

Fat-specific Protein 27, a Novel Lipid Droplet Protein That Enhances Triglyceride Storage^{*[S]}

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Fat-specific protein (FSP)27/Cidec is most highly expressed in white and brown adipose tissues and increases in abundance by over 50-fold during adipogenesis. However, its function in adipocytes has remained elusive since its discovery over 15 years ago. Here we demonstrate that FSP27/Cidec localizes to lipid droplets in cultured adipocytes and functions to promote lipid accumulation. Ectopically expressed FSP27-GFP surrounds lipid droplets in 3T3-L1 adipocytes and colocalizes with the known lipid droplet protein perilipin. Immunostaining of endogenous FSP27 in 3T3-L1 adipocytes also confirmed its presence on lipid droplets. FSP27-GFP expression also markedly increases lipid droplet size and enhances accumulation of total neutral lipids in 3T3-L1 preadipocytes as well as other cell types such as COS cells. Conversely, RNA interference-based FSP27/Cidec depletion in mature adipocytes significantly stimulates lipolysis and reduces the size of lipid droplets. These data reveal FSP27/Cidec as a novel adipocyte lipid droplet protein that negatively regulates lipolysis and promotes triglyceride accumulation.

Adipose tissue is a major determinant of whole body glucose homeostasis and insulin sensitivity, as evidenced by its ability to secrete bioactive peptides and control lipid storage (1–6). The nuclear receptor peroxisome proliferator-activated receptor- γ promotes adipogenesis and enhances these functions, acting in mice and humans to increase insulin signaling and glucose tolerance (7–12). Several proteins that are highly and selectively expressed in adipocytes, such as the secreted proteins adiponectin and leptin as well as adipsin, are under the control of peroxisome proliferator-activated receptor- γ (3–5, 13, 14). These and other adipocyte proteins are highly up-regulated during adipogenesis and confer unique characteristics to these cells, including high capacity to store triglyceride and release fatty acids.

Fat-specific protein (FSP)27, also denoted as CIDEC for the human homolog (15), was discovered over 15 years ago to be strikingly up-regulated during adipogenesis and is highly

expressed in both white and brown adipose tissues (15–18). However, the question of FSP27/Cidec function has remained unsolved. The results we present here demonstrate FSP27/Cidec to be a novel lipid droplet protein that shares many features characteristic of the lipid droplet protein perilipin (19, 20), including the ability to enhance neutral lipid accumulation when expressed in 3T3-L1 preadipocytes or even COS cells. Our data also reveal that a mechanism whereby FSP27/Cidec functions to promote triglyceride deposition in adipocytes is by inhibiting lipolysis. FSP27 is thus a major new modulator of lipid droplet function that is required for optimal storage of triglycerides by adipocytes.

EXPERIMENTAL PROCEDURES

Materials—C57BL/6J (male, 10 weeks old) mice were obtained from The Jackson Laboratory. Human insulin was obtained from Lilly. Fetal bovine serum was purchased from Atlanta Biologicals, Inc. (Lawrenceville, GA). Other reagents used were all of analytical reagent grade.

siRNA—siRNA was purchased from Dharmacon (Chicago). Scrambled was 5'-CAGUCGCGUUUGGACUGG-3'; FSP27 was 5'-CAACUAAGAAGAUCGAUGUUU-3'.

Cell Culture and siRNA Transfection in 3T3-L1 Adipocytes—3T3-L1 fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 μ g/ml streptomycin, and 50 units/ml penicillin and differentiated into adipocytes as described previously (21). 3T3-L1 adipocytes were transfected with siRNA duplexes by electroporation on the 4th or 8th day following the induction of differentiation (day 4 or day 8) as described previously (21–23). The efficiency of siRNA electroporation using this method is greater than 95%, based on fluorescence microscopy of cells electroporated with Cy3-siRNA.

