Supporting Information

Puri *et al.* 10.1073/pnas.0802063105

SI Materials and Methods

Materials. C57 BL/6J (male, 10 weeks old) mice were obtained from Jackson Labs. FBS was purchased from Atlanta Biologicals. Other reagents used were all of analytical reagent grade. Human adipocytes were purchased from Lonza Walkersville. Brown fat preadipocytes were a kind gift from Ron Kahn (Joslin Diabetes Center, Boston, MA).

Cell Culture of Brown Adipocytes. Brown adipocytes were cultured as described previously (1).

Transfections and Plasmids. FSP27 and Cidea plasmid DNA was procured from Open Biosystems. For FSP27, PCR was performed by using 5′-linker having BglII restriction and 3′-linker containing EcoR1 site. For Cidea, PCR was performed by using 5′-linker having BglII restriction and 3′-linker containing BamH1 site. After cutting with the restriction enzymes, purified PCR fragment was cloned into pEGFPC1 vector (Clontech). Cidea-GFP cDNA was transfected in cells using the Lipofectamine Plus Reagent (GIBCO Life Technologies) for COS cells. The adipocytes and preadipocytes were electroporated with 5 μg of cDNA (200,000 cells per 200 μl of PBS) in a 0.4-cm cuvette at 180 V and 950 μF with a time constant of 25 msec on a Bio-Rad Gene Pulser II system.

RNA Isolation and Quantitative RT-PCR. Total RNA was isolated from 3T3-L1 adipocytes or from WAT using TRIzol reagent (Invitrogen). cDNA was synthesized using oligo(dT) primers and AMV reverse transcriptase (Roche Diagnostics) according to the manufacturer's instructions. Quantitative real-time PCR was performed in a LightCycler (Roche Diagnostics) using SYBR Green 1 PCR kit (Roche Diagnostics) and specific primers to amplify the genes. Primer sequences may be obtained on request.

Immunofluorescence in 3T3-L1 and Human Adipocytes. For determination of perilipin, cells were cultured on coverslips. Adipocytes were fixed in 4% paraformaldehyde (10 min), permeabilized with 0.05% Triton X-100 in PBS (15 min), blocked with 1% FBS in PBS (1 h), and treated with guinea pig anti-perilipin polyclonal antibody (1:2,000 dilution; Research Diagnostics) and a rabbit-anti-guinea pig Texas red-labeled secondary antibody (abCAM). Fluorescence imaging was assessed by confocal microscopy. For CIDEA immunofluorescence in human adipocytes same protocol was followed using monoclonal anti-CIDEA (Novus Biologicals) antibody and anti-mouse Alexa Fluor 488 (Molecular Probes) secondary antibody.

Oil Red O Staining for Intracellular Triglycerides. Oil red O (0.4%) in isopropanol solution was freshly made, mixed with one-half volume of H_2O, and filtered through a 0.45-μm filter. To stain for triglycerides or neutral lipids (2), cells in the monolayer were first washed three times with PBS and then fixed in 4% formaldehyde solution in PBS (20 min). After six washes fixed cells were stained with the freshly prepared oil red O solution for 10 min at room temperature, followed by six washes with water.

Glucose Conversion into Triglycerides. Incorporation of radioactive glucose into triacyl glycerol was measured according to the protocol by Rodbell (3). In short, D-[U-^{14}C]-glucose with a specific activity of 306 mCi/mmol (Amersham Biosciences) was used in the glucose conversion to triacyl glycerol. A total of 100 μl of primary fat cell suspension in KRH plus 2.5% BSA (pH 7.4) was added to 900 μl of KRH (pH 7.4) plus 2.5% BSA without pyruvate. After adding 2.5 μCi of D-[U-^{14}C]-glucose and 100 μM cold glucose the cell suspension was incubated with or without 1,000 nM insulin (Eli Lilly) for 1 h at 37°C with shaking at 100 rpm. At the end of 1 h, 5 ml of a modified Dole's extraction mixture (4) consisting of 80 ml of isopropyl alcohol, 20 ml of hexane, and 2 ml of 1 N sulfuric acid was added to each tube. The negative control tubes contained 10 μM cytochalasin B in KRH (pH 7.4) plus 2.5% BSA without pyruvate. All values were adjusted by subtracting the background cytochalasin B counts from the experimental condition counts. Each condition was performed in triplicate, and each experiment was repeated at least three times. The data represent the mean ± SEM of three experiments for each age group and condition.

Morphometric Analysis of Lipid Droplets. The morphometric analysis on lipid droplets was performed on confocal images by using Metamorph version 7.1. The cells were outlined and threshold for oil red intensity. Pixel-to-micrometer ratio was assigned, and the program measured the volume of the oil red-stained lipid droplets in cubic micrometers. In each condition, 20–25 cells were measured for total volume of droplets per cell.

