THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 50, pp. 34538–34544, December 11, 2009

Perilipin Controls Lipolysis by Regulating the Interactions of AB-hydrolase Containing 5 (Abhd5) and Adipose Triglyceride Lipase (Atgl)*[S]

Received for publication, September 21, 2009, and in revised form, October 22, 2009 Published, JBC Papers in Press, October 22, 2009, DOI 10.1074/jbc.M109.068478

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The mobilization of stored lipid by hormones is a fundamental function of fat cells, and there is strong evidence that perilipin (Plin), a lipid droplet scaffold, and adipose tissue triglyceride lipase (Atgl), a triglyceride-specific lipase, play critical roles. Previous work suggested that Abhd5, a protein activator of Atgl, coordinates with Plin in controlling basal and stimulated lipolysis; however, the underlying mechanism is controversial. The present experiments investigated protein trafficking and interactions among Plin, Atgl, and Abhd5 in live cells. The results demonstrate that Plin binds Abhd5 with high affinity and thereby suppresses the interaction of Abhd5 with Atgl. Sequestration of Abhd5 appears to a major mechanism by which Plin reduces basal lipolysis. Phosphorylation of Plin on serine 492 or serine 517 rapidly releases Abhd5 from Plin, allowing Abhd5 to directly interact with Atgl. Imaging experiments demonstrated that the Plin-dependent interaction of Abhd5 and Atgl occurs mainly, but not exclusively, on lipid droplets that contain Plin.

Growing evidence indicates that hormone-stimulated lipolysis involves protein trafficking and specific protein-protein interactions at the surface of lipid droplets (LDs),[2] and there is strong evidence that perilipin A (Plin), a lipid droplet scaffold protein, plays a central role in orchestrating interactions among lipolytic effector proteins (1, 2). For example, phosphorylated Plin provides a docking site by which phosphorylated hormone-sensitive lipase (HSL) gains access to substrates at the surface of LDs (3–5). Adipose triglyceride lipase (Atgl, also known as desnutrin, Pnpla2) also participates in protein kinase A (PKA)-stimulated lipolysis (6, 7), and Plin is thought to be involved in this regulation (8); however, the mechanisms involved are not understood, and proposed models that address this regulation disagree.

Unlike HSL, Atgl is not a direct target of PKA phosphorylation, and therefore, the mechanism of its activation must be indirect. One model whereby Plin could indirectly regulate Atgl activity is by controlling availability of its co-activator, Abhd5 (also known as CGI-58), in a manner that depends on PKA-dependent phosphorylation of Plin (3). According to this "sequestration-release" model, Plin sequesters Abhd5 in the basal state, thereby preventing activation of Atgl and suppressing basal lipolysis. PKA activation leads to Plin phosphorylation, release of Abhd5, and subsequent activation of Atgl. This mechanism is supported by biochemical and dynamic imaging experiments demonstrating that Abhd5 binds Plin in the unstimulated state, and PKA-mediated phosphorylation of Plin leads to rapid release of Abhd5 from Plin (3, 9–11). Additionally, knockdown of Abhd5 reduces both basal and stimulated lipolysis (11, 12), although whether this effect involves interactions with Plin has been questioned (12).

Although these data are consistent with the general model, there are no data demonstrating that 1) the interaction of Abhd5 with Plin and Atgl is mutually exclusive (that is, Plin suppresses the interaction of Abhd5 with Atgl) or that 2) phosphorylation of Plin promotes the physical interaction of Abhd5 with Atgl. In fact, an alternative model has been described whereby Atgl regulates basal but not stimulated lipolysis (1). According to this model, basal lipolysis is stimulated by a ternary complex containing Plin, Abhd5, and Atgl. Furthermore, the model proposes that Abhd5 is not involved in PKA-stimulated activation of Atgl because it is released into the cytoplasm upon Plin phosphorylation.

In the experiments detailed below, we examined the functional interactions among Plin, Abhd5, and Atgl using RNA interference as well as a panel of dynamic protein-protein interaction assays. Results from these experiments indicate the interaction of Plin and Abhd5 regulates basal and stimulated lipolysis. Protein complementation assays indicate that the interaction of Abhd5 with Plin and Atgl is mutually exclusive and that its interaction with Plin predominates in the basal state. We identify the PKA phosphorylation sites on Plin that are necessary for releasing Abhd5 and further demonstrate that phosphorylation of these sites mediates the PKA-dependent interaction of Abhd5 with Atgl. Finally, we show that the PKA-dependent interaction of Abhd5 and Atgl occurs mainly on lipid droplets containing Plin.

EXPERIMENTAL PROCEDURES

Generation of Fluorescent Fusion Proteins and Protein Complementation Constructs—Construction and validation of fluorescently tagged proteins and bimolecular fluorescence

* This work was supported, in whole or in part, by National Institutes of Health Grants DK 62292 and DK76629. This work was also supported by American Diabetes Association Grant RA29 and by a grant from the Department of Veterans Affairs.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3 and Video 1.

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[2] The abbreviations used are: LD, lipid droplet; HSL, hormone-sensitive lipase; BiFC, bimolecular fluorescence complementation; FRET, fluorescence resonance energy transfer; PKA, protein kinase A; EYFP, enhanced yellow fluorescent protein; EYFP, enhanced cyan fluorescent protein; IBMX, isobutylmethylxanthine; siRNA, small interfering RNA.