Transfections and Plasmids—FSP27/Cidec² plasmid DNA was procured from Open Biosystems. PCR was performed by using 5'-linker having BallI restriction and 3'-linker containing EcoRI site. After cutting with the enzymes (BallI and EcoRI), the purified PCR fragment was cloned into pEGFPC1 vector (Clontech). GFP-FSP27 cDNA was transfected in 3T3-L1 adipocytes using the Lipofectamine Plus reagent (Invitrogen). The efficiency of transfection using Lipofectamine Plus reagent was very low. The transfection was also carried out by electroporat-

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[S] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. 1.

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² The abbreviations used are: Cidec, cell death-inducing DNA fragmentation factor, α subunit-like effector C; siRNA, short interfering RNA; PBS, phosphate-buffered saline; BAT, mouse brown adipose tissue; WAT, white adipose tissue; PMSF, phenylmethylsulfonyl fluoride.

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ing the adipocytes with 5 μ g of cDNA (200,000 cells/200 μ l of PBS) in a 0.4-cm cuvette at 180 V and 950 microfarads with a time constant of 20 ms on a Bio-Rad Gene Pulser II system.

Immunofluorescence in 3T3-L1 Adipocytes—For determination of perilipin localization, 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% fetal bovine serum in PBS (1 h) and treated with guinea pig anti-perilipin polyclonal antibody (1:2000 dilution; Research Diagnostics Inc., Flanders, NJ) and a rabbit anti-guinea pig Texas Red-labeled secondary antibody (abCAM, Cambridge, MA). For FSP27 immunostaining, 3T3-L1 adipocytes plated on coverslips were washed twice with PBS and fixed in 4% formaldehyde for 25 min at room temperature, washed three times with PBS, permeabilized with 0.5% Triton X-100 for 5 min, and then washed three times again with PBS. The cells were then exposed to affinity-purified rabbit polyclonal FSP27 antibodies (obtained from Drs. Kasuga and Nishino, Kobe University Graduate School of Medicine, Kobe, Japan) for 60 min at room temperature after blocking with 2% bovine serum albumin in PBS for 60 min at room temperature. Following three washes with PBS, the cells were treated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes; 1:1000 dilution) for 60 min at room temperature. Fluorescence imaging was assessed by confocal microscopy.

Oil Red-O Staining for Intracellular Triglycerides—Oil Red-O (0.4%) in isopropyl alcohol solution was freshly made, mixed with one-half volume of H₂O, and filtered through 0.45- μ m filter. To stain for triglycerides or neutral lipids, 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. For quantitating Oil Red-O staining, imaging was performed using a wide field fluorescence microscope with a \times 63 Nikon PlanApo objective. In any given experiment, all pictomicrographs were exposed identically and subtracted for background where the background was determined by measuring the fluorescence intensity of a section of the image without cells. The fluorescence intensity of Oil Red-O was measured using Axiovision digital imaging software version 4.1 (Carl Zeiss) by marking the periphery of individual cells.

Assay to Measure Glycerol Release—The cultured adipocytes were washed twice with PBS and incubated in Krebs-Ringer bicarbonate HEPES buffer supplemented with 4% bovine serum albumin in the presence or absence of epinephrine and theophylline. After 3 h of incubation, the buffer was collected, and glycerol contents were measured using free glycerol determination kit (Sigma) by following the manufacturer's protocol.

RNA Isolation and Quantitative Reverse Transcription-PCR—Total RNA was isolated from 3T3-L1 adipocytes or from WAT using TRIzol reagent (Invitrogen). cDNA was synthesized using oligo(dT) primers and avian myeloblastosis virus 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.

Confocal Microscopy—Images were taken on a Zeiss Axio-phot microscope equipped with a Hamamatsu digital camera and processed using Metamorph imaging software, version 6.1 (Universal Imaging, Downingtown, PA).

