

Optic Atrophy 1 is an A-kinase anchoring protein that mediates adrenergic control of lipolysis

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Abstract

Adrenergic stimulation of adipocytes yields a cAMP signal that activates protein kinase A (PKA). PKA phosphorylates perilipin, a protein localised on the surface of lipid droplets that serves as a gate-keeper to regulate access of lipases converting stored triglycerides to free fatty acids and glycerol in a phosphorylation-dependent manner. Here, we report a new function for Optic Atrophy 1 (OPA1), a protein known to regulate mitochondrial dynamics, as a dual-specificity A-kinase anchoring protein associated with lipid droplets. By a variety of protein interaction assays, immunoprecipitation and immunolocalization experiments, we show that OPA1 organizes a supramolecular complex containing both PKA and perilipin. Furthermore, by a combination of siRNA-mediated knockdown, reconstitution experiments using full-length OPA1 with or without the ability to bind PKA **or truncated OPA1 fused to a lipid droplet targeting domain** and cellular delivery of PKA anchoring disruptor peptides, we demonstrate that OPA1 targeting of PKA to lipid droplets is necessary for hormonal control of perilipin phosphorylation and lipolysis.

Introduction

In the fed state, the normal physiological response of white adipose tissue (WAT) to insulin involves generation of triacylglycerol (TAG) from free, nonesterified fatty acids (FFA) and glycerol and storage of TAG in intracellular lipid droplets. Lipid droplets consist of a hydrophobic core of TAG, surrounded by a phospholipid and cholesterol monolayer to which numerous proteins are attached. In the fasting state, stimulation of β -adrenergic receptors (β -ARs) with catecholamines either through sympathetic nervous control (noradrenalin), or through hormonal stress responses (adrenalin) or triggering of glucagon receptors on adipocytes initiate a precisely regulated process of lipolysis which liberates FFA and glycerol to be used as energy substrates by other tissues. The activation of lipolysis proceeds by generation of cAMP leading to activation of protein kinase A (PKA). Subsequently, PKA phosphorylates perilipin (PAT-family member perilipin 1, *PLIN1*; see (Kimmel et al, 2009) for review of nomenclature), a protein localised on the surface of the TAG-containing lipid droplets. Perilipin serves as a gate-keeper that controls access of lipases to lipid droplets in a phosphorylation-dependent fashion (Blanchette-Mackie et al, 1995; Fain & Garcija-Sainz, 1983; Greenberg et al, 1991; Greenberg et al, 1993). In the absence of any stimulus, unphosphorylated perilipin on the surface of lipid droplets blocks access of lipases, thus protecting TAG from lipolysis. Upon phosphorylation perilipin alters conformation allowing lipases to access lipid droplets and degrade TAG (Brasaemle et al, 2000b; Souza et al, 2002; Tansey et al, 2003). Hence, at times of stress, exercise or fasting, hormone-induced lipolysis is triggered by making lipid droplets accessible for lipases and liberated FFA serves as fuel for the peripheral tissues of the body (such as the heart and skeletal muscle) (Brasaemle et al, 2000a; Londos et al, 1999). Furthermore, PKA phosphorylates hormone-sensitive lipase (HSL), which was the first substrate for PKA shown to be regulated in hormone-induced lipolysis. HSL was until recently thought to be the enzyme responsible for hydrolysis of

stored TAG and to control the rate-limiting step in regulation of lipolysis (Fain & Garcija-Sainz, 1983). However, HSL null-mutant mice clearly demonstrated that HSL is dispensable as a substantial fraction of catecholamine-stimulated lipolysis as well as basal lipolysis remain unaffected (Haemmerle et al, 2002; Osuga et al, 2000; Wang et al, 2001). Even more recent studies have identified desnutrin / adipocyte triglyceride lipase (ATGL) as the most important enzyme for degradation of TAG (Jenkins et al, 2004; Villena et al, 2004; Zimmermann et al, 2003; Zimmermann et al, 2004). Triacylglycerol hydrolase (TGH) may also contribute to non-HSL TAG lipolysis (Soni et al, 2004). Desnutrin/ATGL and TGH are, however, not regulated by phosphorylation. Together with the fact that HSL is dispensable in catecholamine-stimulated lipolysis, these results point to the gate-keeper function of perilipin as an important regulatory mechanism. However, HSL appears to be the only enzyme degrading diacylglycerol to FFA and monoacylglycerol which is then hydrolysed to FFA and glycerol by monoglyceride lipase, but these reactions proceed comparably faster than the TAG hydrolysis which remains the rate-limiting step in the control of lipolysis (Fredrikson et al, 1986; Giudicelli et al, 1974; Tornqvist & Belfrage, 1976).

