf{y} \mid \mathbf{b}, \mathbf{s}, \mathbf{R} \sim MVN\left(\mathbf{Xb} + \mathbf{Zs}, \mathbf{R}\right),$

where $\mathbf{y}$ is an ny×5 matrix of phenotypic values; $\mathbf{b}$ is the vector of location parameters for effects that differed between the data sets; $\mathbf{s}$ is the vector of location parameters for ns seasons of dissection defined as year-month interaction, and $\mathbf{R} = \mathbf{I}_{n_y} \otimes \mathbf{R}_0$ is residual covariance matrix. For congenic lines the vector $\mathbf{b}$ involved the effect of parity (1, 2, and 3+), the number of pups per litter (1-2, 3, 4, 5, 6, 7, and 8+), and line (Fat, V, W, P, Z). For F_2 congenic intercrosses the vector $\mathbf{b}$ involved the effect of sex (males and females), parity (1, 2, and 3+), the number of pups per litter (1-2, 3, 4, 5, 6, and 7+), line (V and P), and genotype within line (homozygotes Fat/Fat, V/V, P/P and heterozygotes Fat/V or Fat/Ps). For the latter the additive and dominance effect was tested using the DIC statistic, which revealed that the dominance model had a better fit that the additive model. Posterior distributions were summarized with mean and standard deviation for each line or genotype as evaluated at the first parity and five pups in the litter. Posterior probabilities that the congenic lines or homozygous genotypes from F_2 congenic intercrosses differ were computed (high probability (0.95) suggests a significant difference and presence of a *Fob3a* QTL effect).

Bioinformatics analyses of candidate genes in the *Fob3a* interval

A set of 1289 SNPs located within the *Fob3a* interval reduced by congenic mapping was used to genotype congenic Fat , V, W and P lines. Interval-specific haplotype analysis was carried out to identify haplotype blocks that are not identical by descent (non-IBD) between congenic lines. Such regions are likely to contain the causal polymorphism(s) that cause differences in phenotype between the observed lines (DiPetrillo et al., 2005). 29 genes mapped to non-IBD regions. Seven elements were excluded from our analysis (spliceosomal RNAs, hypothetical proteins, nucleolar RNAs). Mouse SNP Wizard tool was employed for strain comparisons. With the Bio GPS Expression database (http://biogps.gnf.org), genes were selected as a hit based on their expression levels in metabolic tissues. Genes expressed in any metabolic tissue (brain/pituitary, WAT, brown adipose tissue, liver, muscle, or intestine) 3 times above the Median expression were considered as hits. Gene ontology (GO) (http://amigo.geneontology.org) database provided molecular function annotations. Any function that could relate to adipose tissue development and/or cell growth/metabolism was considered as a hit. Mouse Genome Informatics (MGI) (http://www.informatics.jax.org) was used to search for existence of candidate gene's mouse knockout and transgenic models and affected anatomical systems. Any engineered mouse model showing variation in body weight or alteration in food intake or metabolism was considered as a hit. The expression of all candidate genes was measured in WAT, liver or brain. The differential expression of a gene between the congenic line V and FAT and V and Z was considered as a hit.

Microarray analyses of F_2 congenic lines

RNA was extracted from WAT of female F_2 littermates of *Fob3a*$^{Fat/Fat}$ and *Fob3a*$^{V/V}$ genotypes (n=5 per genotype) using RNeasy Lipid Tissue Mini Kit (Qiagen, 74804). RNA integrity numbers (RIN) assessed by Agilent 2100 Bioanalyzer (Agilent Technologies,USA) ranged between 9.0 to 9.3 suggesting low variability and very high RNA quality. Affymetrix GeneChip Mouse 1.0 ST

Arrays and protocol (Affymetrix, Santa Clara, ZDA) was employed. Microarray data were analyzed using different R/Bioconductor packages and normalized using RMA algorithm from XPS package. The raw and normalized gene expression data of 10 arrays together with experimental information are deposited in Gene Expression Omnibus database in compliance with MIAME standards (Brazma et al., 2001). Differential expression of genes between the *Fob3a*$^{Fat/Fat}$ and *Fob3a*$^{V/V}$ mice was assessed using modified t-test from LIMMA package by controlling the false discovery rate at level α=0.05. Complete microarray data is available at Gene Expression Omnibus under the accession GSE38321: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE38321.