Immunoblotting. Cells or tissue were solubilized with lysis buffer (1% SDS in phosphate buffer saline containing protease inhibitors) and centrifuged in a microcentrifuge at maximum speed (13,000 rpm) for 15 min, and the top fat layer was removed. Protein was quantified by using the BCA protein assay kit (Pierce) and then resolved by 10% SDS/PAGE, electrotransferred to nitrocellulose, blocked with 5% BSA and 5% nonfat milk in TBST (0.05% Tween 20 in TBS), washed with TBST, and incubated overnight at 4°C with a 1:500 dilution of Cidea antibody in TBST containing 1% BSA. The blots were then washed with TBST and treated with horseradish peroxidase anti-mouse or anti-rabbit secondary antibody for 1 h. Proteins were visualized by using an enhanced chemiluminescent substrate kit (Amersham Pharmacia Biotech).

Affymetrix GeneChip. ***Adipocytes day 0, day 6, day 9, and day 9 plus rosiglitazone.*** 3T3-L1 fibroblast cells were grown in culture for 7 days to a quiescent state (adipocyte day 0 of differentiation) before inducing the differentiation. RNA was isolated from three different cultures (grown on different days) for each condition (in duplicate) by using TRIzol reagent. Total RNA was further purified by using the RNeasy Kit from Qiagen. Affymetrix One-Cycle cDNA synthesis kit and IVT kits were used to prepare cDNA and cRNA, respectively. Affymetrix GeneChip Mouse Expression 430 A and B Arrays were used. For rosiglitazone treatment, adipocytes were treated 7 days after differentiation with 1 μM rosiglitazone for 48 h. At day 9, RNA was harvested by TRIzol reagent from treated cells as well as untreated control cells. Total RNA was further purified by using the RNeasy Kit from Qiagen. Affymetrix One-Cycle cDNA synthesis kit and IVT kits were used to prepare cDNA and cRNA, respectively. Affymetrix GeneChip Mouse Genome 430 2.0 Arrays were used.

26-week-old obese mice with or without rosiglitazone. B6V.Lepob male mice were purchased at 10 weeks of age from The Jackson Laboratory and housed in the University of Massachusetts Medical School Animal Medicine Department. Animals were treated with or without 5 mg/kg rosiglitazone each day for 2 weeks starting at 24 weeks of age. At 26 weeks of age the animals

were fasted overnight for 18 h before they were killed, and primary fat cells were isolated from epididymal fat pads as previously published (5). Total RNA was isolated from primary fat cells, purified, and treated with Affymetrix One-Cycle cDNA synthesis kit and IVT kits as previously described. Affymetrix Genechips MgU74Av2, MgU74Bv2, and MgU74Cv2 were used.

Chow, chow plus rosiglitazone, or high-fat diet. C57BL/6J mice were obtained from The Jackson Laboratory at 4 weeks of age and housed in the University of Massachusetts Medical School Animal Medicine Department. Animals were fed a normal chow diet or a high-fat diet (55% kcal from fat; Harlan Teklad catalog no. 93075) for 20 weeks treated with or without 5 mg/kg rosiglitazone each day for 2 weeks. Animals were fasted overnight for 18 h before sacrifice. Primary fat cells were isolated from epididymal fat pads as previously described. Total RNA was isolated from primary fat cells, purified, and treated with Affymetrix One-Cycle cDNA synthesis kit and IVT kits as described above. Affymetrix GeneChip Mouse Expression 430 2.0 Arrays were used.

Omental and s.c. Human Fat Tissue. Fresh human omental and s.c. tissues were procured according to procedures outlined by the University of Massachusetts Medical School Institutional Review Board, with the informed consent of patients undergoing gastric bypass surgery. Samples were obtained from fasted patients during gastric bypass surgery and frozen in liquid nitrogen. Total RNA was isolated from frozen human tissues by using TRIzol and was processed for experiments by using the methods described previously. Affymetrix GeneChip Human Genome U133 Plus 2.0 arrays were used.

GeneChip Expression Array Analysis was performed as previously published (6).

1. Fasshauer M, *et al.* (2000) Essential role of insulin receptor substrate-2 in insulin stimulation of Glut4 translocation and glucose uptake in brown adipocytes. *J Biol Chem* 275:25494–25501.
2. Tobe K, *et al.* (1987) Differential effects of DNA tumor virus nuclear oncogene products on adipocyte differentiation. *FEBS Lett* 215:345–349.
3. Rodbell M (1964) Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. *J Biol Chem* 239:375–380.
4. Dole VP (1956) A relation between non-esterified fatty acids in plasma and the metabolism of glucose. *J Clin Invest* 35:150–154.
5. Wilson-Fritch L, *et al.* (2004) Mitochondrial remodeling in adipose tissue associated with obesity and treatment with rosiglitazone. *Clin Invest* 114:1281–1289.
6. Tang X, *et al.* (2006) An RNA interference-based screen identifies MAP4K4/NIK as a negative regulator of PPARgamma, adipogenesis, and insulin-responsive hexose transport. *Proc Natl Acad Sci USA* 103:2087–2092.