The localisation of PKA holoenzyme within the cell is controlled by interactions between the regulatory (R)-subunit dimer of PKA and A-kinase anchoring proteins (AKAPs) (Diviani & Scott, 2001; Michel & Scott, 2002; Tasken & Aandahl, 2004). AKAPs target pools of PKA type I and type II (distinguished by the type I or type II R subunit, RI or RII) to distinct subcellular loci and facilitate discrete spatial and temporal control of phosphorylation of specific substrates. They also scaffold supramolecular signalling complexes with multiple signalling enzymes. All AKAPs contain an A kinase binding (AKB) domain and a unique targeting domain directing the PKA-AKAP complex to defined subcellular structures, membranes or organelles (Carr et al, 1992; Gold et al, 2006; Kinderman et al, 2006; Pidoux & Tasken, 2010);(Wong & Scott, 2004).

Some studies have suggested the presence of AKAPs associated with lipid droplets to trigger lipolysis by discrete control of perilipin phosphorylation (Bridges et al, 2006; Zhang et al, 2005). However, no AKAP targeting a pool of PKA to lipid droplets and controlling the induction of lipolysis via phosphorylation of perilipin has been identified to date. In this study we show that the previously identified mitochondrial protein Optic Atrophy 1 (OPA1) also functions as a dual-specificity AKAP (binding both type I and type II PKA) associated with lipid droplets. Mutations in the *opal* gene is the predominant cause of autosomal dominant optic atrophy (ADOA), a progressive form of bilateral blindness caused by loss of retinal ganglion cells and atrophy of the optic nerve (Alexander et al, 2000; Delettre et al, 2000). Previous studies have shown OPA1 to be a dynamin-related GTPase required for mitochondrial fusion, fission and regulation of apoptosis and localized both on the inner membrane of mitochondria and on cristae (Cipolat et al, 2004; Frezza et al, 2006; Ishihara et al, 2006; Sesaki et al, 2003). Here, we report the presence of OPA1 in adipocytes where it is associated both with mitochondria and lipid droplets. We find that OPA1 forms a complex with PKA and perilipin on lipid droplets. Finally, we assign a new function to OPA1 by showing that it is involved in the control of lipolysis in response to adrenergic stimuli by anchoring a pool of PKA that phosphorylates perilipin and thereby triggers lipolysis.

Results

OPA1 is an AKAP associated with lipid droplets

To assess the effect of PKA anchoring in adrenergic regulation of lipolysis, 3T3-L1 cells were differentiated into adipocytes and transfected with constructs directing expression of a HA-tagged soluble fragment of AKAP-Lbc encompassing the PKA binding site (Ht31 anchoring disruptor) or the corresponding control construct with proline substitutions (Ht31-P) that does not bind PKA (Ct in Figure 1A). Subsequently, transfected cells were stimulated with isoproterenol to activate PKA and perilipin phosphorylation status examined in immunoprecipitates from total cell extracts using an anti-RRXpS/T antibody detecting phosphorylated PKA substrates (Figure 1A). While isoproterenol-induced perilipin phosphorylation was observed in Ht31-P transfected cells, expression of the Ht31 anchoring disruptor abolished phosphorylation without affecting the level of immunoprecipitated perilipin. This suggests that an AKAP targets PKA to facilitate discrete adrenergic control of perilipin phosphorylation. In search of an AKAP for perilipin, lipid droplets were purified by sucrose gradient fractionation of lysates from differentiated 3T3-L1 adipocytes and lipid droplet protein extracts submitted to overlay with radiolabeled RII in the absence and presence of Ht31 anchoring disruptor peptide (Figure 1B). Five bands with molecular masses of approximately 110, 90, 75, 50 and 40 kDa detected by RII-overlay and competed by Ht31 peptide appeared to be enriched in lipid droplets compared to cell lysates from 3T3-L1 cells. Proteins in the regions of corresponding mobility from parallel lanes in the gel were excised, subjected to tryptic digestion and analyzed by mass spectrometry (Supplementary Table I).

