

# Adipocyte differentiation-related protein reduces the lipid droplet association of adipose triglyceride lipase and slows triacylglycerol turnover<sup>S</sup>

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**Abstract** Although neutral lipid storage droplets are ubiquitous in eukaryotic cells, very little is known about how their synthesis and turnover are controlled. Adipocyte differentiation-related protein (ADRP; also known as adipophilin) is found on the surface of lipid droplets in most mammalian cell types. To learn how ADRP affects lipid storage, we stably expressed the protein in human embryonic kidney 293 (HEK 293) cells, which express little endogenous ADRP. As expected, ADRP was targeted to the surface of lipid droplets and caused an increase in triacylglycerol (TAG) mass under both basal and oleate-supplemented conditions. At least part of the increased mass resulted from a 50% decrease in the rate of TAG hydrolysis in ADRP-expressing cells. Furthermore, ADRP expression increased the fraction of total cellular TAG that was stored in lipid droplets. ADRP expression induced a striking decrease in the association of adipose triglyceride lipase (ATGL) and mannose-6-phosphate receptor tail-interacting protein of 47 kDa with lipid droplets and also decreased the lipid droplet association of several other unknown proteins. Transient expression of ADRP in two other cell lines also reduced the lipid droplet association of catalytically inactive ATGL. **■** We conclude that the reduced lipid droplet association of ATGL and/or other lipases may explain the decrease in TAG turnover observed in ADRP-expressing HEK 293 cells.—Listenberger, L. L., A. G. Ostermeyer-Fay, E. B. Goldberg, W. J. Brown, and D. A. Brown. Adipocyte differentiation-related protein reduces the lipid droplet association of adipose triglyceride lipase and slows triacylglycerol turnover. *J. Lipid Res.* 2007. 48: 2751–2761.

**Supplementary key words** adipophilin • PAT proteins • ADRP • ADFP • PLIN2

Eukaryotes store lipid in cytosolic lipid droplets, which consist of neutral lipid cores surrounded by phospholipid monolayers (1–3). In mammals, lipid droplets are most abundant in adipose tissue, where stored triacylglycerol

(TAG) provides the primary energy reserve for the organism. Lipid droplets in steroidogenic cells contain cholesterol esters used in the synthesis of steroid hormones. Most other mammalian cells contain smaller lipid droplets, whose function remains unclear. They may serve as local energy reserves or sources of lipid for membrane synthesis. Furthermore, they may protect cells from the harmful effects of excess lipid accumulation by sequestering toxic lipid species away from pathways that lead to cell death (4, 5).

Mechanisms controlling the synthesis and turnover of lipid droplets are only partially understood. According to one model of lipid droplet biogenesis, newly synthesized neutral lipids accumulate inside the endoplasmic reticulum membrane, forming a disk that eventually buds into the cytoplasm surrounded by an endoplasmic reticulum-derived phospholipid monolayer (2, 6). Conversely, lipid droplet turnover occurs via the hydrolysis of stored neutral lipids by cytosolic lipases. Much of what we know about the regulation of lipolysis stems from studies in adipocytes. In response to hormone stimulation, protein kinase A phosphorylates two key substrates: hormone-sensitive lipase (HSL) (7) and perilipins (8, 9). Phosphorylation of HSL stimulates both its activity and its association with lipid droplets, in a manner that depends on perilipins.

Perilipins regulate TAG hydrolysis in two ways (1, 9, 10). First, they greatly enhance TAG turnover under lipolytic conditions by recruiting HSL to lipid droplets and/or by activating it on the lipid droplet surface (7, 11–13). Second, perilipins inhibit TAG turnover under basal con-

Abbreviations: ADRP, adipocyte differentiation-related protein; ATGL, adipose triglyceride lipase; BHK, baby hamster kidney; GFP, green fluorescent protein; HEK 293, human embryonic kidney 293; HSL, hormone-sensitive lipase; TAG, triacylglycerol; TIP47, mannose-6-phosphate receptor tail-interacting protein of 47 kDa; 293/ADRP, ADRP-expressing HEK 293.

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ditions, at least in part by reducing lipid droplet-associated lipase activity. As a result, perilipin-deficient mice are lean and resistant to diet-induced obesity (14, 15). Furthermore, exogenous expression of perilipin A, the predominant isoform, increases lipid accumulation by reducing the basal rate of TAG turnover (16–18). Londos and colleagues (16) have proposed that perilipins may prevent hydrolysis by coating lipid droplets, sterically blocking cytosolic lipases from accessing the surface of lipid droplets in resting adipocytes.

Although HSL has long been considered the key regulator of lipid metabolism in adipocytes, HSL-deficient mice are not obese and accumulate diacylglycerol instead of TAG (19). This surprising finding stimulated a search for other lipases important in TAG turnover. Several observations support a role for one such enzyme, adipocyte triglyceride lipase (ATGL; also known as desnutrin, patatin-like phospholipase domain-containing 2, and calcium-independent phospholipase A2- $\zeta$ ) (20, 21). ATGL hydrolyzes TAG efficiently and is expressed at high levels in adipocytes, at moderate levels in cardiac muscle, and at lower levels in other tissues (22, 23). Orthologs of ATGL are present in *Drosophila melanogaster* (24) and *Saccharomyces cerevisiae* (25, 26), and defects in either lead to excess lipid accumulation. Moreover, genetic ablation of ATGL in mice increases adipose tissue mass and promotes lipid storage in multiple tissues (27). Identifying the mechanisms of ATGL regulation is an active area of research. It is not yet known whether ATGL is activated by perilipins or by any other proteins.

Several proteins with sequence similarity to perilipins have been identified. These form the PAT family, named for its founding members (Perilipin, ADRP, and TIP47). All known PAT family proteins associate with the surface of lipid droplets (9, 28, 29), although mannose-6-phosphate receptor tail-interacting protein of 47 kDa (TIP47) is also present in the cytosol and assists in trafficking of the mannose 6-phosphate receptor (30). In contrast to perilipins, which are expressed only in adipocytes and steroidogenic cells (9, 10), adipocyte differentiation-related protein (ADRP) and TIP47 are widely expressed in mammalian cells (28, 30, 31). The key role of perilipins in adipocytes suggests that at least some PAT family proteins may regulate lipid storage in other cells.