Formatted Alignments

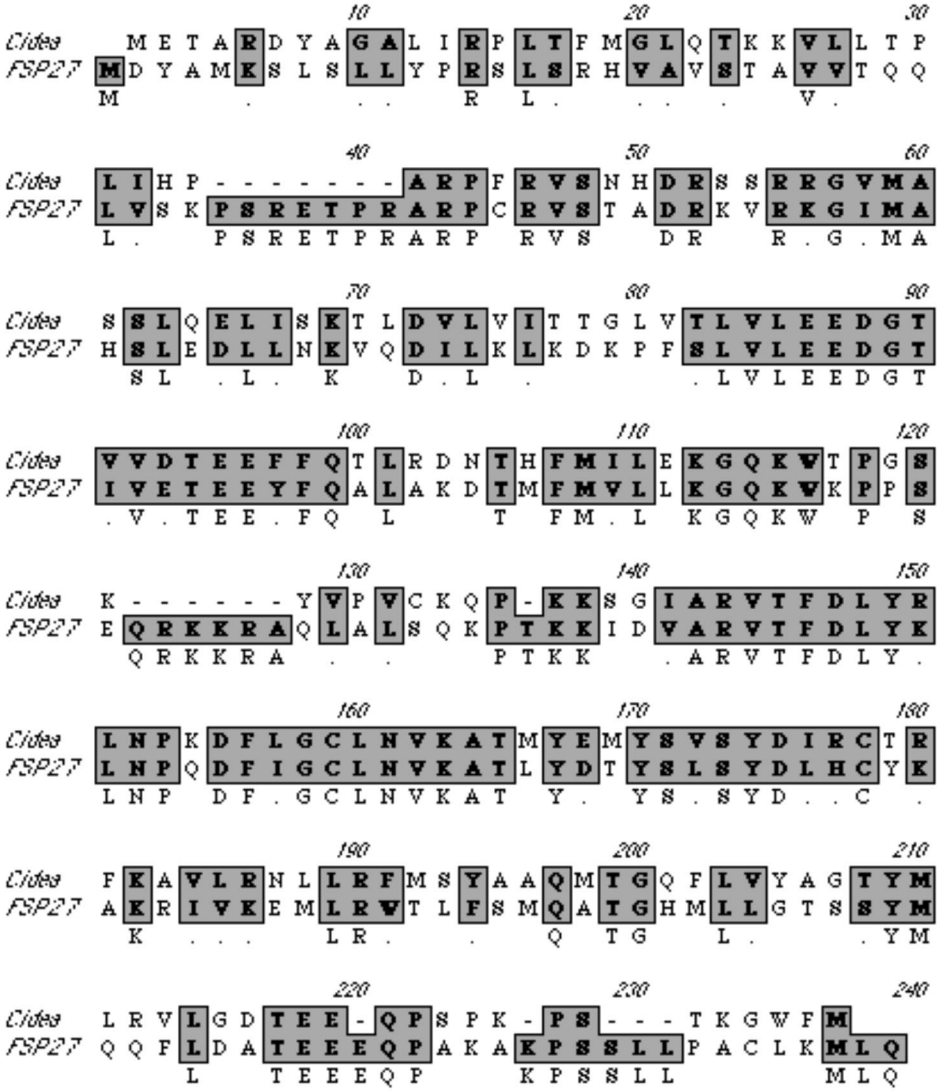

Fig. S1. Formatted alignments of protein sequences of mouse Cidea and Cidec/FSP27 isoforms using MacVector 8.1 shows ≈61% sequence similarity.