Curation of lists of identified proteins from several experiments and bioinformatic analysis to identify proteins with putative PKA binding sites resulted in a list of nine AKAP candidates. Libraries of overlapping peptides covering the putative PKA binding sites of the

candidates were synthesized on solid phase and subjected to RII-overlay in absence and presence of Ht31 peptide (Figure 1C). The amphipathic helix AKB from AKAP-KL was included as positive a control. This analysis identified a sequence in the C-terminal part of a protein called optic atrophy 1 (OPA1) that bound RII in a Ht31-dependent manner. This suggests that OPA1 is a putative AKAP and that, in addition to its mitochondrial localization, OPA1 can also be associated with lipid droplets in adipocytes. To confirm and extend these results, the C-terminal part of the OPA1 sequence mapped in Figure 1C was synthesized as 19-mer overlapping peptides with 1-amino-acid offset on solid phase and analyzed for PKA binding by RI and RII-overlay with or without Ht31. This identified a sequence binding both RI and RII in the C-terminal part of OPA1 (amino acids 940 to 958, Figure 1D). Modeling the sequence of amino acids 940 to 958 in OPA1 as an α -helix in a helical wheel configuration suggested that the AKB sequence is an amphipathic helix with one clearly defined hydrophobic face and a negatively charged polar face, which is characteristic of AKB regions in AKAPs (Figure 1E, left). Furthermore, proline substitutions in the sequence (V942P, I945P, L949P; peptide OPA1 940-958-3P) that distort the α -helical configuration abolished binding to PKA (Figure 1E, right).

We have previously developed an amplified luminescence ligand proximity assay (AlphaScreen) based on RI binding to D-AKAP1 that can be used to characterize AKB regions in AKAPs as competitors (Jarnaess et al, 2008; Stokka et al, 2006). Since OPA1 appeared to bind both RI and RII, we used this RI competition binding platform to evaluate the binding properties of OPA1. As shown in Figure 1F, a peptide corresponding to amino acids 940 to 958 in OPA1 as well as Ht31 competed RI binding to D-AKAP1 with IC_{50} -values of 8.1 ± 0.13 and 9.5 ± 0.16 μ M, respectively, in line with earlier observations with RI using this assay (Jarnaess et al, 2008). In contrast, the OPA1 940-958-3P control peptide was not able to compete the D-AKAP-RI interaction confirming the observation in Figure 1E.

Taken together our results suggest that OPA1 is an AKAP with a PKA binding domain encompassing amino acids 940-958 in the C-terminal part of the protein that can bind both RI and RII.