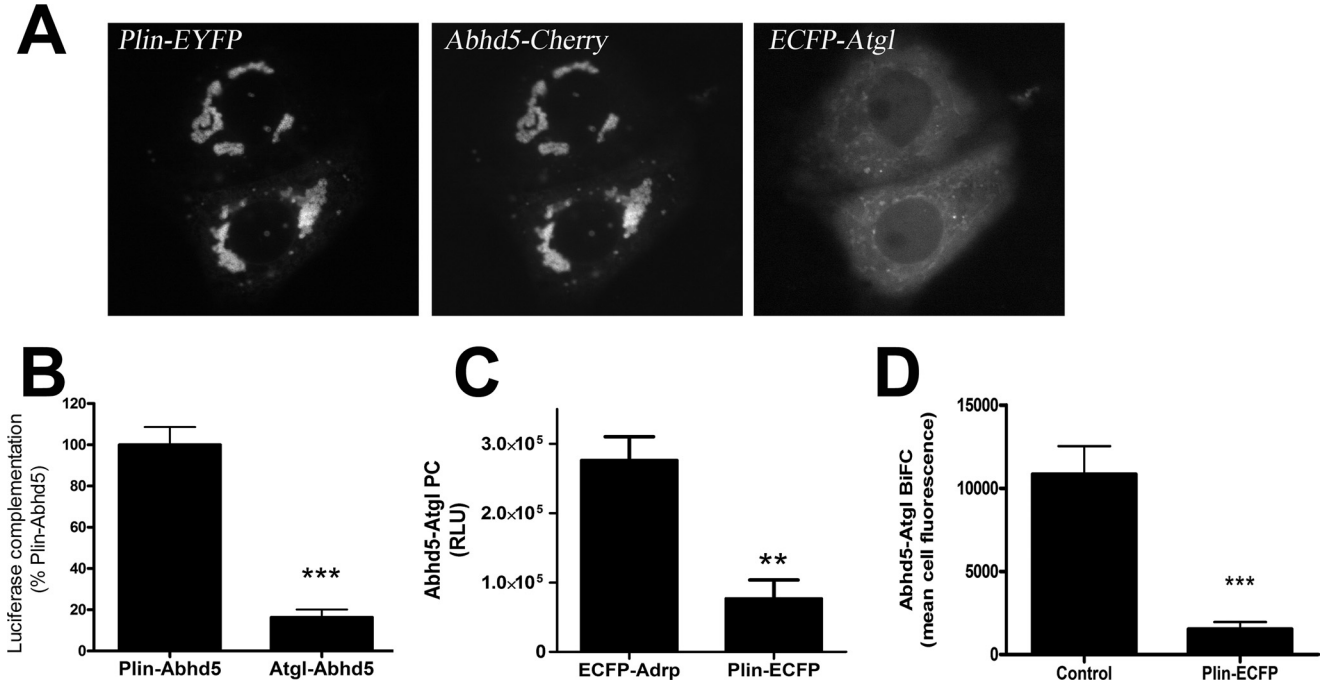

FIGURE 1. **Interaction of Abhd5 with Plin predominates in unstimulated cells.** *A*, in the absence of stimulation, Plin-EYFP and Abhd5-Cherry were highly colocalized on lipid droplets, whereas ECFP-Atgl was mostly cytosolic. *B*, Cos7 cells were transfected with complementary fragments of luciferase fused to Abhd5 and Plin or Atgl. Abhd5 interacted with both Plin and Atgl; however, the luciferase activity generated by the interaction of Abhd5 with Plin was 6 times greater than that produced by Abhd5 with Atgl ($p < 0.001$). Values are the means ± S.E., $n = 3$ experiments, each performed in quadruplicate. *C*, Plin-ECFP significantly suppressed ($p < 0.01$) the interaction of Abhd5 with Atgl as detected by protein complementation. *RLU*, relative light units. Plin-ECFP expression was 104 ± 28% of ECFP-Adrp as determined by ECFP fluorescence. Values are the means ± S.E. of three independent experiments. *D*, Plin-ECFP reduced ($p < 0.01$) the interaction of Abhd5 and Atgl, as determined by BiFC. Plin-ECFP expression was less than 20% that of the ECFP control. Values are the mean ± S.E., $n = 5$.

complementation (BiFC) partners have been described (3, 13). The Venus variant (14) of the EYFP C-terminal complementation fragment was generated by PCR using plasmid DNA as a template (supplied by Dr. Michael Davidson, Florida State University). The generation of *Gaussia princeps* luciferase protein complementation constructs (15) has been described (16). Mutation of PKA phosphorylation sites 1–4 (Ser-81, Ser-222, Ser-276, Ser-433) and site 5 (Ser-492) and site 6 (Ser-517) in Plin was performed by PCR.

Subcellular Colocalization of Fluorescently Tagged Proteins—Cos7 were transfected with Plin-EYFP, Abhd5-Cherry, and ECFP-Atgl, incubated with oleic acid (200 μM complexed to bovine serum albumin) overnight to promote lipid droplet formation, and imaged live the next day.

Luciferase Protein Complementation Analysis—Cos7 or 3T3-L1 preadipocytes were grown in 24-well plates and transfected in quadruplicate with N- and C-luciferase fragments fused to Atgl and Abhd5 along with wild type- or phosphorylation-defective Plin or various controls as specified in the figure legends. For competition experiments, N and C complementation probes were cotransfected with competitors (ECFP-Adrp or ECFP or Plin-ECFP) at a plasmid ratio of 2:2:1. After transfection, cells were cultured for 18–24 h in media containing oleic acid.

For experiments examining the effects of PKA activation, media was replaced with 1 ml of Dulbecco's modified Eagle's medium lacking phenol red, and cells were treated with DMSO (vehicle control) or with a mixture of forskolin/IBMX (2 μM/200 μM final concentration) for 1 h. Luciferase activity was determined as previously described (16).

Fluorescence Resonance Energy Transfer (FRET) in Live Cells—FRET analysis was performed as previously described (3). Briefly, 3T3-L1 cells were transfected with ECFP-Abhd5 and wild type or mutant Plin-EYFP, then loaded with oleic acid overnight. Experiments were performed at room temperature in HEPES-buffered Krebs-Ringer solution containing 1% bovine serum albumin. Images were captured one time per min under basal conditions and after application of forskolin and IBMX to yield a final concentration of 2 and 200 μM.

BiFC in Live Cells—Cos7 were transfected with complementary BiFC fragments fused to Atgl and Abhd5 along with wild type or phosphorylation-defective Plin-ECFP. In some experiments cells were transfected with complementary BiFC fragments fused to Atgl and E262K Abhd5 in the presence of wild type Plin-ECFP. Cells were incubated overnight at 30 °C in media supplemented with oleic acid, then transferred to a recording chamber containing Krebs buffer and bovine serum albumin. Cells were recorded at time = 0 and at 15-min intervals after stimulation with forskolin/IBMX.