RESULTS AND DISCUSSION

FSP27/Cidec is a member of the CIDE family of proteins that share a conserved amino-terminal CIDE-N domain and COOH-terminal CIDE-C domain (Fig. 1A). Three CIDEs have been reported in mouse (Cidea, Cideb, and FSP27/Cidec) and human (CIDEA, CIDEB, and CIDE C) (24). The CIDE-N domain of CIDE proteins has significant homology to the regulatory domains of the apoptotic DNA fragmentation factors, DFF40 (caspase-activated nuclease) and DFF45 (inhibitor) (16, 25). The CIDE proteins, including FSP27/Cidec, can promote apoptosis when highly expressed ectopically in cultured cells (24). However, FSP27/Cidec was originally identified as a highly expressed, endogenous adipocyte-specific protein by subtractive cloning techniques that provided no insight into its function in these cells (16, 25). We confirmed that FSP27/Cidec is highly expressed in mouse WAT and BAT, although detectable expression was also observed in mouse skeletal muscle and liver (data not shown). Our analysis of FSP27/Cidec expression in 3T3-L1 cells also indicated a 59-fold increase in FSP27/Cidec mRNA expression during adipogenesis, consistent with previous findings (16). The Novartis GNF gene atlas shows that the human homolog of FSP27, CIDE C, is highly expressed in human white adipose tissue (15), with little or no expression detectable in other tissues.

Previous studies have suggested FSP27/Cidec may be partially localized with mitochondria in COS cells, based on expression of a fusion protein of FSP27/Cidec and green fluorescent protein (FSP27-GFP) (24). To obtain clues to FSP27/Cidec function specifically in adipose cells, we prepared a similar FSP27-GFP construct and expressed this protein in fully differentiated 3T3-L1 adipocytes. Fig. 1B shows a typical adipocyte expressing this construct. FSP27-GFP is strikingly localized to distinct spherical structures, as visualized by confocal microscopy at multiple optical planes and restored as a projected image. In the adipocyte shown in Fig. 1B, it is also apparent that the spherical structures surrounded by FSP27-GFP are lipid droplets heavily stained with Oil Red-O, which partitions in neutral lipid (26, 27). The efficiency of transfection using Lipofectamine was very low. We also used an electroporation method for transfecting the cDNA, which increased the transfection efficiency with no difference in the results. Interestingly, a previous report listed FSP27/Cidec as one of many proteins found to be present in preparations of lipid droplets after their isolation from adipocytes (28). However, in those studies microscopic examination of FSP27/Cidec localization was not performed.

To confirm that endogenous FSP27/Cidec also localized to lipid droplets, immunofluorescence microscopy was performed on 3T3-L1 adipocytes using affinity-purified FSP27/Cidec antibody. As shown in Fig. 1, C and D, FSP27/Cidec was observed concentrated around the lipid droplets. The pattern of FSP27/Cidec localization around the lipid droplets appeared to be discontinuous rather than a continuous ring (Fig. 1E).