Several studies have linked ADRP to neutral lipid accumulation. ADRP is generally upregulated in parallel with stored lipid during lipid droplet formation and is present on the surface of lipid droplets from the earliest time of their synthesis (28, 32, 33). Moreover, exogenous expression of ADRP leads to an increase in neutral lipid mass and lipid droplet overaccumulation (32, 34, 35). ADRP overexpression in cultured hepatocytes slows TAG turnover and simultaneously decreases TAG secretion in VLDL (35). On the other hand, suppression of ADRP expression in mice significantly reduces hepatic TAG accumulation (36). Together, these findings suggest a role for ADRP in lipid droplet homeostasis. However, the mechanism by which the protein promotes lipid storage is not known.

To further probe the effect of ADRP on lipid storage, we expressed the protein in human embryonic kidney 293 (HEK 293) cells, which normally express ADRP at very low levels. ADRP expression increased cellular TAG levels and reduced the rate of TAG turnover. Moreover, ADRP expression reduced the association of ATGL with lipid droplets. Transiently overexpressed ADRP had the same effect on ATGL in two other cell lines. These data raise the possibility that ADRP may slow TAG hydrolysis in HEK 293 cells at least in part by reducing the association of hydrolases with lipid droplets.

## MATERIALS AND METHODS

### Antibodies

Guinea pig polyclonal antibodies against ADRP and TIP47 and mouse monoclonal anti-ADRP antibodies were from the RDI Division of Fitzgerald Industries (Concord, MA). Other mouse monoclonal antibodies were supplied as follows: anti-green fluorescent protein (GFP) antibodies were from Clontech (Mountain View, CA); anti-HSP90 $\alpha/\beta$  antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-transferrin receptor antibodies were from Zymed (South San Francisco, CA); and anti-GM130 antibodies were from BD Transduction Laboratories (San Jose, CA). Rabbit polyclonal anti-ATGL antibodies were generated as described (37). Rabbit polyclonal anti-calnexin antibodies (38) were a gift from J. Trimmer (University of California, Davis). All fluorescent dye- and peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA).

### Cell culture

HEK 293, baby hamster kidney (BHK), NIH 3T3, and SKBr3 human breast cancer cells were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% iron-supplemented calf serum (JRH Biosciences, Lenexa, KS), 100 U/ml penicillin G sodium, and 100  $\mu$ g/ml streptomycin sulfate (Invitrogen). For MCF7 human breast cancer cells and HeLa cells, calf serum was replaced with 10% FBS (Hyclone, Logan, UT). Where indicated, cell culture medium was supplemented with 400  $\mu$ M oleic acid (Nu-Check Prep, Elysian, MN) complexed to BSA at a 6.6:1 M ratio [prepared as described previously (39)].

### Exogenous expression and detection of ADRP

HEK 293 cells were transfected with murine ADRP in pcDNA3 (Invitrogen; a gift from J. McManaman, University of Colorado Health Sciences Center, Denver) (40) via calcium phosphate. Stable clones were selected by culturing transfected cells with 0.5 mg/ml G418 (Cellgro, Herndon, VA) and maintained with 0.2 mg/ml G418. To assess ADRP expression levels, cells were lysed in 1% Triton X-100, 150 mM NaCl, 50 mM Tris, pH 7.4, 2 mM EDTA, 1  $\mu$ g/ml pepstatin, and 1  $\mu$ g/ml leupeptin. Aliquots of postnuclear supernatants containing equal amounts of protein were subjected to SDS-PAGE and Western blotting.

### Immunofluorescence detection of lipid droplets and associated proteins

Cells were fixed with 3% paraformaldehyde in phosphate-buffered saline (150 mM NaCl and 20 mM phosphate buffer, pH 7.4) for 20 min at room temperature. ADRP or ATGL was detected essentially as described for ADRP (41), except that 0.5 mg/ml goat IgG in antibody diluent and blocking buffer was

replaced, respectively, with 0.1% and 3% BSA. To visualize neutral lipids, 1  $\mu$ g/ml BODIPY 493/503 (Molecular Probes, Carlsbad, CA) was added to cells during staining with secondary antibody. Stained cells were visualized with a Zeiss Axiovision 200 fluorescence microscope. Out-of-focus fluorescence was removed with deconvolution software using the inverse filter algorithm (Axiovision version 4.4). Single sections of a Z-stack are shown.

### Quantification of lipid mass

Cells were cultured in medium alone or with 400  $\mu$ M oleate complexed to BSA for 15 h. Cells were washed with 150 mM NaCl and 50 mM Tris, pH 7.4, and lipids were extracted with hexane-isopropanol (3:2) as described (42). Five micrograms of oleyl alcohol (Sigma, St. Louis, MO) was added to each sample as a recovery standard. Lipids were dried under nitrogen, resuspended in  $\text{CHCl}_3$ /methanol (1:1), and separated by TLC using hexane-isopropyl ether-acetic acid (65:35:2) as the solvent. Lipids were visualized by cupric acetate staining and compared with a standard curve [1–8  $\mu$ g of cholesterol, triolein, and cholesteryl oleate (43)]. Proteins were extracted from cell residues after lipid extraction with 0.1 M NaOH and quantified using a protein assay kit from Bio-Rad (Hercules, CA).

### TAG turnover

One million HEK 293 or ADRP-expressing HEK 293 (293/ADRP) B8 cells were plated in 10 cm dishes (triplicate plates for each time point) and incubated overnight with medium containing 400  $\mu$ M oleate complexed to BSA to promote lipid accumulation. At time zero, oleate-supplemented medium was removed and cells were incubated with medium containing 6  $\mu$ M triacsin C (Biomol, Plymouth Meeting, MA). At the indicated times, lipids were extracted, separated by TLC, and compared with a standard curve as described above. TAG mass was normalized to a recovery standard (oleyl alcohol) and expressed relative to protein levels at time zero.

### Detection of ATGL, TIP47, and TAG after subcellular fractionation

Eight subconfluent 35 mm plates each of HEK 293 and 293/ADRP B8 cells were transfected with GFP-tagged ATGL (a gift from C. Jackson, National Institutes of Health, Bethesda, MD) (37) using Lipofectamine 2000 (Invitrogen). ATGL-transfected cells (and one confluent 10 cm plate of untransfected cells as a carrier to facilitate lipid droplet recovery) were cultured with medium containing 400  $\mu$ M oleate overnight. Cells were scraped into cold phosphate-buffered saline, pelleted, and resuspended in HEPES buffer (10 mM HEPES, 5 mM EDTA, pH 7.4, 1  $\mu$ g/ml pepstatin, and 1  $\mu$ g/ml leupeptin). After incubating on ice for 10 min, cells were homogenized with a 25 gauge needle. The homogenate was adjusted to 10% sucrose, transferred to a Beckman centrifuge tube (number 344062), and overlaid with  $\sim$ 3.1 ml of HEPES buffer to fill the tubes. Samples were spun in a Beckman L8-55 centrifuge (SW60 rotor) at 280,000  $g$ , 4°C, for 3.5 h. Fractions of  $\sim$ 0.6 ml were collected from the top, and volumes were adjusted to 0.7 ml with HEPES buffer. Pellets were washed three times with HEPES buffer before resuspending in gel-loading buffer. Equivalent volumes of each gradient fraction were subjected to SDS-PAGE and Western blotting using an antibody against the GFP tag. Additional Western blots using antibodies against ADRP, HSP90, and calnexin confirmed that lipid droplets mostly segregated in fraction 1, cytosolic proteins were largely in the void volume (fractions 6 and 7) or pellet, and membrane proteins were efficiently pelleted.