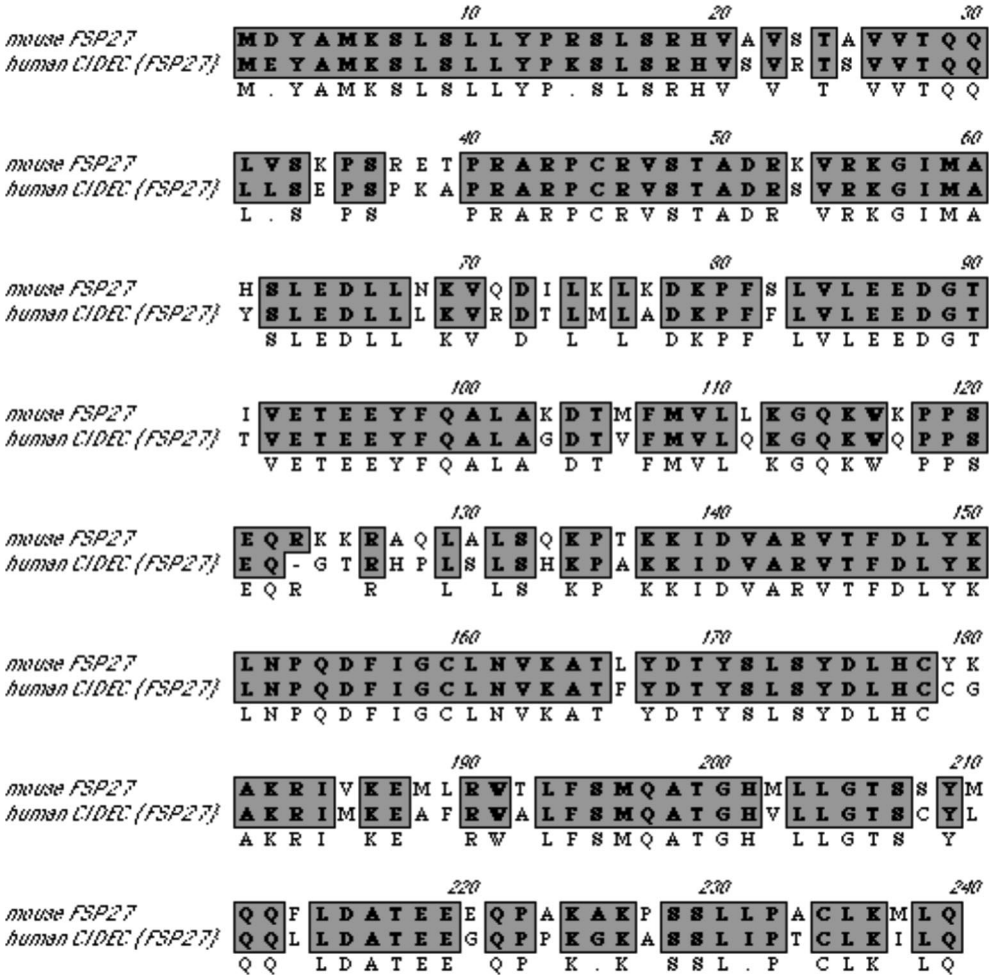

Fig. S2. Formatted alignments of protein sequences of mouse (FSP27) and human Cidec isoforms using MacVector 8.1 shows ≈90% sequence similarity.