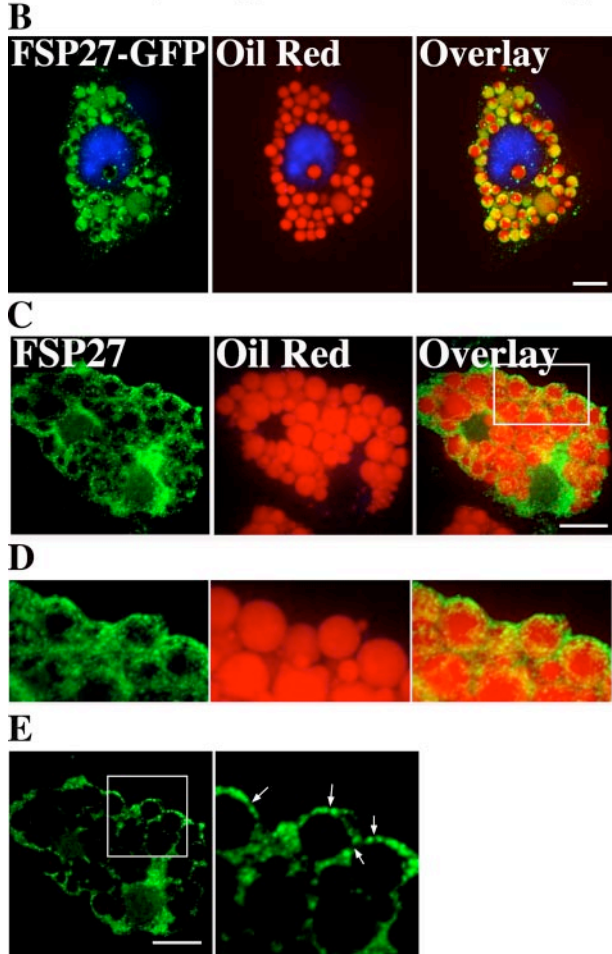
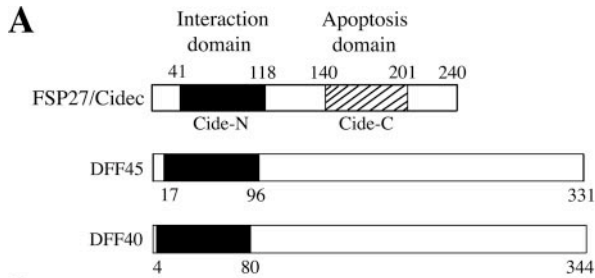


FIGURE 1. Expressed FSP27-GFP localizes to the surface of lipid droplets in 3T3-L1 adipocytes. *A*, graphic representation of FSP27 showing amino-terminal CIDE-N domain and COOH-terminal CIDE-C domain. CIDE-N domain of FSP27 has a significant homology to the CIDE-N domain of DFF-45 and DFF-40. *B*, confocal microscopic image of mature 3T3-L1 adipocyte (day 8) expressing FSP27-GFP (left panel) after 48 h of transfecting FSP27-GFP cDNA. The middle panel shows lipid droplets stained with Oil Red-O. Bar, 10 μ m. *C*, immunostaining of endogenous FSP27/Cidec in a 3T3-L1 adipocyte (day 9) using primary affinity-purified antibodies against mouse FSP27. Bar, 10 μ m. *D*, inset from Fig. 1C is enlarged to show the localization of FSP27 around the lipid droplets. *E*, Z-section (1 μ m) representing FSP27/Cidec immunostaining in a 3T3-L1 adipocyte. The inset from the left panel is enlarged in the right-hand side panel showing discontinuous staining pattern of FSP27/Cidec (arrows). Bar, 10 μ m.

Some punctate cytoplasmic staining was also observed in these experiments, although this could be due to some nonspecific binding of the antibody.

We next attempted to resolve the discrepancy between the data shown in Fig. 1 suggesting FSP27/Cidec is a lipid droplet protein in adipocytes versus the published data indicating it partially colocalizes with mitochondria in COS cells (24). Care-