For quantification of TIP47 in subcellular fractions, four 10 cm plates each of HEK 293 and 293/ADRP B8 cells were grown,

processed, and subjected to SDS-PAGE and Western blotting as for ATGL localization.

For quantification of TAG mass in subcellular fractions, four confluent plates each of HEK 293 or 293/ADRP B8 cells were grown, processed for sucrose gradient centrifugation, and fractionated as described for ATGL localization. A recovery standard (oleyl alcohol) was added to each fraction, and lipids were extracted according to Bligh and Dyer (44). Lipids in fraction 1 (floatable lipid droplets), fractions 6 and 7 (cytosol), the membrane pellet, and whole cell lysate were dried under nitrogen, resuspended in  $\text{CHCl}_3$ /methanol (1:1), and separated by TLC as described above for quantification of lipid mass.

### Silver staining of lipid droplet-associated proteins

Eight 10 cm plates of HEK 293 or 293/ADRP cells were incubated with medium containing 400  $\mu$ M oleate overnight. Cells were collected and homogenized in HEPES buffer as described above. An aliquot of cell lysate was removed for protein analysis. The remaining homogenate was transferred to a Beckman centrifuge tube (number 331372) and overlaid with  $\sim$ 11 ml of HEPES buffer. Samples were spun in a Beckman L8-55 centrifuge (SW41 rotor) at 26,000  $g$  for 30 min at 4°C. To reduce contamination with cytosolic proteins, the floating fat layer was adjusted to 25% sucrose and transferred to a centrifuge tube on top of a 1 ml 60% sucrose cushion. The homogenate was overlaid with HEPES buffer to fill the tube and centrifuged in a SW41 rotor at 26,000  $g$  for 30 min at 4°C. The floating fat layer was collected and spun in a microcentrifuge at maximum speed for 20 min at 4°C. Infranatant was removed from below the floating fat layer until  $\sim$ 200  $\mu$ l remained. Lipid droplet fractions isolated from 293/ADRP cells in this manner contained ADRP but were free of contaminating membrane (calnexin, transferrin receptor, GM130) and cytosolic (HSP90) proteins (data not shown). Proteins from isolated lipid droplet fractions were separated on 7.5% or 15% polyacrylamide gels. The amount loaded was normalized to the protein concentrations of whole cell lysates. Proteins were visualized by silver staining.

### Statistical analysis

Mean values were compared with GraphPad Prism software (Instat; GraphPad Software, Inc., San Diego, CA) using a two-tailed *t*-test.

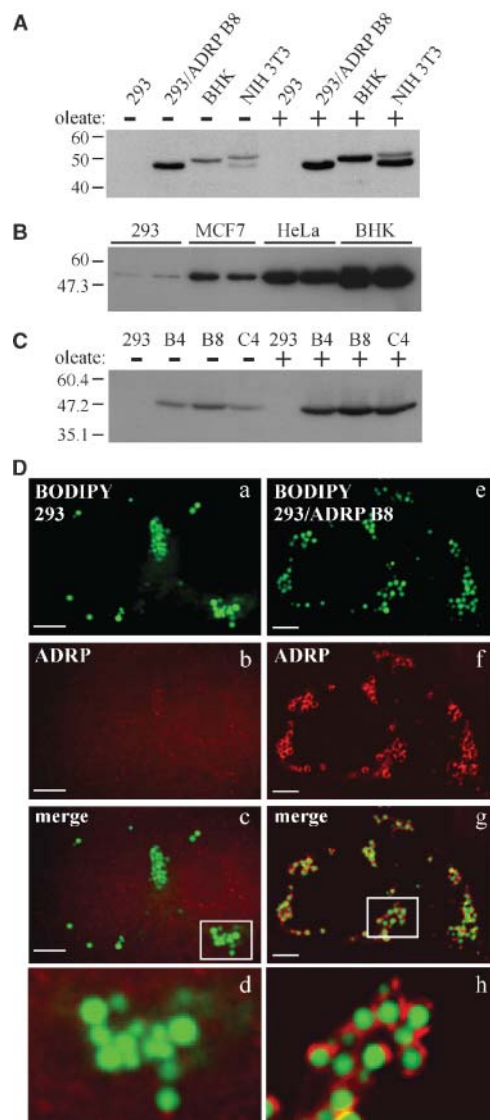
## RESULTS

### Exogenous expression of ADRP in HEK 293 cells

Endogenous ADRP was expressed at very low levels in HEK 293 cells. It was undetectable by Western blotting of whole cell lysates with our antibodies, even after oleate feeding (Fig. 1A), and was visible in <1% of cells by immunofluorescence microscopy (Fig. 1D, panel b). ADRP was detectable by Western blotting of isolated lipid droplets (Fig. 1B). However, HEK 293 cell lipid droplets contained less ADRP than lipid droplets from two other human cell lines, MCF7 and HeLa, or from BHK cells (Fig. 1B). All three human cell lines had less detectable ADRP than BHK or NIH 3T3 cells (Fig. 1A, B). This could result either from suboptimal reactivity of human ADRP with our antibodies or from species-specific differences in ADRP expression.