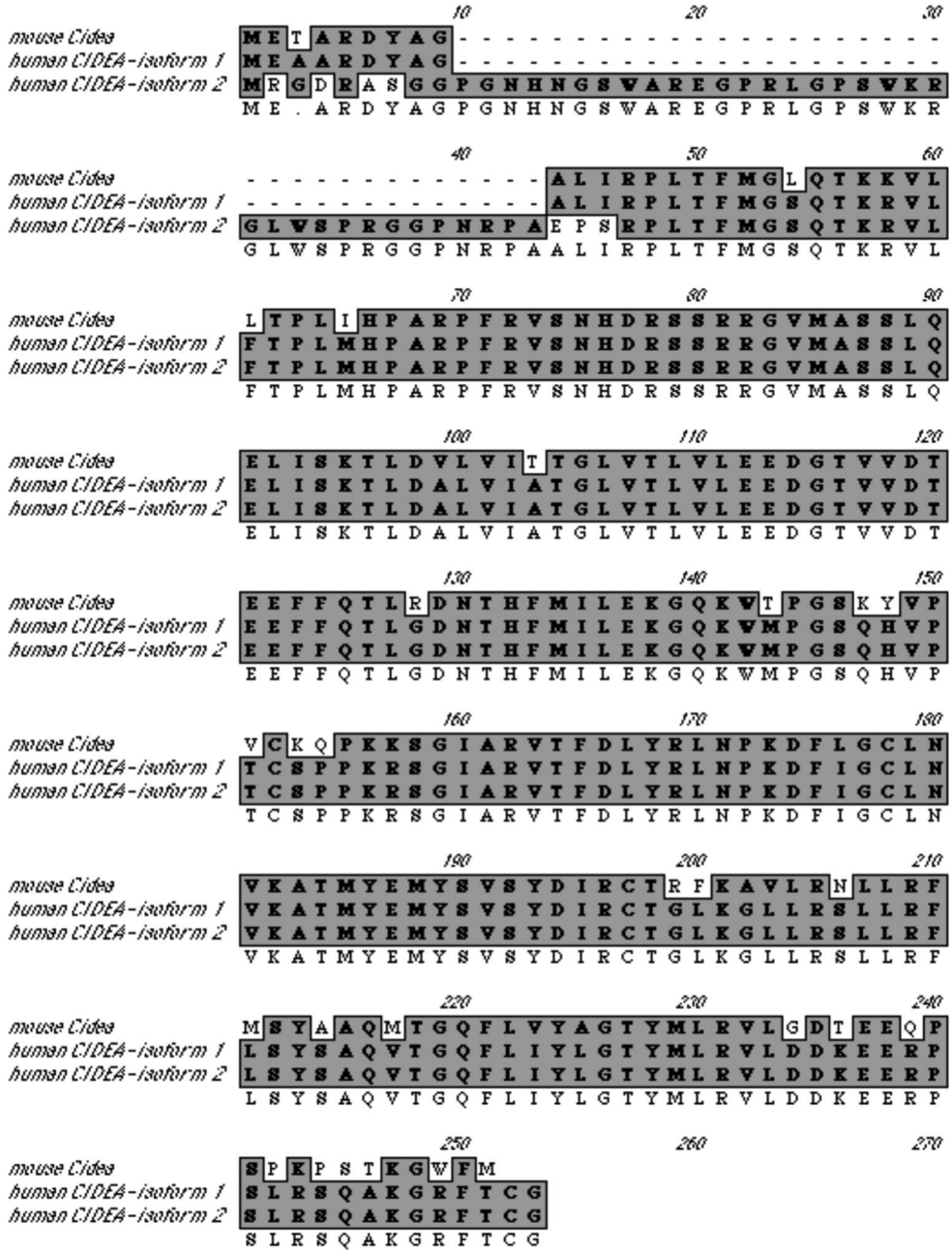

Fig. S3. Formatted alignments of protein sequences of mouse and human Cidea isoforms using MacVector 8.1 shows ≈90% sequence similarity.

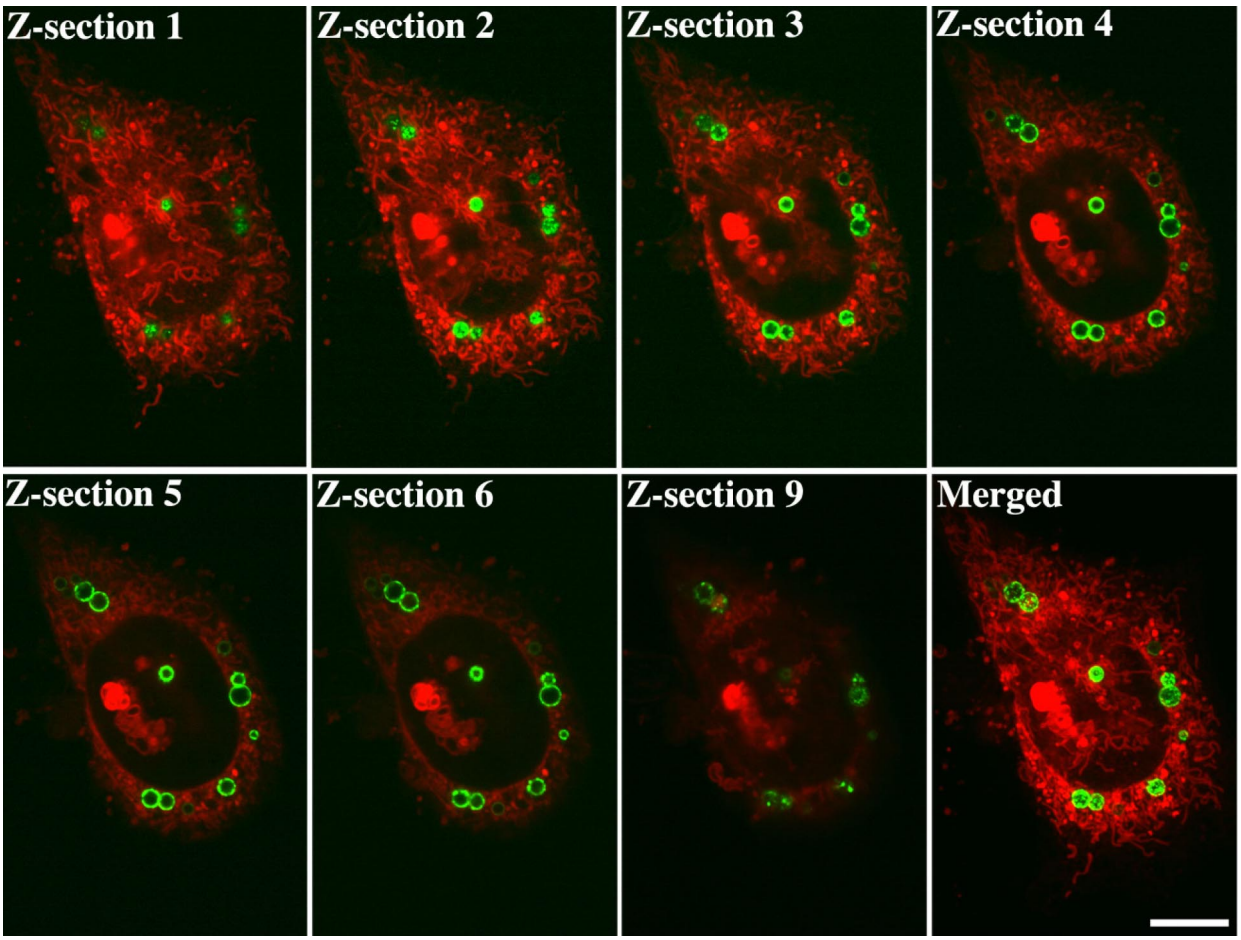

Fig. S4. Confocal Z-sections (4 μm each) of a 3T3-L1 adipocyte (day 4) representing Cidea-GFP and mitochondrial localization (MitoTracker Deep Red 633). (Scale bar: 10 μm.)