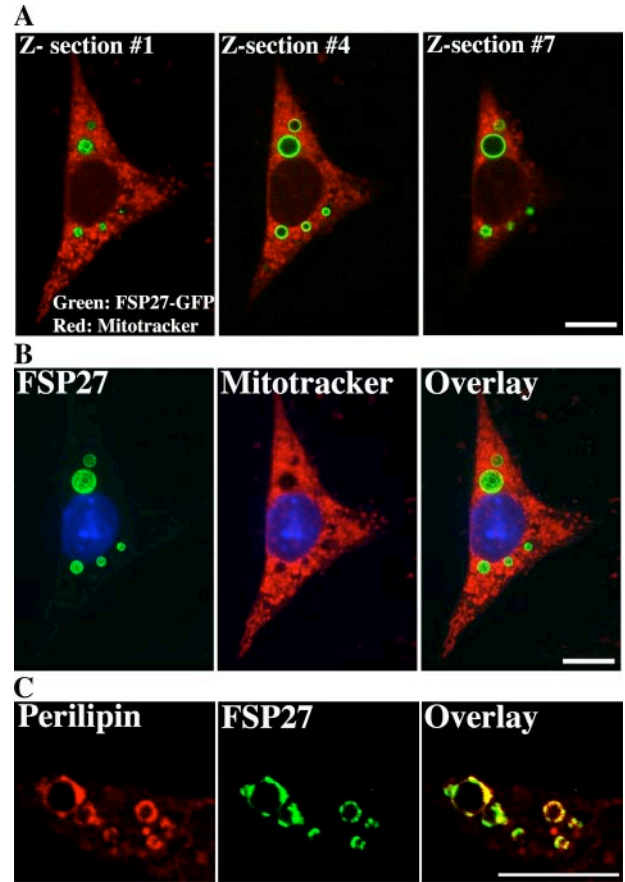


FIGURE 2. Expressed FSP27-GFP localizes on the surface of lipid droplets and colocalizes with perilipin in 3T3-L1 adipocytes. *A*, selected confocal Z-sections (3 μ m each) of a 3T3-L1 adipocyte (day 4) expressing FSP27-GFP after 48 h of transfecting FSP27-GFP cDNA. Mitochondria were stained with MitoTracker Deep Red 633 (Molecular Probes, Inc., Eugene, OR) according to the manufacturer's protocol. Total 13 Z-sections of 3 μ m each were obtained (see supplemental Fig. 1). Bar, 10 μ m. *B*, confocal images (merged optical planes) of a 3T3-L1 adipocyte (day 4) after 48 h of expressing FSP27-GFP (left panel) and mitochondria stained with MitoTracker Deep Red 633 (middle panel). Bar, 10 μ m. All the Z-sections are displayed individually in supplemental Fig. 1. *C*, 4 μ m Z-section of a confocal image of 3T3-L1 adipocyte showing colocalization of FSP27-GFP and perilipin. Bar, 10 μ m.

ful high resolution confocal microscopy was performed on adipocytes expressing both FSP27-GFP and the mitochondria-localized MitoTracker Red 633 dye. As shown in Fig. 2A, FSP27-GFP is strikingly localized to distinct spherical structures visualized by confocal microscopy at various optical planes (Fig. 2A; also see supplemental Fig. 1). Merged images at all optical planes through the cultured adipocytes again confirmed FSP27-GFP localizes to large spherical structures (Fig. 2B). Virtually no colocalization of FSP27-GFP with MitoTracker could be observed. We also failed to observe significant colocalization of expressed FSP27-GFP with mitochondria in COS cells (data not shown). This disposition of FSP27-GFP is reminiscent of the first described endogenous lipid droplet-associated protein perilipin, which also localizes to the periphery of intracellular neutral lipid storage droplets in adipocytes (20, 29, 30). The localization of FSP27-GFP in fully differentiated adipocytes was thus compared with that of endogenous perilipin detected by immunofluorescence using anti-perilipin antibody. A 0.4- μ m optical Z-section from a mature adipocyte is shown in Fig. 2C, which depicts extensive colocalization of expressed FSP27-GFP

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with endogenous perilipin. Taken together, the data in Figs. 1 and 2 reveal FSP27/Cidec as a novel lipid droplet protein with a cellular disposition similar to the known lipid droplet protein perilipin.

Perilipin is known to strongly enhance triacylglycerol storage by inhibiting lipolysis in adipocytes (31–36). Therefore, we tested the effect of FSP27-GFP expression in 3T3-L1 preadipocytes on total neutral lipid accumulation by staining the cells with Oil Red-O. These experiments revealed that preadipocytes expressing FSP27-GFP display lipid droplets of increased size compared with neighboring untransfected cells (Fig. 3A). In these transfected 3T3-L1 preadipocytes, FSP27-GFP was either associated with these lipid droplets in punctate structures or appeared as distinct rings around the periphery of lipid droplets (Fig. 3A). Quantification of the Oil Red-O intensity in cells transfected with FSP27/Cidec revealed significantly higher neutral lipid stores as compared with control, untransfected preadipocytes (Fig. 3C). A similar result was obtained when FSP27-GFP was expressed in COS cells (Fig. 3, B and C) or Chinese hamster ovary cells (not shown). In all cases, FSP27-GFP expression increased lipid droplet size and increased total neutral lipid.