Cells and tissue lysates preparation

All cells were rinsed twice with ice-cold PBS before lysis. Cells were lysed with Triton-X 100 containing lysis buffer (50 mM HEPES [pH 7.4], 2 mM EDTA, 10 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 40 mM NaCl, 50 mM NaF, 2 mM sodium orthovanadate, 1% Triton-X 100, and one tablet of EDTA-free protease inhibitors Roche per 25 ml). Tissues were homogenized with the same buffer supplemented with 0.1% of sodium lauryl sulfate (SDS) and 1% of sodium deoxycholate. Cells and tissues were rotated at 4°C for 10 minutes and then the soluble fractions of cell lysates were isolated by centrifugation at 13,000 rpm for 10 min in a microcentrifuge. Protein levels were then quantified using Bradford reagents and analyzed by western blotting. The Licor Odyssey system was used to quantify western blots from human samples.

Plasma metabolite analyses

Plasma metabolites were measured using the following commercial kits; leptin (Crystal Chem Inc., 90030), triglycerides (Thermo Scientific, TR22421) and free fatty acids (Roche, 11383175001), cholesterol (Thermo Scientific, TR13421).

Gene expression analysis

Total RNA was isolated from cells or tissues using the Rneasy Kit (Qiagen, 74106) and reverse-transcription was performed using Superscript III reverse transcriptase (Invitrogen, 18080-044). The resulting cDNA was diluted in Dnase-free water (1:15) before quantification by real-time quantitative PCR. mRNA transcript levels were measured using SYBR Green PCR master mix (Applied Biosystems, 430955) and the Biosystems 7900HT Sequence Detection System v2.3 software. All Data are expressed as the ratio between the expression of target gene to the housekeeping genes *36B4* or *Gapdh*. The following primers obtained from Integrated DNA Technology were used for qRT-PCR:

	Sens primer	Antisens primer
36B4	TAAAGACTGGAGACAAGGTG	GTGTACTCAGTCTCCACAGA
Acadm	GAAGGTTGAACTCGCTAGGC	GCTAGCTGATTGGCAATGTC
Acc	GCCTCTTCCTGACAAACGAG	TGACTGCCGAAACATCTCTG
Acs	GCTGATCCAGAAGGGGTTCA	CCACCCCACACTTCTTGCCT
Acly	CTCACACGGAAGCTCATCAA	ACGCCCTCATAGACACCATC
Acox	GCCTGAGCTTCATGCCCTCA	ACCAGAGTTGGCCAGACTGC
Adfp	AAGCATCGGCTACGACGACAC	GGACAGTCTGGCATGTAGTCTGGA
Angpt1	AGCCTGGATTTCCAGAGGGGCTG	GGAAGGGCCACAGGCATCGAAC
Ap2	GACGACAGGAAGGTGAAGAG	ACATTCCACCACCAGCTTGT
C/EBP-α	CAAGAACAGCAACGAGTACCG	GTCACTGGTCAACTCCAGCAC
C/EBP-β	GCACAAGGTGCTGGAGCTGAC	CTTGAACAAGTTCCGCAGGGT
C/EBP-δ	ATCGACTTCAGCGCCTACA	GCTTTGTGGTTGCTGTTGAA
Colec10	AGTGGGACGCCAGGGACCAAA	GGGCATGGCTAGCATCCCTCCT
Cpt1	CCATGAAGCCCTCAAACAGATC	ATCACACCCACCACCACGATA
CytC	TGGACCAAATCTCCACGGTCTGTT	TAGGTCTGCCCTTTCTCCCTTCTT
Deptor	AGCAGAGAGAGCTGGAACGC	CAGAGGCCTCCTTATGTTCA
Dgat1	GGCCTGCCCCATGCGTGATTAT	CCCCACTGACCTTCTTCCCTGTAGA
Dgat2	GAAGCTGCCCGCAGCGAAAA	TCTTGGGCGTGTTCCAGTCAA
Dscc1	GCCTGCAGGAGCTTGGACCTT	CGGGAACACTCTGCTGCCACAC
Ebag9	TACGGGCCTCGCACCTCTCG	CCGAAACTGAGTGATGGCCATGGT
Eif3E	GATGTGCGGAAACGCCGGCA	TTGCTGGTAGGGCGAGACTGC