Kinetics of the OPA1-PKA interaction

We next determined the rate and affinity constants of the OPA1-PKA association. Real-time analyses were carried out by surface plasmon resonance (SPR) technology (Figure 2). Specifically, cAMP-free RI and RII were immobilized on the surface of a CM5 sensor chip coated with 8-AHA-cAMP and a purified, MBP-fused full-length OPA1 protein (containing the AKB region) was injected over the surfaces. The sensorgrams of MBP-OPA1 binding to RI and RII loaded surfaces (Figure 2A and 2C, respectively) as well as the corresponding steady state binding isotherms (Figure 2B and 2D) clearly demonstrated a concentration-dependent binding with nanomolar affinities for both RI (K_D 12.5 ± 2.8 nM) and RII (K_D 14.0 ± 1.4 nM) and with K_D -values consistently somewhat lower for RI than for RII in all experiments. Furthermore, in contrast to observations made with other RI-binding AKAPs which typically display a faster off-rate for RI, the on- and off-rates of RI and RII binding to OPA1 were similar (RI: $K_a = 6.6 \cdot 10^5$ $M^{-1}s^{-1}$, $K_d = 3.7 \cdot 10^{-4}$ s^{-1} ; RII: $K_a = 1.1 \cdot 10^6$ $M^{-1}s^{-1}$, $K_d = 4.4 \cdot 10^{-4}$ s^{-1}). Binding of MBP-OPA1 to RI and RII was displaced in the presence of increasing concentrations (10 to 50 μ M) of the OPA1 940-958 peptide characterized in Figure 1F (Figure 2E and 2F), whereas no displacement was observed with the proline-substituted OPA1 940-958-3P peptide. These results suggest that OPA1 may be a true dual-specificity AKAP.

OPA1 and PKA co-localize on lipid droplets in 3T3-L1 adipocytes

OPA1 expression in brown (BAT) and white (WAT) adipose tissue, liver, muscle and 3T3-L1 adipocytes was analyzed by immunoblotting (Figure 3A). This showed high levels of expression of three different forms in brown adipose tissue (BAT) and 3T3-L1 adipocytes whereas only the high and low molecular weight isoforms were detected at lower levels of expression in WAT. Liver and muscle also expressed OPA1 isoforms of different mobility. In contrast, perilipin was only expressed in adipose tissue and 3T3-L1 adipocytes. The level of expression of OPA1 could relate to the number of mitochondria e.g. in BAT versus WAT as OPA1 is previously reported to be localized in mitochondria.

The expression of OPA1 was next analyzed during differentiation of 3T3-L1 preadipocytes into adipocytes (Figure 3B). Whereas the expression of the different R isoforms RI α , RII β remained constant during the differentiation process, the expression of OPA1 was up-regulated somewhat prior to or concomitantly with the appearance of perilipin which could indicate regulation of OPA1 during adipocyte development by transcription factors such as PPAR γ to match that of other lipid droplet proteins. **In contrast, mitochondrial proteins such as mitofilin and mitofusin 2 were not regulated with the same kinetics or amplitude, suggesting that the observed regulation of OPA1 is not just a function of an increase in the number of mitochondria.**

The subcellular localization of OPA1 versus perilipin and PKA in adipocytes was analyzed by dual immunofluorescent labeling followed by laser confocal analysis and the extent of overlap was measured using line plot profile and intensity correlation coefficient-based analysis (Bolte & Cordelieres, 2006). OPA1 showed extensive co-localization with perilipin on lipid droplets (Figure 3C-E'; R=0.91). Moreover, studies of the localization of PKA type I (RI α) and type II (RII β) in 3T3-L1 adipocytes demonstrated RI α to have strong

co-localization with perilipin on the surface of lipid droplets (Figure 3F-H'; R=0.85). In contrast, RII β appeared more localized in the cytoplasm and showed only partial overlap with perilipin on lipid droplets (Figure 3I-K'; R=0.67). In agreement with previous studies, the localization of HSL was found to be mainly in the cytoplasm, but showed also some extent of co-localization with perilipin on lipid droplets (Figure 3L-N'; R=0.76) (Brasaemle et al, 2000a; Londos et al, 1999).

To explore the possibility of a direct physical interaction between OPA1 and RI α on lipid droplets, we next solubilized the lipid droplet protein content of differentiated 3T3-L1 cells in a buffer containing detergent and N-octyl- β -d-glucoside. Immunoprecipitation of OPA1 revealed the presence of both perilipin and OPA1 in the precipitate (Figure 4A). The reverse immunoprecipitation experiment using a perilipin antibody likewise showed co-precipitation of OPA1 (Figure 4B). Interestingly, immunoprecipitation of RI α co-precipitated OPA1 (Figure 4C) and conversely, RI α was detected in OPA1 immunoprecipitates (Figure 4D). In contrast, RII β could not be co-precipitated with OPA1 from 3T3-L1 cells (not shown), indicating either that it does not bind in the presence of RI or that the fraction associated with OPA1 is below the level of detection. Moreover, HSL and phospho-HSL were not detected after immunoprecipitation of OPA1 in adipocytes (Figure 4E). Together our results indicate that OPA1 forms a complex with perilipin and RI α on lipid droplets in 3T3-L1 adipocytes.