Consistent with the concept that perilipin has little or no effect on the triacylglycerol synthetic pathway while inhibiting triglyceride hydrolysis (31), adipocytes from perilipin null mice exhibit increased basal lipolysis and a blunted response to lipolytic agents such as catecholamines (37). We thus tested the effects of either perilipin or FSP27/Cidec depletion on lipolysis in cultured adipocytes. As shown in Fig. 4B, siRNA-directed silencing of either of these proteins elicited significant increases in glycerol release under basal conditions, as has been observed previously in perilipin-deficient adipocytes (32). About an 80% decrease in FSP27 mRNA and a 90% decrease in perilipin mRNA expression was observed (Fig. 4C). Previous studies have revealed that upon stimulation of adipocytes by β -adrenergic agents, perilipin is rapidly phosphorylated and appears to be required for optimal responsiveness to lipolytic agents (32, 38, 39). Surprisingly, FSP27/Cidec or perilipin depletion did not compromise the responsiveness of 3T3-L1 adipocytes to either sub-maximal or maximal concentrations of epinephrine plus theophylline in the present experiments (Fig. 4B). It is possible that the failure of perilipin or FSP27/Cidec depletion to attenuate the stimulated lipolytic rates observed in response to catecholamine and theophylline is because of incomplete depletion of these proteins. Nonetheless, basal lipolysis is clearly up-regulated by the partial loss of either of these proteins under the conditions of these experiments.

Interestingly, when FSP27/Cidec is depleted from fully differentiated, cultured adipocytes, the lipid droplets were uniformly dispersed into smaller structures than those displayed by control cells (Fig. 4A). This phenomenon also occurs upon chronic stimulation of lipolysis by catecholamines, but it appears not to be required for optimal lipolytic rates under those conditions (40, 41). These data are consistent with the hypothesis that FSP27/Cidec may function to promote the formation of the large lipid droplets seen in primary fat cells. The formation of lipid droplets from the endoplasmic reticulum