Microscopy—Images were acquired with an Olympus IX-81 microscope equipped with a spinning disc confocal unit. Images were captured using a 60× 1.2 NA plan apo water immersion lens and a Hamamatsu ORCA cooled CCD camera. The following Chroma filter sets were used for the indicated fluorophores: mCherry, 41043; EYFP and Venus BiFC, 31044; ECFP, 41028; EYFP FRET, exciter from 41028 and the dichroic/

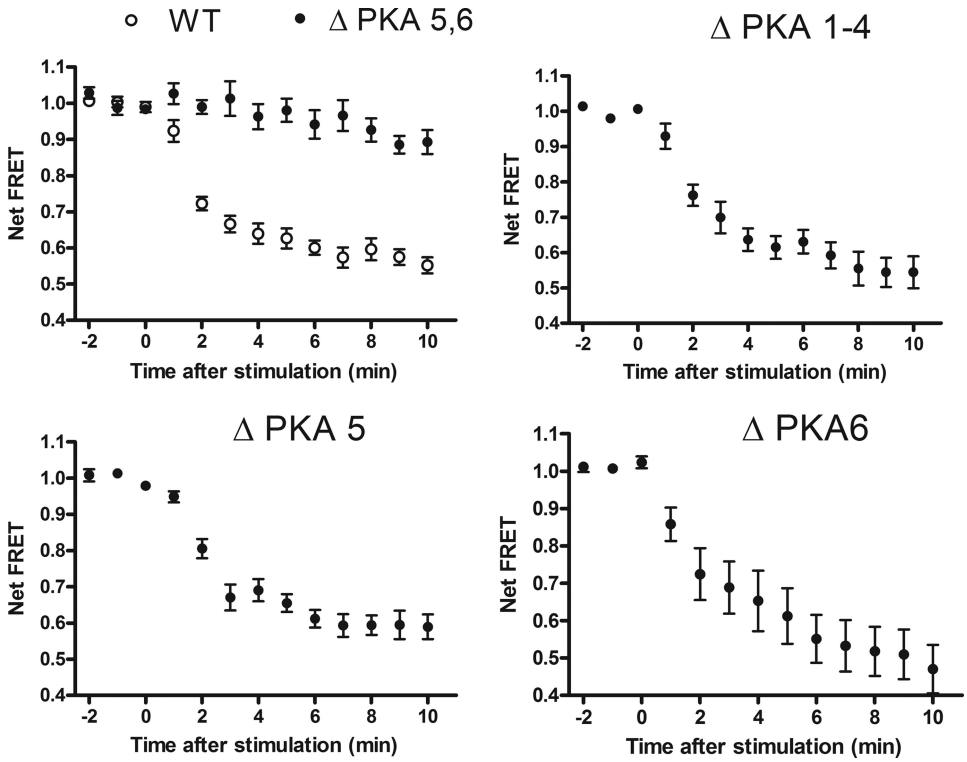

FIGURE 2. **Ser492 (site 5) and Ser-517 (site 6) of Plin control the PKA-dependent interaction of Plin with Abhd5.** FRET between ECFP-Abhd5 and Plin-EYFP was monitored in live 3T3-L1 cells before and after PKA activation with forskolin/IBMX. *Upper left*, PKA activation rapidly reduced energy transfer between ECFP-Abhd5 and wild type (*WT*) Plin-EYFP. Mutations of PKA sites 5 and 6 (Δ*5,6*) in Plin eliminated the effects of forskolin/IBMX. Mutation of Plin PKA sites 1, 2, 3 and 4 (Δ*1–4*, *upper right*) or individual mutation of sites 5 (Δ*5*) or 6 (Δ*6*) failed to interfere with forskolin/IBMX-induced disruption of Plin-Abhd5 complexes. Values are the means ± S.E., $n = 6–17$.

emitter from 31044. Microscope control and data acquisition were performed using IPlabs (Scanalytics, BD Biosciences) software.

Image Analysis—FRET was performed using the three-filter method and net FRET calculated using the FRET extension of IPlabs software as previously described (3). To quantify the BiFC signals, the subcellular domain containing Plin-ECFP LDs was defined for each frame using the autosegmentation tool of IPlabs, and the net (total minus background) fluorescence intensities of those pixels in the EYFP (BiFC) channel was summed for each frame. Background fluorescence was determined by the mean fluorescence intensity of an area immediately adjacent to the cell of interest. Total intensities were normalized to basal BiFC value for each cell, then averaged for presentation.

siRNA Knockdown and Lipolysis in 3T3-L1 Adipocytes—3T3-L1 adipocytes were maintained and differentiated in 10-cm plates as previously described (13). Four days after induction of differentiation, cells were trypsinized and replated (1.5×10^5 cells/well; 3–6 wells/siRNA) in collagen-coated 24-well plates containing media and 100 nM siRNA and DharmaFECT Duo Reagent (Dharmacon). For single gene knockdown, cells were transfected with scrambled siRNA (siCon, Dharmacon D-056623-04) or Abhd5 siRNA (siAbhd5, Dharmacon D-04227-01/04, mixed 1:1). For double knockdown experiments, cells were transfected with 50 nM siCon or siAbhd5 or siPlin and 50 nM siGlo (Dharmacon D001600-01) or 50 nM each of siAbhd5 and siPlin for double knockdown.

Transfection media was changed to Dulbecco's modified Eagle's medium plus 10% fetal bovine serum on the following day, and cells were incubated for 3 days before testing. For experiments examining the effects of Abhd5 and Plin siRNA on basal lipolysis, cells were washed and incubated in Krebs-Ringer buffer with 10 mM HEPES and 2% fatty acid free bovine serum albumin containing 3 nM phenylisopropyladenosine. For experiments examining the effects of Abhd5 siRNA on Atgl activity, cells were incubated with 3 nM phenylisopropyladenosine (basal) or 10 μM forskolin and 1 mM IBMX (stimulated) in the presence of 2 μM BAY59–9435, a selective HSL inhibitor. The medium was collected at 3 h, and triplicate wells analyzed for free fatty acid content using a Wako reagent kit. Immunoblotting for Abhd5 (16) and Plin (13) was performed on the same wells used in lipolysis assay to confirm the level of knockdown.

Statistical Analyses—Statistical analysis was performed by analysis of variance, and group means were compared by the Bonferroni test.

RESULTS

The Interaction of Plin with Abhd5 Predominates in Unstimulated Cells and Inhibits the Interaction of Abhd5 with Atgl—Previous immunochemical analyses have shown that endogenous Plin and Abhd5 are colocalized on LDs in the basal state, whereas Atgl is found both in the cytosol and on LDs (3, 9, 10). Furthermore, PKA activators reduce the colocalization of Abhd5 with Plin while improving its colocalization with Atgl. Although these morphological results are generally consistent with the sequestration-release model, they did not provide proof for the model because they fail to address specific protein-protein interactions at the molecular level. Thus, many key tenets and predictions of the sequestration-release model remain untested.