While 3T3-L1 adipocytes appear to have an abundance of PKA type I and RI is associated with OPA1, WAT from wild type mice has been reported to contain mainly PKA type II that regulates lipolysis and with RII β being the predominant RII subunit (Cederberg et al, 2001; Cummings et al, 1996). Therefore, we also examined the pool of PKA associated with OPA1 in total protein extracts from WAT. Here, we found that RII (Figure 4F and G) and not RI (not-shown) co-immunoprecipitated with OPA1 and *vice versa*, which is compatible with the notion of OPA1 as a dual-specificity AKAP where the association with

type I or type II PKA is determined by the availability of the two isozymes. Furthermore, OPA1 forms a complex with perilipin also in WAT (Figure 4F and 4H).

OPA1 distribution between lipid droplets and mitochondria

As reports on OPA1 localization and function in cells without lipid droplets describe a mitochondria-associated protein involved in mitochondrial fusion and fission (Cipolat et al, 2004; Ishihara et al, 2006), it was important to examine the distribution of OPA1 between lipid droplets and mitochondria in adipocytes. Immunostaining of 3T3-L1 adipocytes with perilipin as a marker of lipid droplets was performed together with transfection of the targeting domain of the MDDX28 helicase tagged with the fluorescent protein mCherry (construct Δ MDDX28mCherry) as a marker of the inner mitochondrial membrane, or with immunostaining for mitofusin 2 as a marker of the outer mitochondrial membrane. Laser confocal analysis and intensity correlation coefficient-based analysis revealed little overlap between the lipid droplet marker and mitochondrial markers (Figure 5, A-C and D-F and Supplemental Figure S1A and S1B, $R=0.22$ and 0.43 , respectively). In contrast, transfection of 3T3-L1 adipocytes with a construct directing the expression of the full-length OPA1 tagged with green fluorescent protein (GFP-FL-OPA1[#]) showed specific import of the tagged OPA1 to lipid droplets as evident from a strong overlap with perilipin immunostaining as a marker for lipid droplets (Figure 5G-I and Supplemental Figure S1C, $R=0.89$) whereas comparably lower intensity signals were detected over mitochondria. This, together with the extensive co-localization of endogenous OPA1 with perilipin (Figure 3C-E'; $R=0.91$) strongly suggest that OPA1 distribution in adipocytes is not strictly limited to mitochondria and that the main fraction is targeted to lipid droplets together with perilipin.

To determine in more detail the distribution of OPA1 between mitochondria and lipid droplets in adipocytes, post-nuclear supernatants were fractionated by sucrose gradient centrifugation to separate light components with high lipid content such as liposomes from cytoplasm and organelles with comparably higher density. Immunoblotting of the resulting fractions (Figure 5J) and quantification by densitometry of the relative distribution from several experiments (Figure 5K) showed perilipin exclusively in fraction 1 corresponding to the lipid droplet position in the density gradient. In contrast, mitochondrial markers for the inner membrane (mitofilin), outer membrane (mitofusin 2) and mitochondrial matrix (pyruvate dehydrogenase E1 β subunit) were mainly localized in the bottom fractions, although 10% of mitofilin, 5% of mitofusin 2 and 4% of pyruvate dehydrogenase E1 β subunit were also found in the top fraction. These mitochondrial contaminations observed in the lipid droplets fraction could represent mitochondrial membranes from ruptured mitochondria or possibly intact mitochondria weakly associated with lipid droplets. Interestingly, although OPA1 is reported to be a mitochondrial protein in other cell types, its distribution in 3T3-L1 adipocytes with more than 80% in the top fractions and less than 20% in the bottom fractions is distinctly different from the distribution of the mitochondrial markers in the same experiments. Altogether, our results strongly suggest that a significant portion of OPA1 is associated with the lipid droplet fraction in a mitochondria-independent manner. Furthermore, we also observed a strong co-localization between lipid droplets (perilipin immunolabeling) and GFP-FL-OPA1 (Figure 5G-H and Supplemental Figure S1C; Supplemental Figure S2A-C') and GFP-FL-OPA1 with 3 proline substitutions (V942P, I945P, L949P) inside the AKB domain (GFP-OPA1[#] AKBmut) (Supplemental Figure S2D-F'). In addition, deletion of the OPA1 mitochondrial targeting sequences (amino acids 1-87; Δ MTS) as defined in several reports (Kita et al, 2009; Misaka et al, 2002; Olichon et al, 2002; Satoh et al, 2003) or deletion of the 30 first critical amino acids of the MTS (Δ 1-30) abolish targeting of OPA1 to lipid