We transfected HEK 293 cells with murine ADRP and selected several stably transfected lines. Transfected ADRP was easily detectable by blotting, and ADRP levels were





**Fig. 1.** Comparison of adipocyte differentiation-related protein (ADRP) expression levels in multiple cell lines. A, C: Untransfected human embryonic kidney 293 (HEK 293), ADRP-expressing 293 clones (293/ADRP B4, B8, or C4), baby hamster kidney (BHK), and NIH 3T3 cells were cultured with (+) or without (–) 400  $\mu$ M oleate overnight before homogenization. Fifteen micrograms of protein from postnuclear supernatants was separated by SDS-PAGE and probed with a polyclonal antibody to ADRP. The heterogeneity in ADRP size between cell lines may result from amino acid sequence differences or from posttranslational modifications. B: Untransfected HEK 293, MCF7, HeLa, and BHK cells were cultured with 400  $\mu$ M oleate overnight and floatable lipid droplets were isolated by subcellular fractionation. Lipid droplets from equivalent amounts of whole cell lysate (for HEK 293, MCF7, and HeLa) or from half as much whole cell lysate (for BHK), normalized to whole cell lysate protein, were subjected to SDS-PAGE and Western blotting, probing with a monoclonal anti-ADRP antibody. Duplicate lanes for each cell type are shown. Blots shown are representative of three independent experiments. D: Neutral lipid (detected with BODIPY 493/503; a, e) and ADRP (detected by indirect immunofluorescence; b, f) were visualized in untransfected (a–d) or ADRP-expressing (e–h) cells (clone 293/ADRP B8) after overnight incubation with 400  $\mu$ M oleate. The merged images (c, g) show localization of ADRP to the surface of lipid droplets in transfected cells. Panels d and h are enlarged images of the boxed areas in c and g, respectively. Bars = 5  $\mu$ m.

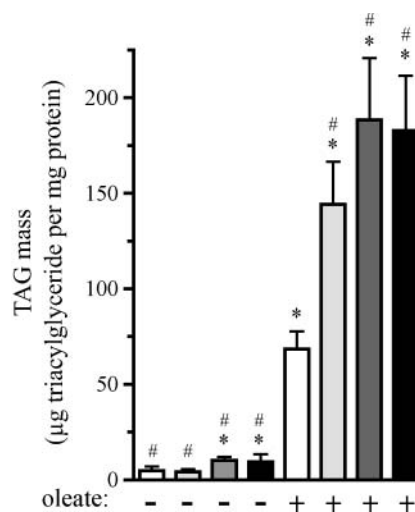
induced after oleate feeding (Fig. 1C). Oleate feeding can induce endogenous ADRP levels both by stimulating transcription and by reducing turnover (45, 46). As transcription of the transfected gene was unlikely to be affected by oleate supplementation, increased ADRP levels in oleate-fed 293/ADRP clones probably resulted from stabilization of the protein. Transfected ADRP in the oleate-fed 293/ADRP clone B8 was about as abundant as the endogenous protein in BHK and NIH 3T3 cells after oleate feeding (Fig. 1A).

### Transfected ADRP is targeted to lipid droplets in 293/ADRP cells

Lipid droplets stained with the lipophilic dye BODIPY 493/503 were seen in both HEK 293 and 293/ADRP B8 cells after oleate feeding (Fig. 1D, panels a, e), although they appeared more highly clustered in parental than in transfected cells. ADRP staining was essentially undetectable by immunofluorescence in HEK 293 cells (Fig. 1D, panel b) but was readily observed on lipid droplets in 293/ADRP B8 cells (Fig. 1D, panels f–h).

### ADRP enhances neutral lipid storage

To determine the effect of ADRP on lipid accumulation, neutral lipids were isolated from parental HEK 293 cells or 293/ADRP clones cultured with or without oleate-supplemented medium. As expected, oleate supplementation substantially increased TAG storage in all cells (Fig. 2). Furthermore, as reported previously in other cell types (32, 34), exogenous ADRP expression increased



**Fig. 2.** Quantification of total triacylglycerol (TAG) mass in ADRP-expressing cells. Untransfected HEK 293 cells (white bars) or three independent clones of ADRP-expressing 293 cells (293/ADRP B4, light gray bars; 293/ADRP B8, dark gray bars; or 293/ADRP C4, black bars) were cultured with (+) or without (–) 400  $\mu$ M oleate overnight before lipid extraction. TAG mass was determined by quantitative TLC and is expressed relative to milligrams of protein. Data are means  $\pm$  SD from at least three independent experiments, and each experiment included duplicate or triplicate samples. \*  $P < 0.002$  compared with unsupplemented HEK 293 cells; #  $P < 0.0001$  compared with oleate-supplemented HEK 293 cells.

TAG levels in HEK 293 cells, both with and without oleate supplementation. In contrast, cholesteryl ester mass was low and did not increase with exogenous ADRP expression (data not shown).

Chang et al. (36) found that a reduced fraction of total cellular TAG was present in lipid droplets in liver extracts from ADRP-deficient mice compared with wild-type controls. To determine whether ADRP expression in HEK 293 cells had a similar effect on TAG distribution, homogenates of parental or 293/ADRP B8 cells were fractionated on sucrose density gradients. TAG mass in floating lipid droplets, pelleted cell membranes, and cytosol was determined by TLC (**Fig. 3**). A higher fraction ( $63.0 \pm 14.3\%$ ) of total TAG was present in lipid droplets isolated from 293/ADRP B8 cells than from parental cells ( $46.6 \pm 11.1\%$ ). Conversely, a higher fraction of total TAG was present in the membrane pellet of HEK 293 cells ( $23.8 \pm 10.2\%$ ) than in 293/ADRP membranes ( $9.6 \pm 2.3\%$ ). Thus, in agreement with previous results (36), ADRP ex-

pression in HEK 293 cells increased the fraction of total TAG present in floatable lipid droplets. Together, these results suggest that ADRP may have an as yet uncharacterized role in lipid droplet synthesis.

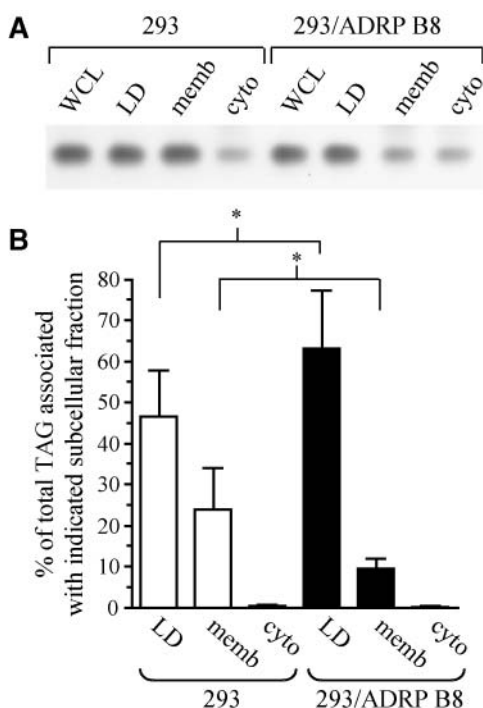
### ADRP expression reduces TAG turnover

We next determined whether ADRP, like perilipin (16–18), reduced the rate of TAG turnover in oleate-fed cells. To do this, we measured TAG hydrolysis while inhibiting TAG synthesis with  $6 \mu\text{M}$  triacsin C. [Because triacsin C does not inhibit all acyl-CoA synthetases (47), we first verified that it blocked TAG synthesis in HEK 293 cells. Six micromolar triacsin C completely inhibited the oleate-induced increase in TAG accumulation in HEK 293 cells (see supplementary Fig. I).] TAG was hydrolyzed substantially more slowly in 293/ADRP B8 cells than in parental cells (**Fig. 4**). That is, the half-time of TAG turnover was 5.6 h in parental cells and 12.7 h in 293/ADRP B8 cells.