Enpp2	AGCTCGAGGCTGGGAGTGCA	GGAGGGCGGACAAACCCTGC
Eny2	*CCCGCGGTGATGGTGGTTAGCA*	AAGGCTGGCATGCTGAGCAAGGAA
Dgat2	GAAGCTGCCCGCAGCGAAAA	TCTTGGGCGTGTTCCAGTCAA
Fabpl	GCCCGAGGACCTCATCCAGAAA	CTCTCTTGTAGACAATGTCGCCCA
Fasn	CTGGCCCCGGAGTCGCTTGAGTATA	GGAGCCTCCGAAGCCAAATGA
FAT/CD36	GTCCTGGCTGTGTTTGGAGG	GCTGCTACAGCCAGATTCAG
Fatp1	CCAGACGGACGTGGCTGTGTAT	GTCCCTGCTTCAGGTCTAGAAAGA
Fsp27	CTGGAGGAAGATGGCACAATCGTG	CAGCCAATAAAGTCCTGAGGGTTCA
Gapdh	TCACCATCTTCCAGGAGGGA	GCATTGCTGACAATCTTGAGTGAG
Gyk	AATCCGTTACTCCACATGGA	ACCCGATCTTAACTGTCAAT
Hl	AAATCCCCTCCAAATCTCCA	CGCACTCACTATCTTCCAGAT
Lpl	GCACTTTCCAGCCAGGATGC	GGCCTGGTTGTGTTGCTTGC
Mal2	CGCTGGCCCCGACATCCTAA	AGGTGGCCGCTGCTTCCAGTA
Nov	GCTGCTCCTGCTGTCCGGTG	GCAGCACTCCCCTGGCACTG
Nudcd1	CCCCGAGAGGTGTTCCGCCT	TGTGGCACTGACTTTCCACGG
Pepck	CGATGACATCGCCTGGATGA	TCTTGCCCTTGTGTTCTGCA
Perilipin	TGCTGGATGGAGACCTC	ACCGGCTCCATGCTCCA
Pgc1a	TCACGTTCAAGGTCACCCTA	TGCTCTTTGCGGTATTCATC
Pgc1b	GGCTCTTCCGCTCACA	ACCTGGCAGTAGTCGTGGTC
Pkhd1l1	TGGGGCTGCTCCCACTCAGT	GGCTGCCACTGGCTGAGCAA
Ppar-a	AGAGCCCCATCTGTCCTCTC	ACTGGTAGTCTGCAAAACCAAA
Ppar-d	AACCGCAACAAGTGTCAGTACTGC	ATGCTCCGGGCCTTCTTTT
Pppr-g2	ACTGCCTATGAGCACTTCAC	CAATCGGATGGTTCTTCGGA
Redd1	TCTTCGCTGACCGCGCTAGCT	TCCAGGTATGAGGAGTCTTCCTCCG
Rspo3	TGCCAAGGAGGCTGTGCAACG	CTCGGACCCGTGTTTCAGTCCC
Scd1	GCCCACCACAAGTTCTCAGA	GGGCGATATCCATAGAGATG
Taf2	TCACGCTGTTTGGCCTCAGCA	AGCCGGACGGCCGGTACTTA
Tmem74	GGACTTTCTCCGGGTGCCATGC	GTAGGGAGGCCTGCTCCTCTGG
Tnfrsf11b	TGGGCTGCAGAGACGCACCTA	GGTGCGGTTGCACTCCTGCTT
Trsp1	TGCCCACCTGGCACGACCTA	GCTTGGCTGACCAGGACTGGC
Ttc35	CGCCCTCTCTCCTCGCCGAC	CTCGGATGGCCTCCACGGTT

RNA quantification by northern blotting

Northern blotting for the quantification of *Trhr* was performed as previously described (Stylianou et al., 2005). The sequences of the primers used to synthesize the probe are 5'-GGC CAC TGT GCT TTA TGG AT-3' and 5'-TTA CAA CCA CTG CGA GCA TC-3'.

Tissue histology

Mouse tissues were collected and immediately fixed in 10% formalin for 24 hours. Tissues were then transferred to a solution of 70% ethanol for storage. Tissues were embedded in paraffin, cut, mounted, and stained using hematoxylin-eosine. Slides were analyzed by light microscopy using an Olympus BX41 microscope. The size of hepatocytes and adipocyte was measured using image J and the imaging software cellSens from Olympus.

Glucose uptake and incorporation into triglycerides

Glucose uptake was quantified with labeled ^{3}H-2deoxy-D-glucose in MEFs treated with doxycycline (100ng/ml) or 3T3-L1 cells with DEPTOR knockdown. Briefly, MEFs were incubated in serum free media for 4 hours and then stimulated with insulin (100nM) for 30 minutes. Because the viability of 3T3-L1 was rapidly compromised by serum deprivation, cells growing in normal DMEM 10% FBS were used. Following stimulation with insulin or 10% serum, cells were rinsed and incubated with warmed HEPES solution containing labeled 2-deoxy-D-glucose for 8 minutes (MEFs) or 20 minutes (3T3-L1). Cells were then rinsed, lysed with NaOH (50 mM) and radioactivity was measured. Radioactive counts were corrected to protein content as measured using Bradford reagent. For the measurement of glucose incorporation into total triglycerides and in the glycerol or free fatty acids fractions of triglycerides and phospholipids, MEFs were differentiated as described above for 8 days and then incubated with tritiated water with or without insulin (100nM) overnight. Cells were washed and triglycerides were extracted as described above. One small aliquot of the lower chloroformic phase (total cell lipids) was transferred into a scintillation vial and