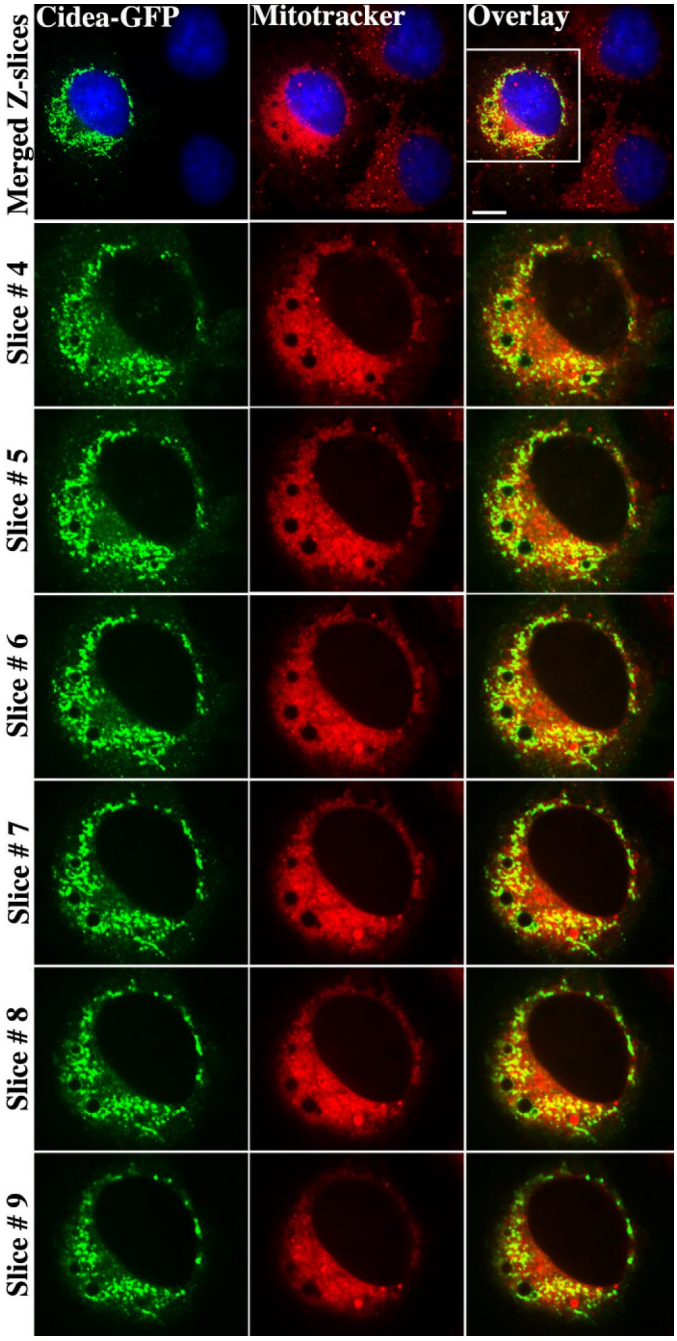

Fig. S5. Confocal Z-sections (3 μm each) of a COS cell representing Cidea-GFP and mitochondrial localization (MitoTracker Deep Red 633). Please note that, after careful analysis of all of the Z-sections, almost no colocalization of Cidea-GFP with mitochondria was observed. (Scale bar: 10 μm.)