In this study we generated a panel of fluorescently tagged proteins and devised sensitive protein-protein interaction assays to investigate the interactions of Plin, Abhd5, and Atgl in live cells. Cos7 cells were triple-transfected with Plin, Abhd5, and lipase-inactive (S47A) Atgl that had been tagged with distinct fluorescent proteins. Wild type and lipase-inactive Atgl are targeted identically (16, 17), whereas use of inactive Atgl avoids confounding effects of lipase activity on intracellular lipid droplet content (16). As expected from previous analysis of endogenous proteins, Plin-EYFP and Abhd5-Cherry were highly colocalized on LDs (Fig. 1*A*). In contrast, ECFP-Atgl was

both cytosolic and on LDs; however, its colocalization with Abhd5-Cherry was very poor. Thus, although Abhd5 can interact with both Plin and Atgl, its interaction with Plin predominates in cells. To explore this directly, we compared binary interaction of Abhd5 with Plin or Atgl using a luciferase protein complementation assay. As shown in Fig. 1*B*, the interaction of Abhd5 and Plin reconstituted 6 times more activity than did the interaction of Abhd5 with Atgl ($p < 0.001$) and reflected differences in the subcellular colocalization of the fluorescent proteins.

The above results indicate that the interaction of Abhd5 with Plin predominates over its interaction with Atgl under basal conditions and suggests that the interaction of Abhd5 with Plin and Atgl might be mutually exclusive. If the interaction of Abhd5 with Plin and Atgl is mutually exclusive, then expression of Plin should reduce the interaction of Abhd5 with Atgl. If, however, Abhd5 can bind Plin and Atgl simultaneously, then the presence of Plin should not influence Abhd5-Atgl interactions. We tested the mode of Abhd5 interactions using luciferase and fluorescence complementation assays in transfected Cos7 cells. As shown in Fig. 1*C*, expression of Plin-ECFP suppressed Abhd5-Atgl luciferase complementation by 72% compared with the lipid droplet protein Adrp ($p < 0.01$). Similarly, Plin-ECFP suppressed Abhd5-Atgl fluorescence complementation by 85% compared with ECFP (Fig. 1*D*, $p < 0.01$). These data strongly indicate that the interaction of Abhd5 with Plin and Atgl is mutually exclusive in cells.

Mutation of PlinA PKA Sites 5 (Ser-492) and 6 (Ser-517) Prevents Rapid Dissociation of Abhd5 from Plin after Forskolin/IBMX Treatment—Plin phosphorylation rapidly decreases the molecular proximity between Plin and Abhd5 on LDs, as determined by FRET (3). Mouse PlinA has six consensus PKA phosphorylation sites that are thought to contribute to various aspects of Plin function (1, 4, 18). To gain further insight into this regulation, we mutated specific PKA phosphorylation sites on Plin and examined the effects on Plin-Abhd5 interactions in live cell 3T3-L1 cells. As shown in Fig. 2, forskolin/IBMX rapidly decreased energy transfer between ECFP-Abhd5 and Plin-EYFP. Mutation of PKA sites 1–4 had no effect on forskolin-induced disruption of FRET between Abhd5 and Plin, indicating involvement of PKA sites 5 and/or 6. Independent mutation of site 5 or 6 had no effect on forskolin-induced inhibition of FRET; however, forskolin-induced changes in energy transfer were absent when both sites were mutated. These results identify sites 5 or 6 of PlinA as necessary and likely sufficient determinants of PKA-dependent regulation of Plin-Abhd5 interaction.

Mutation of PlinA PKA Sites 5 (Ser-492) and 6 (Ser-517) Prevents Forskolin/IBMX-dependent Association of Abhd5 and Atgl—The sequestration-release model predicts that phosphorylation-dependent release of Abhd5 by Plin will facilitate the interaction of Abhd5 with its target lipase. This hypothesis was tested in protein complementation assays using wild type and phosphorylation-defective Plin mutants as well as a mutant Abhd5 (E262K) that has greatly reduced interaction with Plin (Ref. 10 and supplemental Fig. S1).

As shown in Fig. 3*A*, forskolin/IBMX treatment increased the interaction of Abhd5 with Atgl by nearly 3-fold ($p < 0.001$).

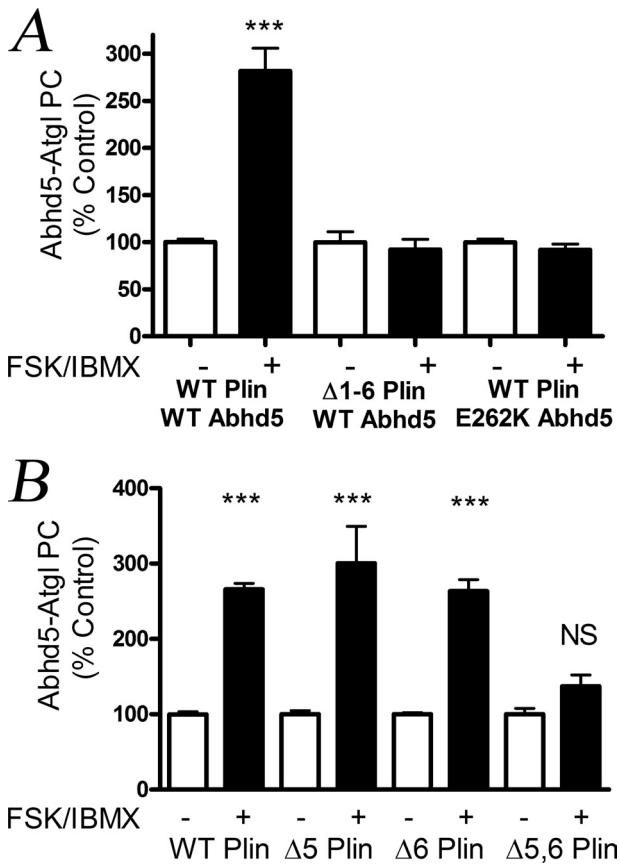

FIGURE 3. **Plin regulates the molecular interaction of Abhd5 and Atgl in Cos7 cells.** *A*, forskolin (*FSK*)/IBMX increased the interaction of Abhd5 and Atgl in cells expressing wild type (*WT*) Plin ($p < 0.001$), and this effect was absent in cells expressing phosphorylation-defective Plin (Δ1–6) and in cells expressing mutant Abhd5 that has defective interactions with Plin. Values are the means ± S.E. of three independent experiments, each performed in triplicate. *PC*, protein complementation. *B*, combined mutation of PKA sites 5 and 6 of Plin disrupted PKA-mediated regulation of Abhd5-Atgl interactions, but individual mutations did not. Values are the means ± S.E. of three independent experiments, each performed in triplicate.