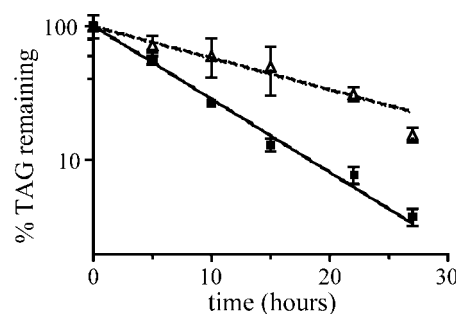
We also attempted to determine whether ADRP affected the rate of TAG synthesis by examining the incorporation of  $^{14}\text{C}$ -labeled oleic acid into TAG. In fact, ADRP expression led to a small increase in the amount of  $^{14}\text{C}$ -labeled TAG present in 293/ADRP B8 cells over 2 h of labeling (data not shown). However, as we were unable to completely block TAG hydrolysis in these cells, we do not know whether this resulted solely from the ADRP-induced decrease in the rate of TAG hydrolysis.

### ADRP reduces the lipid droplet association of ATGL

We hypothesized that ADRP might reduce TAG hydrolysis at least in part by inhibiting the binding of lipases to the surface of lipid droplets. Because ATGL plays a role in TAG hydrolysis in several cell types (22, 23, 37), we examined the localization of endogenous ATGL in HEK 293 and 293/ADRP B8 cells by indirect immunofluorescence. ATGL was predominantly localized to the surface of BODIPY 493/503-stained lipid droplets in HEK 293 cells



**Fig. 3.** Quantification of TAG mass in subcellular fractions of ADRP-expressing cells. Floatable lipid droplets, cytosol, and membrane pellets were isolated from oleate-supplemented HEK 293 and 293/ADRP B8 cells as described. Lipids in each fraction were extracted and separated by TLC. A: A representative chromatogram from one experiment is shown comparing TAG levels in whole cell lysate (WCL), lipid droplets (LD), pelleted membranes (memb), and cytosol (cyto). To ensure that TAG levels fell within the standard curve (not shown), the amount of lipid from isolated lipid droplets, pelleted membranes, and cytosol loaded per lane was  $1.87\times$ ,  $7.5\times$ , and  $112.5\times$  the amount loaded for whole cell lysate lanes. B: TAG accumulating in lipid droplets, pelleted membranes, or cytosol fractions was quantified from four independent experiments (duplicate samples in each experiment) and expressed as the percentage of total TAG isolated in whole cell lysate (means  $\pm$  SD). \*  $P < 0.05$  for comparison of similar fractions from HEK 293 and 293/ADRP B8 cells.