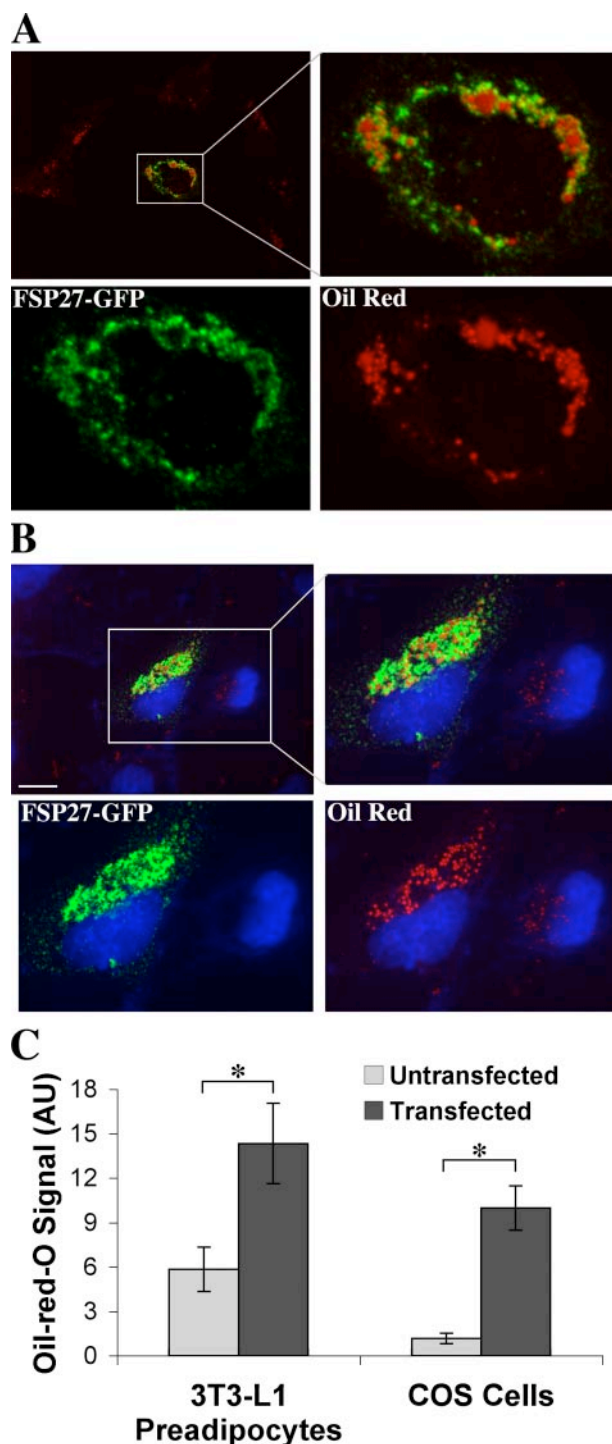


FIGURE 3. The expression of FSP27-GFP in 3T3-L1 preadipocytes and COS cells augments intracellular neutral lipid accumulation. A and B, confocal microscopic images of 3T3-preadipocytes (A) and COS cells (B) transfected with the FSP27-GFP cDNA construct. The cells were fixed and stained with Oil Red-O that stains neutral lipids. Bar, 10 μ m. C, semi-quantitative analysis of neutral lipids calculated by measuring the Oil Red-O signal. Imaging was performed using a wide field microscope with a $\times 63$ Nikon PlanApo objective. The fluorescence intensity was determined using Axiovision digital imaging software version 4.1 (Carl Zeiss). Results are an average of 25 or more cells from different fields in each case from three independent experiments \pm S.E. (*, $p < 0.0001$; paired t test). AU, arbitrary units.

requires a complex series of steps and components, and the size of droplets likely depends on a carefully controlled pathway (42, 43). Our data to date do not permit us to determine the step or