The effect of PKA activation was absent in cells expressing Plin that cannot be phosphorylated by PKA as well as in cells expressing E262K Abhd5, which binds poorly to Plin. These data demonstrate that the PKA-dependent regulation requires the interaction of Abhd5 with Plin and the phosphorylation of one or more PKA sites on Plin. We next investigated the Plin phosphorylation sites that mediate the increase in Abhd5-Atgl interaction and found that single mutation of sites 5 or 6 had no effect, whereas mutation of both of these sites prevented stimulation of Abhd5-Atgl interaction by forskolin/IBMX (Fig. 3*B*). Similar results were obtained in 3T3-L1 preadipocytes in which FRET experiments were performed (supplemental Fig. S2). These data demonstrate a common molecular basis for the PKA-induced dissociation of Abhd5 from Plin, as determined by FRET, and its association with Atgl, as determined by luciferase complementation.

The Plin-dependent Interaction of Abhd5 and Atgl Occurs Mainly on Lipid Droplets—It is not known where the PKA-induced interaction of Abhd5 and Atgl takes place, and hypothetical models that address this point disagree (1, 19). Attempts to observe FRET between tagged Abhd5 and Atgl were not successful. Therefore, we turned to BiFC, which is

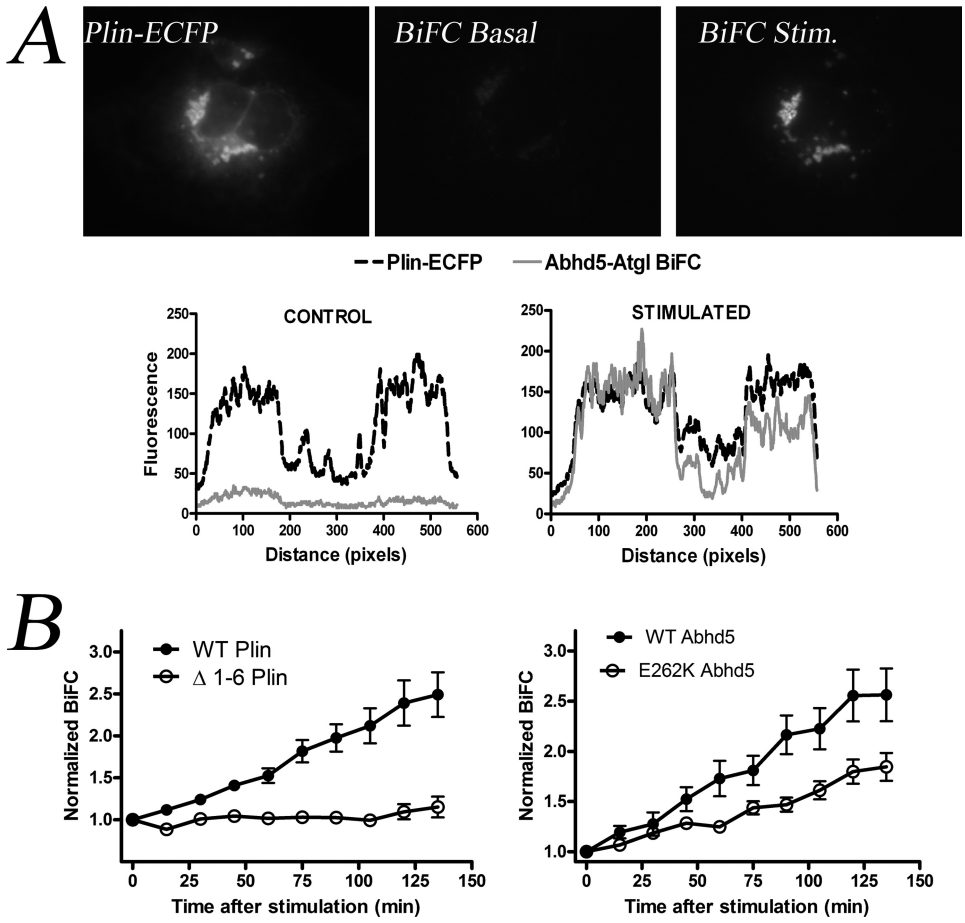

FIGURE 4. **Plin-dependent interaction of Abhd5 and Atgl occurs on lipid droplets in Cos7 cells.** *A*, subcellular localization of the PKA-dependent Abhd5 and Atgl BiFC is shown. Fluorescence imaging was performed on live cells expressing complementary EYFP fragments fused to Abhd5 and Atgl along with wild type (*WT*) Plin-ECFP before ($t = 0$) and during stimulation with forskolin/IBMX. *Top*, shown is a fluorescence image of Plin-ECFP and BiFC signals before and after stimulation. *Bottom*, shown is a line scan analysis of Plin-EYFP and Abhd5-Atgl BiFC fluorescence before and after stimulation showing nearly perfect registration of increased BiFC signals with Plin fluorescence. *B*, PKA-stimulated Abhd5-Atgl BiFC is absent in cells expressing phosphorylation-defective Plin (*left*, $p < 0.001$) and is greatly reduced ($p < 0.01$) in cells expressing E262K Abhd5, which interacts poorly with Plin (*right*). Values are means ± S.E., $n = 7–13$.

highly sensitive because the complemented fluorescence, indicative of Abhd5-Atgl interaction, is nearly irreversible and, thus, accumulates over time (20). Cos7 cells were transiently transfected with Plin-ECFP and complementary fragments of Venus fused to Abhd5 and Atgl (Fig. 4*A*). Cells in the resting state had low levels of Venus fluorescence, and increased fluorescence complementation was detected within 15 min of forskolin/IBMX stimulation and accumulated and linearly over time (see supplemental Video 1). Although some fluorescence complementation was observed in the cytoplasm, the greatest increase in BiFC was observed on LDs containing Plin, as determined by line scans (Fig. 4*A*, *lower*). Forskolin/IBMX-induced Abhd5-Atgl BiFC was absent in cells expressing phosphorylation-defective Plin (Fig. 4*B*, *left*). Furthermore, PKA-induced Abhd5-Atgl BiFC was significantly reduced (but not completely eliminated) in cells expressing the E262K Abhd5 which poorly interacts with Plin (Fig. 4*B*, *right*).