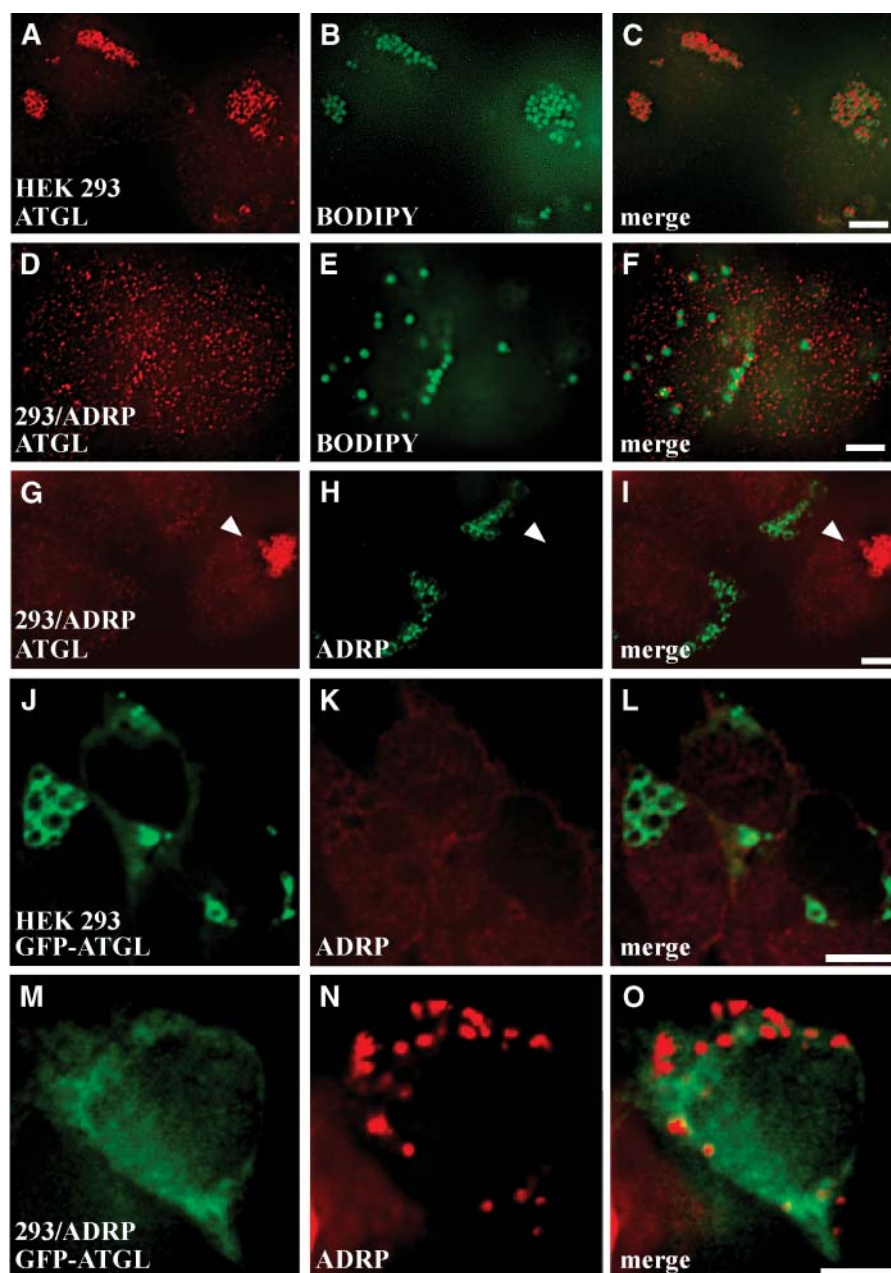


**Fig. 4.** TAG turnover in ADRP-expressing cells. Lipid accumulation was induced by overnight incubation with  $400 \mu\text{M}$  oleate. At time zero, oleate was removed and further TAG accumulation was blocked with triacsin C. TAG mass in untransfected HEK 293 cells (closed squares) and 293/ADRP B8 cells (open triangles) was measured at subsequent time points with quantitative TLC. Data are expressed as means  $\pm$  SD ( $n = 3$ ) and are representative of three independent experiments. The lines represent the best fit to these data (as determined by GraphPad Prism 4 software) using the equation for one-phase exponential decay (top to zero).

(Fig. 5A–C). By contrast, ATGL localization was dramatically different in 293/ADRP B8 cells (Fig. 5D–I). ATGL ring staining of BODIPY 493/503-labeled lipid droplets was much less frequent in these cells, although some ATGL localized to puncta close to lipid droplets (Fig. 5D–F). ATGL association with lipid droplets was similarly low in two additional 293/ADRP clones (see supplementary Fig. II). Occasionally, transfected cells lost ADRP expression (Fig. 5G–I, arrowheads). In these cells, significant

ATGL was found associated with lipid droplets. Thus, immunofluorescence analysis suggested that ADRP expression greatly reduced the association of ATGL with lipid droplets.

We next aimed to quantify the effect of ADRP on ATGL localization by subcellular fractionation and Western blotting. However, our ATGL antibody did not detect endogenous ATGL by Western blotting (data not shown). Thus, we transiently expressed GFP-tagged ATGL in HEK

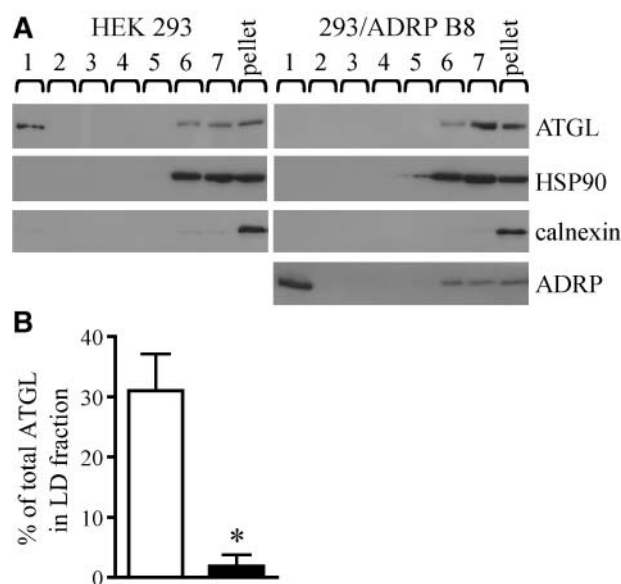


**Fig. 5.** Fluorescence detection of adipose triglyceride lipase (ATGL) lipid droplet association. Localization of endogenous ATGL (A, D, G) or green fluorescent protein (GFP)-tagged ATGL (J, M) is shown in untransfected (A–C, J–L) and ADRP-expressing (D–I, M–O) HEK 293 cells (clone B8) in relation to BODIPY 493/503-stained neutral lipid (B, E) or ADRP immunostaining (H, K, N). The merged images (C, F, I, L, O) show ATGL on the surface of lipid droplets in those cells lacking ADRP expression. The arrowheads indicate ATGL localized to lipid droplets in a transfected cell that lost ADRP expression. Bars = 10  $\mu$ m (C, F) or 5  $\mu$ m (I, L, O).



293 and 293/ADRP B8 cells. To ensure that the GFP tag did not affect ATGL localization, we first examined the transfected cells by fluorescence microscopy. As seen for the endogenous protein, GFP-tagged ATGL was largely localized to lipid droplets in HEK 293 cells but appeared mostly cytosolic in 293/ADRP B8 cells (Fig. 5J–O). We next subjected homogenates of GFP-ATGL-transfected HEK 293 or 293/ADRP B8 cells to sucrose gradient centrifugation and determined the distribution of GFP-ATGL across the gradient (Fig. 6A). HSP90, calnexin, and ADRP served as markers of cytosol, endoplasmic reticulum, and lipid droplets, respectively. More than 30% of GFP-ATGL from HEK 293 cells was present in the lipid droplet fraction, whereas <2% of the protein from 293/ADRP B8 cells floated to this position (Fig. 6B). Together with the immunofluorescence data (Fig. 5), this result strongly suggested that ADRP expression reduced the association of ATGL with lipid droplets.

To generalize our findings, we examined the effect of ADRP overexpression on ATGL localization in two additional cell lines. Both SKBr3 and MCF7 cells expressed



**Fig. 6.** Western analysis of GFP-tagged ATGL location after sub-cellular fractionation. **A:** After transient transfection with GFP-tagged ATGL, HEK 293 and 293/ADRP B8 cells were homogenized and fractionated by ultracentrifugation as described in Materials and Methods. Fractions were collected from the top of each sample, with fraction 1 corresponding to floating lipid droplets and fractions 6 and 7 corresponding to the void volume. Equivalent volumes of each fraction were run on SDS-PAGE. GFP-ATGL in each subcellular fraction was detected by Western blotting with antibodies directed against the GFP tag and compared with markers for cytosolic protein (HSP90), endoplasmic reticulum membrane protein (calnexin), and lipid droplet-associated protein (ADRP). **B:** The amount of GFP-tagged ATGL in the floating lipid droplet (LD) fraction (fraction 1) from untransfected HEK 293 cells (white bar) and 293/ADRP B8 cells (black bar) was quantified by densitometry and is expressed as the percentage of total ATGL (the sum of ATGL in all lanes). Data are means  $\pm$  SD from four independent experiments. \*  $P < 0.0001$  compared with HEK 293 cells.

more endogenous ADRP than HEK 293 cells but less than BHK and NIH 3T3 cells (data for MCF7 shown in Fig. 1B). However, as observed previously in HeLa cells (37), GFP-ATGL expression in these cells reduced the size of lipid droplets (data not shown). This suggested that GFP-ATGL hydrolyzed significant amounts of TAG, altering lipid droplet morphology and making it difficult to score lipid droplet association of the protein. To examine lipid droplet targeting in the absence of this effect, we expressed the catalytically inactive ATGL-S47A mutant (37) in SKBr3 and MCF7 cells with or without exogenous ADRP. GFP-ATGL-S47A was predominantly lipid droplet-associated in both cell types (see supplementary Fig. III). However, coexpression of GFP-ATGL-S47A with ADRP dramatically reduced the lipid droplet association of the mutant lipase in both cell types (see supplementary Fig. III).