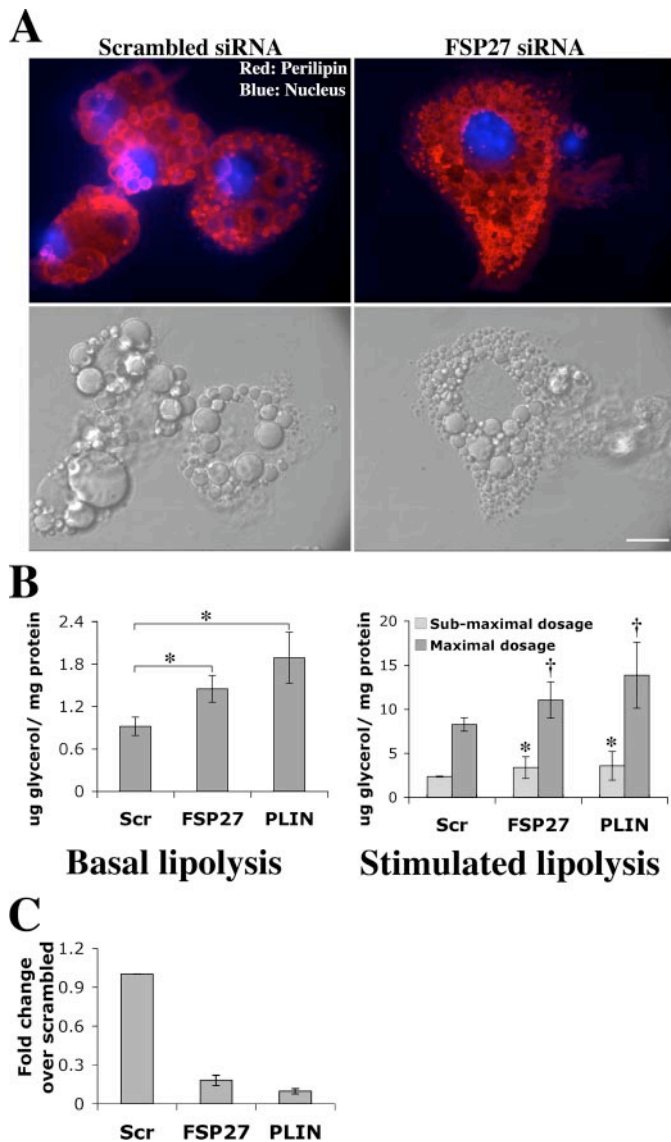


FIGURE 4. FSP27/Cidec depletion causes fragmentation of lipid droplets and increased basal lipolysis in mature adipocytes. *A*, after depleting FSP27/Cidec in mature adipocytes (7 days after initiating differentiation), the cells were immunostained with anti-perilipin. The *top right panel* represents a confocal image of a FSP27-depleted adipocyte with fragmented lipid droplets where perilipin is still associated with the microdroplets. *Top left panel* shows control adipocytes. *Lower panels* are the phase contrast images of the respective cells in the *top panels*. Bar, 10 μm . *B*, glycerol release was measured in 3T3-L1 adipocytes after siRNA-mediated depletion of FSP27 and perilipin. Approximately 70% depletion for FSP27/Cidec and 90% depletion for perilipin mRNA expression was observed. For stimulation, sub-maximal (150 nM epinephrine + 60 μM theophylline) and maximal (1.5 μM epinephrine + 0.6 mM theophylline) doses of agents were used. Data are means \pm S.E. for more than three independent experiments (*, $p \leq 0.05$ (paired *t* test) compared with scrambled with sub-maximal dose; †, $p \leq 0.05$ (paired *t* test) compared with scrambled with maximal dose). *C*, quantitation of FSP27 and perilipin knockdown using quantitative real time PCR analysis. Data are means \pm S.E. for three independent experiments.

steps in this process that FSP27/Cidec regulates, a question that future studies will address. Also of interest is whether the apparent fragmentation of lipid droplets upon loss of FSP27/Cidec is directly related to the increase in lipolysis observed under these conditions. Nonetheless, the data in Fig. 4 show that loss of FSP27/Cidec both enhances basal lipolytic rates and disperses lipid droplets in 3T3-L1 adipocytes. In a similar study,

Nishino *et al.*³ have also observed that FSP27/Cidec contributes to efficient energy storage in WAT by promoting formation of unilocular lipid droplets in adipocytes.

Perilipin and other related lipid droplet proteins such as ADRP and TIP47 contain PAT domains involved in their lipid binding function, but other lipid droplet proteins do not seem to contain this domain (for overview, see Ref. 44). FSP27/Cidec does not contain a highly conserved PAT domain, nor does it apparently display protein kinase A phosphorylation sites characteristic of perilipin. Instead, FSP27/Cidec contains CIDE domains and shares significant sequence similarity to Cidea (25), a BAT protein in mouse that has been reported to be mitochondrial when expressed ectopically in cultured cells (45). Interestingly, Cidea is poorly expressed in WAT in the mouse such that its function seems to be mostly restricted to BAT in this animal. Cidea-null mice have a lean phenotype with resistance to diet-induced obesity and diabetes (45). This phenotype was attributed to the ability of Cidea to apparently inhibit the activity of the mitochondrial uncoupling protein UCP1. However, it will be interesting to further explore this hypothesis in light of the data presented here indicating a Cidea isoform, FSP27/Cidec, is a lipid droplet protein rather than a mitochondrial protein. Our results reveal the important function of FSP27/Cidec to augment lipid storage during and after adipogenesis, which in turn likely plays an important role in whole body energy homeostasis. It will be interesting in future studies to determine the structural basis for interaction of FSP27/Cidec with lipid droplets and to unravel its mechanism of action to negatively regulate lipolysis.

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