counted to measure the incorporation of the radiolabel into total lipids. After evaporation of the remaining chloroformic phase, lipids were saponified with 1ml of ethanol-KOH (1ml of saturated KOH (14.5 mM plus 20 ml of absolute ethanol) at 75ºC for 2 hours. After 2 hours, 1ml of water was added and ethanol was completely evaporated. The non-saponifiable lipids were extracted with 1ml of petroleum ether and discarded. This was repeated twice. After evaporation of the remaining petroleum ether, three droplets of bromocresol green were added as a pH indicator and samples were acidified with perchloric acid (6%). After incubation at 60ºC to dissolve the lipids, fatty acids were extracted with 2ml of ether petroleum and transferred to a scintillation vial. This last step was repeated 3 times. The petroleum was evaporated and the radioactivity counted. This measurement corresponds to the incorporation of the tracer in the fatty acids fraction of triglycerides and phospholipids. The incorporation of tracers into the glycerol fraction of triglycerides and phospholipids was determined by calculating the difference between the incorporation of the tracer into total lipids and fatty acids.

Triglyceride extraction and quantification

Triglycerides were extracted from adipocytes following the method of Folch (Folch and Lees, 1957). Briefly, cells were scraped in 600 μl of PBS and transferred to a glass tube. After addition of 3 ml of a chloroform:methanol (2:1) solution, the tubes were vortexed and centrifuged for 10 minutes. The superior, aqueous phase was collected and discarded. The remaining lower phase was washed 3 times with water, evaporated, dissolved in 100 μl of isopropanol, and evaluated for triglycerides content using a standard assay kit from Thermo Scientific (Thermo Scientific, TR22421).

Chromatin immunoprecipitation assays (ChIP)

Sub-confluent MEFS (90%) were treated with dexamethasone (1μM) or vehicle (ethanol) for 16 hours and then fixed in PFA (1%) during 30 minutes. Cells were washed twice with PBS+protease inhibitor, scraped, and centrifuged

(5000rpm, 7 minutes). The pellet was resuspended in 750 μl of ChIP lysis buffer (HEPES 50 mM, NaCl 140 mM, triton 1%, protease inhibitor) and cells were kept on ice for 30 minutes. Cell lysates were sonicated (15 pulse, 9 seconds each). The lysates were put on ice for 30 seconds between each pulse. Following the sonication, 50μl of the sample was collected and stored at 4°C (input). Two fractions of the cell lysate (200 μl) were used for the following steps. 50 μl of G protein and 20 μg of salmon sperm were added to each fraction and incubated at 4°C for 2 hours. The samples were centrifuged and the supernatant collected. Antibodies against the glucocorticoid receptor (2.5 μg) or IgGs (2.5 μg) were added to each fraction and incubated overnight. The next day, 50 μl of G protein and 20 μg of salmon sperm were added to each fraction and incubated at 4°C for 2 hours. Beads were washed twice with ChIP lysis buffer, once with ChIP wash high salt buffer (HEPES 50 mM, NaCl 500 mM, triton 1%), once with ChIP wash buffer (Tris 10 mM, LiCl 250 mM, NP40 0.5%, EDTA 0.1 mM). Beads were resuspended in 75 μl of elution buffer (Tris 50 mM, SDS 1%, EDTA 10 mM) and incubated at 65°C for 10 minutes. The supernatant was collected and the previous step was reapeated once. The samples (150 μl) were incubated at 65°C overnight. The input collected previously was also placed at 65°C. The next day, samples were purified using the PCR purification kit (Qiagen) and the samples were used for qPCR analysis using the following primers:

Deptor promoter Set -69 to -193bp
5'-CACGCCCCCAAGCCGAAGTT-3'
5'-CCGCCCCGGCCATCTTTGTT-3'

Deptor promoter Set -431 to -531bp
5'-TTTTTGAACCGCAGGGCAGGA-3'
5'-TGCTGAAGGGCATTTCAGGCTACC-3'