### ADRP reduces the lipid droplet association of TIP47

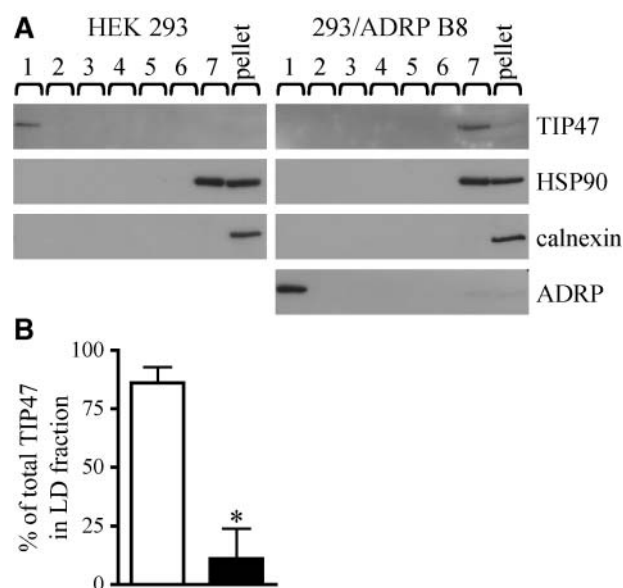
We next examined the effect of ADRP expression on the lipid droplet association of TIP47, a widely expressed PAT family member. We subjected HEK 293 and 293/ADRP B8 cell homogenates to sucrose gradient centrifugation and detected endogenous TIP47 distribution across the gradient by Western blotting. As was true of GFP-ATGL, more TIP47 floated to the top of the gradient with lipid droplets from HEK 293 cells, whereas most TIP47 from 293/ADRP B8 cells was present in cytosolic fractions (Fig. 7). Similarly, suppression of ADRP synthesis was recently shown to increase the lipid droplet association of TIP47 in Huh7 cells (48).

### ADRP expression in HEK 293 cells reduces the lipid droplet localization of several proteins

We next analyzed the lipid droplet protein profile from parental cells and two 293/ADRP clones by SDS-PAGE and silver staining (Fig. 8). One of the darkest bands in the HEK 293 cell lipid droplet profile was present at  $\sim 47$  kDa (Fig. 8A, top arrow) and might correspond to endogenous TIP47. [Consistent with this possibility, endogenous TIP47 migrated slightly faster than transfected murine ADRP on Western blots of 293/ADRP cells (data not shown)]. ADRP expression reduced the intensity of this band as well as that of several other bands (Fig. 8A, B, arrows). The bands indicated had molecular masses of  $<50$  kDa, smaller than the predicted molecular mass of ATGL (54 kDa). Together, these data show that ADRP expression reduced the lipid droplet association of several other proteins in addition to TIP47 and ATGL. Further work will be required to determine the identity of these proteins.

## DISCUSSION

We found that ADRP expression significantly affected TAG storage in HEK 293 cells. In agreement with earlier reports (32, 34), ADRP increased cellular TAG levels both with and without oleate feeding. Furthermore, we found that ADRP expression slowed TAG hydrolysis. This may partially explain the reduced TAG content in livers of

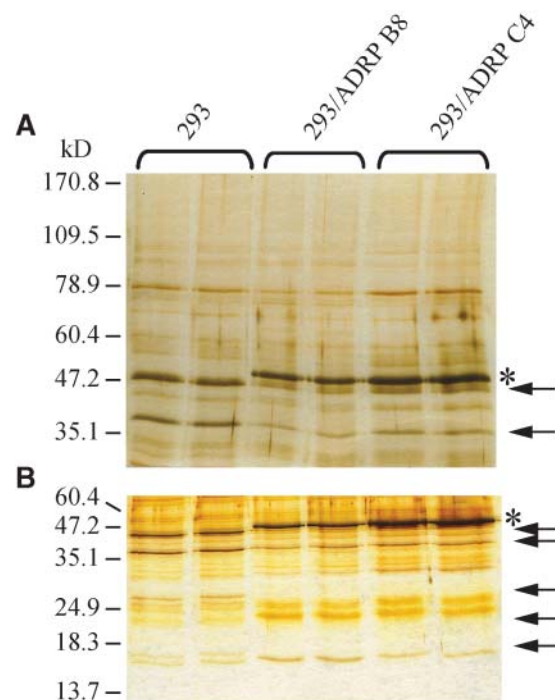


**Fig. 7.** Western analysis of mannose-6-phosphate receptor tail-interacting protein of 47 kDa (TIP47) location after subcellular fractionation. HEK 293 and 293/ADRP B8 cells were homogenized and fractionated by ultracentrifugation as described for Fig. 6. A: TIP47, HSP90, calnexin, and ADRP were detected by Western blotting in equivalent volumes of each subcellular fraction. B: The amount of TIP47 in the floating lipid droplet (LD) fraction (fraction 1) from untransfected HEK 293 cells (white bar) and 293/ADRP B8 cells (black bar) was quantified by densitometry and is expressed as the percentage of total TIP47 (the sum of TIP47 in all lanes). Data are means  $\pm$  SD from four independent experiments. \*  $P < 0.0001$  compared with HEK 293 cells.

ADRP-null mice and their resistance to diet-induced fatty liver (36). This phenotype is analogous to the resistance of perilipin-deficient mice to diet-induced obesity (14, 15). Thus, suppression of TAG turnover in liver (and possibly other tissues) by ADRP may play an important physiological role, in the same way that suppression of basal TAG hydrolysis by perilipins is important in adipocytes.