Abhd5 and Plin Contribute to the Control of Basal and PKA-stimulated Lipolysis in 3T3-L1 Adipocytes—The experiments above indicate that Plin is a negative regulator of basal lipolysis due to its ability to sequester Abhd5. To validate this prediction in adipocytes, we examined the effects of single and double siRNA knockdown of Abhd5 and Plin on lipolysis in differentiated 3T3-L1 adipocytes. As shown in Fig. 5*A*, knockdown of Plin greatly increased basal lipolysis. Knockdown of Abhd5 reduced basal lipolysis somewhat in the presence of Plin. Importantly, Abhd5 knockdown largely eliminated the rise in lipolysis produced by loss of Plin, indicating that Abhd5 lies downstream of Plin in the control of basal lipolysis.

Plin phosphorylation is required for PKA-stimulated lipolysis in adipocytes, and this effect appears to involve both Atgl and HSL (4, 8). To better gauge the role of Abhd5/Atgl during PKA activation we examined lipolysis in the presence of BAY59–9435, a selective and potent inhibitor of HSL that does not inhibit Atgl (21). As shown in Fig. 5*B*, knockdown of Abhd5 reduced both basal and stimulated lipolysis ($p = 0.007$), with the greatest absolute reduction occurring under stimulated conditions. Together, these results indicate that Plin regulates adipocyte lipolysis in part through reversible interactions with Abhd5.

DISCUSSION

Growing evidence indicates Plin plays a central role in organizing lipolytic effector proteins and directing their interactions in a PKA-dependent fashion (19, 22). Loss of function experiments have clearly demonstrated the importance of Atgl and Abhd5 in the control of basal and stimulated lipolysis (11, 12, 23); nonetheless, the mechanisms involved are not understood as the specific interactions among molecular effectors of lipolysis have not been directly investigated in cells. We previously proposed a model whereby Plin indirectly regulates Atgl activity by controlling accessibility of its co-activator, Abhd5, in a manner that depends on PKA-dependent phosphorylation of Plin (supplemental Fig. S3). According to this model, Abhd5 is sequestered by Plin in the basal state, thereby preventing activation of Atgl and reducing basal lipolysis. PKA activation leads to Plin phosphorylation, release of Abhd5, and subsequent activation of Atgl. This model is consistent with several observations (19), including the demonstration that Abhd5 is a potent activator of Atgl and that Plin binds Abhd5 in the basal state and releases it after phosphorylation. Nonetheless, several key elements and predictions of the proposed model have not been established. In fact, an alternative model has been described whereby Atgl reg-

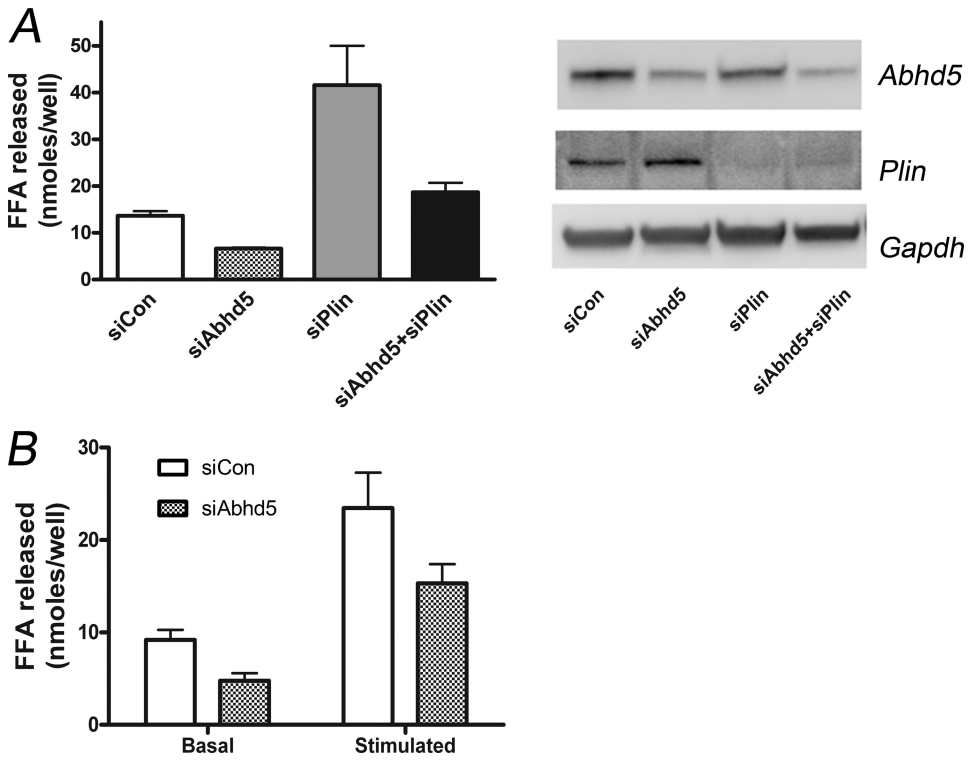

FIGURE 5. **Effect of Abhd5 knockdown on lipolysis in 3T3-L1 adipocytes.** *A*, Abhd5 mediates elevated lipolysis produced by Plin knockdown. Abhd5 and Plin expression were suppressed by siRNA separately and in combination in differentiated 3T3-L1 adipocytes, as detailed under "Experimental Procedures." Average knockdown was 56 ± 12% for Abhd5 and 68 ± 15% for Plin. *Left*, knockdown of Plin greatly increased basal lipolysis ($p = 0.022$), and this effect was significantly inhibited by knockdown of Abhd5 ($p = 0.012$). Values are the means ± S.E. for three independent experiments, each performed in triplicate. *FFA*, free fatty acid. *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase. *B*, Abhd5 contributes to HSL-independent lipolysis. 3T3-L1 adipocytes were transfected with control siRNA or siRNA for Abhd5. Basal and forskolin/IBMX-stimulated lipolysis was measured over 3 h in the presence of the HSL inhibitor BAY59–9435. Knockdown of Abhd5 significantly reduced basal and stimulated lipolysis ($p = 0.007$). Values are the means ± S.E. for three independent experiments, each performed in triplicate.

ulates basal but not hormone-sensitive lipolysis (1, 22). According to this model, basal lipolysis is stimulated by a ternary complex containing Plin, Abhd5, and Atgl. Furthermore, the model proposes that Abhd5 is not involved in PKA-stimulated activation of Atgl because it is released into the cytoplasm upon Plin phosphorylation.