droplets as well as mitochondria (Supplemental Figure S2G-P'). Furthermore, deletion of the 57 last amino acids of the MTS (Δ 30-87) also reduced targeting of OPA1 to both lipid droplets and mitochondria, although to a greater extent for the mitochondrial localization (Supplemental Figure S2Q-V', $R=0.48$ versus $R=0.26$). These observations suggest that the MTS in OPA1 is important for the targeting of OPA1 also to lipid droplets and that an independent lipid droplet targeting sequence cannot readily be defined.

OPA1 is implicated in the adrenergic control of lipolysis

To address the function of OPA1 as a putative AKAP for perilipin we employed a combined strategy of RNA interference and rescue with wild type or modified forms of OPA1 and examined the effect on adrenergic regulation of lipolysis. First, 3T3-L1 adipocytes were transfected with specific OPA1 siRNA or scrambled control, incubated for 24 h, re-transfected with OPA1 siRNA and incubated for another 48 h. As shown by immunoblot analysis (Figure 6A) siRNA-mediated knock down of OPA1 markedly reduced OPA1 protein level by approximately 85% after 48 to 72 h of culture compared to cells transfected with scrambled siRNA ($p < 0.025$, $n=3$, see also Supplemental Figure S3A). No difference was seen between control-transfected and untransfected cells, indicating that the method of transfection did not interfere with OPA1 expression nor was it toxic to the differentiated adipocytes. The expression of RI α , RI β and HSL remained constant in cells that received OPA1 siRNA, indicating that removal of OPA1 does not affect other components of the PKA signaling pathway. Interestingly, perilipin expression was significantly reduced after siRNA-mediated knock down of OPA1 by up to 75% at 48 h and 55% at 72 h post transfection (Supplemental Figure S3B; $p < 0.05$). The effect of OPA1 knock down on perilipin expression may indicate that OPA1 and perilipin interact to stabilize each other and that loss

of perilipin expression could result from destabilization of a supramolecular complex. Interestingly, expression of the PAT-family protein ADRP (perilipin 2), but not TIP47 (perilipin 3), appeared to increase 2 to 3-fold at 48 h, maybe as a rescue mechanism for perilipin downregulation to help preserve lipid droplets (Supplemental Fig. S4).