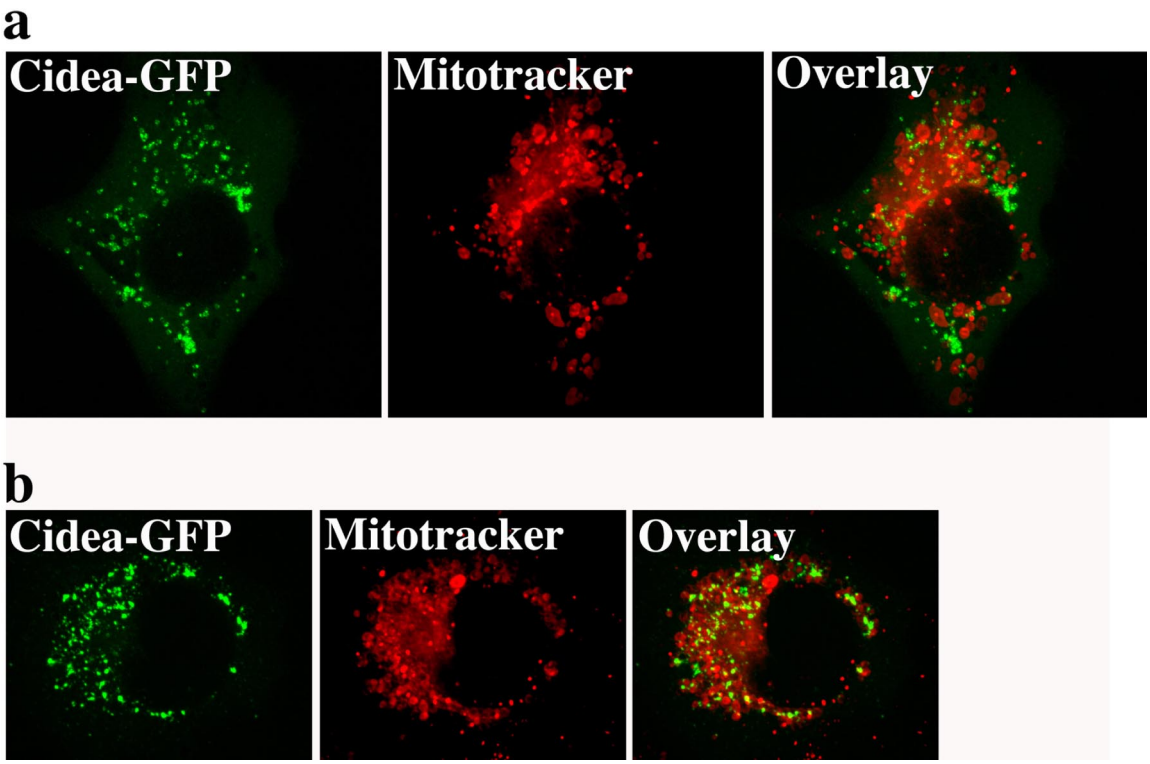

Fig. S6. Confocal microscopic images of COS cells transfected with Cidea-GFP and Mitotracker Deep Red 633. Almost no colocalization was observed between Cidea and mitochondria.

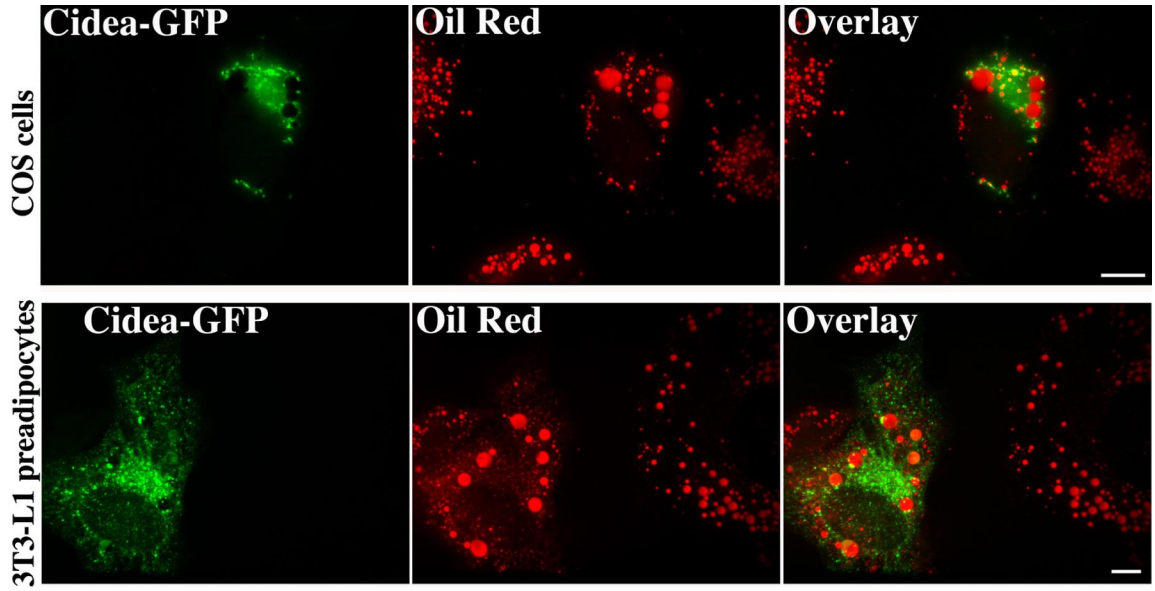

Fig. S7. Confocal images of COS cells and 3T3-L1 preadipocytes after 24 h of expression of Cidea-GFP. Eight hours after transfection a 400 μM oleic acid/BSA mixture was added to the medium. Oil red staining of lipid droplets shows enhanced lipid droplet size in Cidea-GFP expressed cells.