The present work explored specific mechanistic relationships among lipolytic effectors with a panel of fluorescent reporters and novel cellular protein-protein interaction assays. Protein complementation assays established that interaction of Abhd5 with Plin and Atgl in live cells is mutually exclusive and that the interaction of Abhd5 with Plin predominates under basal conditions.

It is well known that Plin suppresses lipolysis (24), although the mechanisms involved are poorly understood. The results of protein interaction assays predict that Plin suppresses lipolysis in part by sequestering Abhd5 from Atgl. We verified that knockdown of Plin greatly increases basal lipolysis in 3T3-L1 adipocytes. Importantly, this increase in lipolysis was nearly eliminated by knockdown of Abhd5, indicating that Abhd5 acts downstream of Plin in the control of basal lipolysis. These data do not support a model in which basal lipolysis is mediated by a ternary complex of Plin, Abhd5, and Atgl as loss of Plin promotes basal lipolysis and the interaction of Abhd5 with Plin and Atgl is mutually exclusive.

Previous investigations of the dynamic interactions between Plin and Abhd5 by FRET demonstrated that PKA activation rapidly decreases the molecular proximity between Abhd5 and Plin in a manner dependent upon phosphorylation of one or more PKA sites in Plin (3). Plin is a multifunctional protein, and recent work indicates that phosphorylation of specific sites controls distinct functional responses (1). For example, of the six consensus PKA sites, site 5 (Ser-492) is thought to control lipase-independent droplet fragmentation (25), whereas site 6 (Ser-517) is required for full lipase activation (8). The present experiments indicate that phosphorylation of either site 5 or 6 of Plin is sufficient to release Abhd5. Although release occurs with similar kinetics, it is unclear whether these sites constitute independent means of decreasing the interaction of Abhd5 with Plin. Regardless, identification of these critical PKA sites provided a powerful means of testing whether dissociation from Plin is mechanistically linked to its association with Atgl.

We tested whether Plin phosphorylation controls the interaction of Abhd5 with Atgl using PKA-defective Plin mutants as well as a mutant of Abhd5 that does not bind Plin. The results establish that PKA activation promotes the interaction of Abhd5 with Atgl, and this effect requires the initial interaction of Abhd5 with Plin as well as the phosphorylation of Plin. Importantly, the molecular requirements of phosphorylation-dependent dissociation of Abhd5 from Plin, as measured by FRET, are exactly those required for association of Abhd5 with Atgl, as measured by luciferase complementation.

The observation that mutation of PlinA Ser-517 did not affect release of Abhd5 or its binding to Atgl was somewhat surprising as this mutation has been reported to disrupt activation of lipolysis in transfected differentiated mouse embryonic fibroblasts (8). This indicates that phosphorylation of Plin Ser-517 might perform another function required for lipolysis in addition to promoting the binding of Abhd5 to Atgl. It is also possible that cell background is important; for example, Plin-dependent activation of hormone-sensitive lipase appears to require Atgl in some cells but not others (8, 18, 26).

Previous immunofluorescence analyses have detailed PKA-induced changes in the subcellular colocalization of endogenous lipolytic effectors (3); however, interpretation of these results was tentative as immunofluorescence analysis was performed on fixed cells and did not detect specific interactions. In contrast, BiFC allows continuous imaging of specific protein-

protein interactions in live cells and is, thus, a powerful means of identifying the subcellular sites of PKA-induced, Plin-dependent Abhd5-Atgl interactions. We established that Abhd5-Atgl BiFC signals were inhibited by Plin in the basal state and that the PKA-dependent interaction of Abhd5 and Atgl required phosphorylation of Plin as well as the initial interaction of Abhd5 with Plin. The PKA-dependent interaction of Abhd5 and Atgl was detected on intracellular structures that lack Plin as well as lipid droplets containing Plin; however, the greatest increase in that interaction occurred on lipid droplets that contain Plin. Thus, although Atgl is mostly cytosolic in the basal state and sustained PKA activation disperses Abhd5 from Plin-containing LDs, PKA activation clearly increases the interaction of Abhd5 and Atgl at the LD surface.

PKA-stimulated lipolysis involves activation of both HSL and Atgl, which together constitute nearly all triglyceride lipase activity in fat cells (27). Previous investigators reported that knockdown of Abhd5 reduced lipolysis mediated by these lipases in 3T3-L1 adipocytes (11). As expected, we found that knockdown of Abhd5 reduced stimulated fatty acid efflux when HSL was inhibited. The magnitude of the reduction reflected the partial knockdown achieved, reinforcing the conclusion that Abhd5 plays a critical role in PKA-activated lipolysis independent of HSL. In addition, Abhd5 contributes to basal lipolysis as its knockdown also reduced HSL-independent fatty acid efflux from unstimulated cells. This is likely due to a small fraction of Abhd5 that is not sequestered by Plin in the basal state. Consistent with this interpretation, we found that expression of Plin did not completely abolish Abhd5-Atgl interaction.

The present work probed the organization and interaction of lipolytic effectors by overexpressing specific reporter constructs. Although this may be viewed as a limitation, we note that the subcellular targeting of the reporters in transfected cells corresponds closely to that of the endogenous counterparts in adipocytes (3). Furthermore, the functional impact of these interactions has been verified here and recently elsewhere (12) using RNA interference of endogenous lipolytic effectors.

How the interactions of Abhd5 and Atgl are regulated in tissues lacking Plin, like muscle and liver, is an important area of future research. Available evidence suggests that specific members of the Plin protein family could contribute to cell-specific regulation of stored triglyceride. In this regard, we have recently shown that Plin5 (also known as MLDP and LSDP5) binds Abhd5 in muscle and coordinates its interaction with Atgl (16). This interaction appears to promote basal lipolysis but may not be involved in stimulated lipolysis as forskolin does not affect FRET between Abhd5 and Plin5.[3]

In summary, our results demonstrate that Plin regulates the molecular interaction of Abhd5 with its target lipase, Atgl, in a fashion that depends on PKA-dependent Plin phosphorylation. The sequestration of Abhd5 by Plin appears to be a major means by which Plin suppresses lipolysis, and we speculate that compounds capable of interfering with this interaction would mimic Plin phosphorylation and, thus, promote lipolysis in fat cells. Efforts are under way to identify such compounds, as they might serve as leads for novel anti-obesity therapeutics.