Deptor promoter Set -675 to -802bp
5'-GCTGAGCCATCTCTCCAGCCC-3'
5'-GGCAGGCATGGCGACACACT-3'

Deptor promoter Set -1203 to 1322bp
5'-TGTGTATGGCCGCGGAAGCC-3'
5'-TTTGAATGGCCGAGCTGGTCTAC-3'

Deptor promoter Set -1564 to 1726bp
5'-CTGAAACCTCTCTCCATTGGTGCAT-3'
5'-AGCCCAGGATAGCATCAAACCCT-3'

36B4 promoter Set -953 to -1104bp
5'-CCCACTGTCTGGCAGGCACA-3'
5'-AGGGCGGGGGCAAAACCAAA-3'

The results were normalized by subtracting the IgG result (blank) from the GR result and by dividing this number by the value of the input.

Nuclear extraction from 3T3-L1 cells

The day before the extraction, $1.7x10^6$ 3T3-L1 cells from shCtrl or shDeptor_1 and _2 were plated on a 10cm dish. Nuclei were extracted using a commercial kit (Pierce, 78833). Following the extraction, proteins were quantified by Bradford, normalized, and used for western blot. Lamin A/C was used as a loading control for nuclear proteins ad PTEN was used as a cytosolic marker. FoxO1 levels were quantified using ImageJ.

Lentiviral shRNAs

shRNA-encoding plasmids were purchased from Sigma-Aldrich. The clone ID of the hairpins used in this study are:

shCtrl (Luc): TRCN0000072246
shCtrl (RFP): TRCN0000072203
shDeptor_1: TRCN0000110157
shDeptor_2: TRCN0000110159

The sequence of these hairpins can be retrieved on the The RNAi Consortium (TRC) website http://www.broadinstitute.org/rnai/public/gene/search. The hairpin

vectors were co-transfected with the Delta VPR envelope and CMV VSV-G packaging plasmids into actively growing HEK-293T using FuGENE 6 transfection reagent as previously described (Sarbassov et al., 2005). Virus containing supernatants were collected at 48 hours after transfection and filtered using a 0.45M filter. 3T3-L1 cells (250,000) were infected for 48 hours in the presence of 8 μg/ml polybrene. After infection, the cells were split into fresh media. Cells were selected on the following day with 1 μg/ml puromycin, a dose sufficient to induce the death of uninfected 3T3-L1 cells.

PPRE-luc reporter assays and Akt overexpression in 3T3-L1 cells

Control or Deptor knock down 3T3-L1 cells were transfected with 2.5μg of PPAR-γ2, 2.5μg of a reporter vector containing peroxisome proliferator response elements (PPRE) upstream of the luciferase gene, and 0.5μg of renilla as an internal control using Fugene HD (Roche 04709691001). Cells were incubated for 12 hours and then split. Cells were lysed 24 hours later and assayed for luciferase activity using the Dual-Luciferase ® Reporter Assay System (Promega, E1960). For the rescue experiment, 3T3-L1 cells infected with shLuc or ShDeptor_1 were infected with P-BABE empty or P-BABE-FLAG-Myr-Akt1 and selected with geneticin. Cells were grown to 2 days post-confluence and were treated with the adipogenic cocktail for one day instead of two. The treatment was shortened because 3T3-L1 cells overexpressing Myr-Akt1 detaches when treated with the adipogenic cocktail for more than 1 day. The cells were treated for the following days with media supplemented with insulin.

Human tissue collection and analysis

Human subcutaneous adipose tissue was collected from obese (BMI>30) Caucasian men from the eastern part of the Province of Quebec, Canada, undergoing a bariatric surgery (biliopancreatic diversion with duodenal switch). The surgical protocol has been described elsewhere (Vohl et al., 2004). Lean individuals (BMI<30) were recruited when undergoing a first cardiac surgery (coronary artery bypass graft and/or aortic valve replacement). All individuals

provided written informed consent before their inclusion in the study. The procedures relative to the treatment of the samples were performed in accordance with the Committee on the Use of Humans as Experimental Subjects (COUHES) of the Massachusetts Institute of Technology. Protein lysates were prepared as described above. DEPTOR and aP2 protein levels were quantified using the Licor Odyssey system and were corrected to the β-ACTIN level.

Reagents

The following chemicals were all purchased from Sigma-Aldrich: RU486 (Mifeprestone) (M8046), cycloheximide (C4859), puromycin (P8833), doxycycline (44577), insulin (I5500), dexamethasone (D4902), IBMX (I5879), rapamycin (R0395), and actinomycin D (856258). Rosiglitazone was purchased from Alexis Biochemicals (ALX-350-125).

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