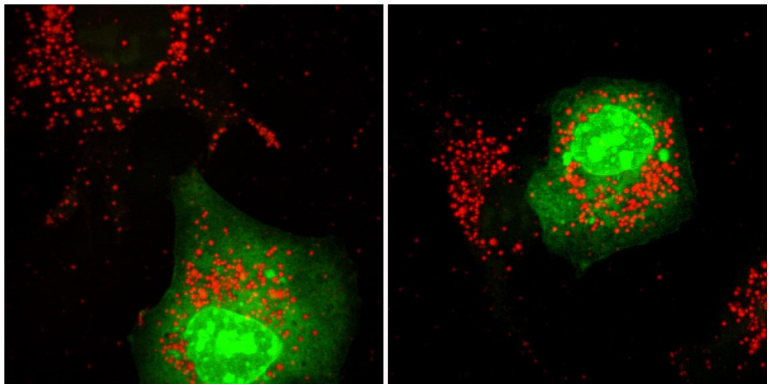

Fig. S8. Confocal images of COS cells transfected with GFP vector alone. Oil red staining shows that there was no difference in lipid droplets of untransfected and GFP-transfected cells.

Table ST1. Rosiglitazone treatment increases Cidea mRNA levels in both *in vitro* and *in vivo* conditions

	Adipocytes day 0 vs. day 6		26-week-old obese vs. 26-week-old obese + rosiglitazone		Chow vs. Chow + rosiglitazone		Adipocytes day 9 vs. day 9 + rosiglitazone		Chow vs. high-fat diet	
Genes	Fold change	*P* value	Fold change	*P* value	Fold change	*P* value	Fold change	*P* value	Fold change	*P* value
Cidea	3.1	0.023	6.0	<.0001	7.8	0.004	5.7	0.006	9.7	<.0001
FSP27	59.0	0.011	1.5	0.008	1.0	0.004	−1.0	0.967	1.0	0.407
PLIN	34.0	0.007	ND	ND	−1.2	0.181	−1.4	0.012	−1.2	0.258
TIP-47	ND	ND	ND	ND	1.24	0.049	1.27	0.061	1.31	0.144
ADRP	2.4	0.004	−1.1	0.273	1.2	0.348	2.0	0.001	1.4	0.030
MLDP	ND	ND	1.8	0.009	2.4	0.039	ND	ND	2.5	0.068
Cav-1	2.7	0.004	1.1	0.165	1.0	0.518	−1.6	0.002	1.1	0.104
CGI-58	4.3	0.093	1.6	0.007	1.2	0.116	1.3	0.026	−1.6	0.002
S3-12	6.1	0.001	−1.1	0.181	−1.2	0.037	−1.1	0.102	−1.2	0.001
Prp19	−1.4	0.094	1.4	0.076	1.1	0.42	−1.2	0.039	1.3	0.024

Table shows fold change in expression of genes encoding known lipid droplet-associated proteins and the *P* values based on Affymetrix GeneChip analysis. Total RNA was isolated from 3T3-L1 adipocytes or primary adipocytes from mouse adipose tissue (epididymal fat pads) from mice after rosiglitazone treatment. 3T3-L1 adipocytes were treated with or without μM rosiglitazone for 48 h, and mice were treated with or without 5 mg/kg rosiglitazone each day for 2 weeks.

Table ST2. Cidea functions to restrain lipolysis in human and mouse adipose tissues

	CIDEA mRNA		Lipolysis		
Conditions	Change	*P* value	Change	*P* value	Citation
Healthy (BMI 24 ± 0.5; $n = 43$) vs. obese (BMI 38 ± 0.4; $n = 143$) human subjects	↓ 50%	0.0004	↑ 200%	<0.001	20
In obese human subjects 2–4 years after bariatric surgery	↑ 200%	0.02	↓ 40%	<0.05	20
In vitro TNF-α treatment of differentiated primary human fat cells	↓ 90%	0.012	↑ 200–300%	ND	20,40
siRNA-based silencing of Cidea in preadipocytes from healthy human subjects	↓ 20–90%	ND	↑ 15–50%	0.013	20
Brown adipose tissue of Cidea-null mice vs. WT mice	↓ 100%	ND	↑ 67%	<0.001	18

Presented are compiled published results from the literature that strongly support a negative correlation of Cidea expression levels with lipolytic rates in human and mouse adipose tissues.

Table ST3. Table shows fold changes in expression of genes encoding lipid droplet-associated proteins and the *P* values based on Affymetrix GeneChip analysis

	Omental insulin-sensitive vs. insulin-resistant		Subcutaneous insulin-sensitive vs. insulin-resistant	
Genes	Fold change	*P* value	Fold change	*P* value
Cidea	−2.0	0.005	−1.4	0.332
FSP27	1.1	0.640	−1.3	0.113
PLIN	−1.0	0.706	−1.2	0.269
TIP-47	1.3	0.034	−1.3	0.297
ADRP	1.3	0.153	−1.1	0.822
CGI-58	−1.4	0.155	−2.0	0.211
S3-12	−1.1	0.298	1.3	0.082
Prp19	−1.0	0.893	−1.0	0.850

Total RNA was isolated from the omental and s.c. adipose tissues of obese patients undergoing bariatric surgery.