Our findings are consistent with a recent report that ADRP expression slows TAG hydrolysis in cultured hepatocytes (35). In apparent contrast, genetic ablation of the ADRP gene does not enhance TAG hydrolysis in adipocytes (36). However, this finding is expected, as mature adipocytes express little ADRP and probably use perilipins to control TAG hydrolysis (28). In this context, it is interesting that perilipin A represses TAG hydrolysis when expressed in cells that endogenously produce ADRP (16–18). Together with our data, these studies suggest that both perilipin A and ADRP can slow TAG turnover but that perilipin A does so more efficiently. Interestingly, experiments by Sztalryd and colleagues (49) show that ADRP-null embryonic fibroblasts have no significant defect in lipolysis. In these cells, increased expression of TIP47 may compensate for the lack of functional ADRP.

ADRP-mediated repression of TAG hydrolysis in HEK 293 cells correlated with a dramatic loss of ATGL from lipid droplets. Moreover, increasing ADRP expression in SKBr3 and MCF7 cells decreased the lipid droplet



**Fig. 8.** Analysis of lipid droplet protein association in ADRP-expressing cells. Lipid droplets were isolated in the floating fraction from HEK 293 cells and two independent clones of ADRP-expressing 293 cells (B8 and C4) after ultracentrifugation as described in Materials and Methods. Total protein in lipid droplet fractions from HEK 293 and 293/ADRP cells was separated by SDS-PAGE using 7.5% (A) or 15% (B) polyacrylamide and detected by silver staining. Duplicate lanes for each cell type are shown. The amount loaded per lane was normalized to the protein concentration of whole cell lysates. Asterisks indicate protein migrating at the expected molecular mass for murine ADRP. Arrows indicate proteins that were reproducibly decreased in ADRP-expressing HEK 293 cells. Gels are representative of four independent experiments.

association of a catalytically inactive form of ATGL. Further work will show whether the ability of ADRP to limit lipid droplet binding of ATGL and/or other lipases is responsible for its ability to slow TAG turnover. Our finding may explain the variation in ATGL localization among previous reports. ATGL was found to be largely cytosolic in COS7 cells (22), modestly lipid droplet-associated in 3T3-L1 adipocytes (23), and substantially lipid droplet-associated in HeLa cells (37). Variable levels of ADRP and/or other PAT family members in these cells could affect ATGL localization.

#### How ADRP might reduce the lipid droplet association of other proteins

We show that ADRP expression decreased the lipid droplet association of ATGL (Figs. 5, 6), TIP47 (Fig. 7), and other unidentified proteins (Fig. 8). However, the mechanism through which ADRP altered the localization of other proteins is unknown. Perilipin has been proposed to prevent HSL binding to lipid droplets by forming a barrier that sterically blocks the access of other proteins (16). In our experiments, silver staining of lipid droplet-



associated proteins suggested that the amount of ADRP in 293/ADRP cells was unlikely to completely coat lipid droplets (Fig. 8). We propose an alternative explanation as a working model. Phospholipids in the lipid droplet surface monolayer may be less tightly packed than in conventional membrane bilayers, transiently exposing the neutral lipid core and making the lipid droplet surface more hydrophobic than that of cell membranes. Some cytosolic proteins might thus bind lipid droplets through hydrophobic interactions. In doing so, these proteins could crowd the phospholipids, increasing their condensation and reducing lipid droplet surface hydrophobicity. This in turn might affect the ability of other proteins to bind lipid droplets. A recent study of apolipoprotein B provides precedent for the idea that molecular packing on the surface of model lipid droplets can affect protein binding (50). That study showed reversible binding of amphipathic  $\alpha$ -helix-rich domains of apolipoprotein B to the triolein/water interface, depending on the compression of the surface. If proteins varied in their affinity for cellular lipid droplets, proteins that bound most strongly could increase phospholipid packing, displacing other proteins or reducing their ability to bind. In this way, differences in lipid droplet affinity could establish a hierarchy of protein binding to lipid droplets.


This model could explain our results as well as some published data of others. ADRP could reduce the lipid droplet binding of several proteins (including TIP47 and ATGL) if its affinity for lipid droplets were unusually high. Such a high lipid droplet affinity could also explain the puzzling specificity of ADRP and perilipins for lipid droplets. It is not known how these proteins avoid binding to the cytosolic surface of the endoplasmic reticulum membrane, from which lipid droplets are probably derived. These proteins might distinguish lipid droplets from membranes based on their hydrophobicity. It is notable that recent proteomic studies have identified lipid droplet-associated proteins with functions apparently unrelated to lipid metabolism, including a number of Rab proteins (51–53). The lipid droplet association of one of these, Rab18, appears to be physiologically relevant (54, 55). However, the presence of so many Rabs (which usually mark individual organelles or organelle subdomains) in one place is unprecedented. Rabs are dually geranylgeranylated and thus are among the most hydrophobic of cytosolic proteins. A small fraction of these Rabs, and by extension possibly other proteins as well, might associate with lipid droplets through hydrophobic interactions even if they did not function there.

Finally, our model could be relevant to an intriguing proteomic study of adipocyte lipid droplets (51). Although perilipins were by far the most abundant proteins in resting adipocyte lipid droplets, lipolytic stimulation greatly increased the relative abundance of a number of other proteins (51). Conformational changes in perilipin upon lipolytic stimulation might reduce its affinity for lipid droplets or its condensing effect on lipid droplet phospholipids, facilitating the binding of other modestly hydrophobic proteins. Such a mechanism could allow

coordinated recruitment of a variety of proteins to lipid droplets in response to hormonal stimulation.

### Additional roles of ADRP

In addition to its role in slowing lipid droplet TAG hydrolysis, ADRP may also affect lipid storage in other ways. For example, fractionation studies of fibroblasts from ADRP-deficient mice showed a lower fraction of total TAG in floating lipid droplets and a corresponding higher fraction in pelletable membranes than in wild-type controls (36). Similarly, we found in this study that ADRP expression in HEK 293 cells increased the fraction of TAG that could be isolated in floating lipid droplets by gradient centrifugation. These results raise the intriguing possibility that ADRP may play some role in lipid droplet synthesis. Alternatively, however, ADRP-deficient lipid droplets might simply associate nonspecifically with pelletable membranes or cytoskeleton during lysis or gradient fractionation. The increased clustering of lipid droplets in HEK 293 cells compared with 293/ADRP cells (Figs. 1D, 5B, E) could be related to such behavior. Further work will be required to distinguish between these possibilities.

In conclusion, we have demonstrated an important role for ADRP in regulating TAG turnover and the lipase association with lipid droplets. Future work will more fully characterize the physiological roles of ADRP and ATGL in regulating lipid storage in nonadipose tissues. 

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