[3] J. G. Granneman, unpublished information.

Acknowledgments—We thank Z. Zhu, L. Zhou, and R. Granneman for expert technical assistance and Elizabeth Floyd for advice on siRNA transfection.

REFERENCES

1. Brasaemle, D. L. (2007) *J. Lipid Res.* **48,** 2547–2559
2. Londos, C., Brasaemle, D. L., Schultz, C. J., Adler-Wailes, D. C., Levin, D. M., Kimmel, A. R., and Rondinone, C. M. (1999) *Ann. N. Y. Acad. Sci.* **892,** 155–168
3. Granneman, J. G., Moore, H. P., Granneman, R. L., Greenberg, A. S., Obin, M. S., and Zhu, Z. (2007) *J. Biol. Chem.* **282,** 5726–5735
4. Miyoshi, H., Souza, S. C., Zhang, H. H., Strissel, K. J., Christoffolete, M. A., Kovsan, J., Rudich, A., Kraemer, F. B., Bianco, A. C., Obin, M. S., and Greenberg, A. S. (2006) *J. Biol. Chem.* **281,** 15837–15844
5. Sztalryd, C., Xu, G., Dorward, H., Tansey, J. T., Contreras, J. A., Kimmel, A. R., and Londos, C. (2003) *J. Cell Biol.* **161,** 1093–1103
6. Zechner, R., Strauss, J. G., Haemmerle, G., Lass, A., and Zimmermann, R. (2005) *Curr. Opin. Lipidol.* **16,** 333–340
7. Zimmermann, R., Strauss, J. G., Haemmerle, G., Schoiswohl, G., Birner-Gruenberger, R., Riederer, M., Lass, A., Neuberger, G., Eisenhaber, F., Hermetter, A., and Zechner, R. (2004) *Science* **306,** 1383–1386
8. Miyoshi, H., Perfield, J. W., 2nd, Souza, S. C., Shen, W. J., Zhang, H. H., Stancheva, Z. S., Kraemer, F. B., Obin, M. S., and Greenberg, A. S. (2007) *J. Biol. Chem.* **282,** 996–1002
9. Subramanian, V., Rothenberg, A., Gomez, C., Cohen, A. W., Garcia, A., Bhattacharyya, S., Shapiro, L., Dolios, G., Wang, R., Lisanti, M. P., and Brasaemle, D. L. (2004) *J. Biol. Chem.* **279,** 42062–42071
10. Yamaguchi, T., Omatsu, N., Matsushita, S., and Osumi, T. (2004) *J. Biol. Chem.* **279,** 30490–30497
11. Yamaguchi, T., Omatsu, N., Morimoto, E., Nakashima, H., Ueno, K., Tanaka, T., Satouchi, K., Hirose, F., and Osumi, T. (2007) *J. Lipid Res.* **48,** 1078–1089
12. Bezaire, V., Mairal, A., Ribet, C., Lefort, C., Girousse, A., Jocken, J., Laurencikiene, J., Anesia, R., Rodriguez, A. M., Ryden, M., Stenson, B. M., Dani, C., Ailhaud, G., Arner, P., and Langin, D. (2009) *J. Biol. Chem.* **284,** 18282–18291
13. Moore, H. P., Silver, R. B., Mottillo, E. P., Bernlohr, D. A., and Granneman, J. G. (2005) *J. Biol. Chem.* **280,** 43109–43120
14. Nagai, T., Ibata, K., Park, E. S., Kubota, M., Mikoshiba, K., and Miyawaki, A. (2002) *Nat. Biotechnol.* **20,** 87–90
15. Remy, I., and Michnick, S. W. (2006) *Nat. Methods* **3,** 977–979
16. Granneman, J. G., Moore, H. P., Mottillo, E. P., and Zhu, Z. (2009) *J. Biol. Chem.* **284,** 3049–3057
17. Smirnova, E., Goldberg, E. B., Makarova, K. S., Lin, L., Brown, W. J., and Jackson, C. L. (2006) *EMBO Rep.* **7,** 106–113
18. Souza, S. C., Muliro, K. V., Liscum, L., Lien, P., Yamamoto, M. T., Schaffer, J. E., Dallal, G. E., Wang, X., Kraemer, F. B., Obin, M., and Greenberg, A. S. (2002) *J. Biol. Chem.* **277,** 8267–8272
19. Granneman, J. G., and Moore, H. P. (2008) *Trends Endocrinol. Metab.* **19,** 3–9
20. Hu, C. D., Chinenov, Y., and Kerppola, T. K. (2002) *Mol. Cell* **9,** 789–798
21. Mottillo, E. P., Shen, X. J., and Granneman, J. G. (2007) *Am. J. Physiol. Endocrinol. Metab.* **293,** E1188–E1197
22. Brasaemle, D. L., Subramanian, V., Garcia, A., Marcinkiewicz, A., and Rothenberg, A. (2009) *Mol. Cell. Biochem.* **326,** 15–21
23. Lass, A., Zimmermann, R., Haemmerle, G., Riederer, M., Schoiswohl, G., Schweiger, M., Kienesberger, P., Strauss, J. G., Gorkiewicz, G., and Zechner, R. (2006) *Cell Metab.* **3,** 309–319
24. Brasaemle, D. L., Rubin, B., Harten, I. A., Gruia-Gray, J., Kimmel, A. R., and Londos, C. (2000) *J. Biol. Chem.* **275,** 38486–38493
25. Marcinkiewicz, A., Gauthier, D., Garcia, A., and Brasaemle, D. L. (2006) *J. Biol. Chem.* **281,** 11901–11909
26. Zhang, H. H., Souza, S. C., Muliro, K. V., Kraemer, F. B., Obin, M. S., and Greenberg, A. S. (2003) *J. Biol. Chem.* **278,** 51535–51542
27. Schweiger, M., Schreiber, R., Haemmerle, G., Lass, A., Fledelius, C., Jacobsen, P., Tornqvist, H., Zechner, R., and Zimmermann, R. (2006) *J. Biol. Chem.* **281,** 40236–40241