Knock down of OPA1 protein expression was associated with a significant decrease in isoproterenol-induced glycerol release from 3T3-L1 adipocytes with little adrenergic induction of lipolysis and a 60 % reduction in glycerol release compared to scrambled control at 5 or 10 nM isoproterenol (Figure 6B). The inhibition of isoproterenol-induced lipolysis by OPA1 knock down was partially overcome at 100 nM where only 37% inhibition compared to control was observed probably because the high cAMP activates PKA over longer distances inside the cell and reducing the requirement for AKAP targeting. For this reason, further studies of the effect of manipulation of OPA1 levels on lipolysis were examined at concentrations around EC_{50} (10-30 nM). To rescue cells with OPA1 knock down, 3T3-L1 adipocytes were first transfected with OPA1 siRNA to remove endogenous OPA1. This was followed by transfection with mammalian expression vectors encoding wild type full length OPA1 fused to green fluorescent protein (GFP-FL-OPA1), GFP-OPA1 made insensitive to functional siRNA (GFP-FL-OPA1[#]) or GFP-FL-OPA1[#] with three proline substitutions (V942P, I945P, L949P) inside the AKB domain (GFP-FL-OPA1[#] AKBmut) which should abolish binding of both type I and type II PKA as shown in Figure 1E, 1F, 2E and 2F. Whereas cells transfected with an expression vector directing the transcription of an siRNA-sensitive OPA1 did not regain OPA1 expression, adipocytes transfected with vectors directing the expression of siRNA insensitive GFP-FL-OPA1[#] or GFP-FL-OPA1[#] AKBmut presented expression of a GFP-FL-OPA1 fusion protein (Figure 6C). Furthermore, colocalization with lipid droplets was confirmed for both these constructs by perilipin immunofluorescent labeling (Figure 5G-I, Supplemental Figures S1C and S2D-F'). While the expression of RI α ,

RHIB and HSL remained constant, the expression of perilipin was also found to be decreased in cells transfected with OPA1 siRNA (75% reduction) as previously observed in Figure 6A and in Supplemental Figure S3B, and there was a tendency to reversal of this effect in cells where OPA1 expression was rescued (Supplemental Figure S3C, $p=0.07$).

To assess the ability of the exogenously introduced GFP-FL-OPA1 to form a complex with endogenous PKA and perilipin, immunoprecipitations were performed from cell lysates of adipocytes co-transfected with OPA1 siRNA and siRNA resistant GFP-FL-OPA1[#] or GFP-FL-OPA1[#] AKBmut (Figure 6D, left). Immunoprecipitation with RI α revealed the presence of GFP-FL-OPA1 in the precipitate from cells transfected with a vector encoding GFP-FL-OPA1[#] but not from cells expressing GFP-FL-OPA1[#] AKBmut, whereas RI α was precipitated in both cases. Together with the GFP immunoprecipitation experiment, where RI only co-precipitated with GFP-FL-OPA1[#] and not GFP-FL-OPA1[#] AKBmut, this indicates that OPA1 with 3 proline substitutions in the PKA binding site does not interact with PKA *in situ*. However, immunoprecipitation with GFP antibody showed the presence of GFP-FL-OPA1 and perilipin in precipitates from cells expressing either GFP-FL-OPA1[#] or GFP-FL-OPA1[#] AKBmut indicating that both the wild type and substituted protein localize normally and interact with perilipin as also shown in the colocalization studies (Figure 5G-I, Supplemental Figure S1C, S2D-F'). Interestingly, the basal phosphorylation state of perilipin as observed with an anti-RXXpS/T antibody was found to be reduced by more than 90% in immunoprecipitates from cells reconstituted with GFP-FL-OPA1[#] AKBmut as compared to cells expressing GFP-FL-OPA1[#] (Figure 6D, right panel, $p < 0.0025$). This suggests that the absence of PKA anchored to OPA1 affects basal levels of perilipin phosphorylation. Finally, immunoprecipitation of HSL revealed the presence of the same levels of phospho-HSL in precipitates from both GFP-FL-OPA1[#] and GFP-FL-OPA1[#] AKBmut transfected cells,

indicating that the ability of OPA1 to bind PKA does not influence the basal phosphorylation state of HSL.

Isoproterenol-induced lipolysis in cells reconstituted with OPA1 was next assessed as glycerol-release in the media from 3T3-L1 adipocytes transfected with OPA1 siRNA and either GFP-FL-OPA1[#] or GFP-FL-OPA1[#] AKBmut (Figure 6E). In the presence of 10 nM isoproterenol, a robust glycerol release was observed in cells that had received scrambled siRNA (solid bar) whereas significantly less glycerol was released in cultures treated with OPA1 siRNA (open bar, 51% reduction, $p < 0.001$) as also seen in Figure 6B. Moreover, when GFP-FL-OPA1[#] was expressed in cells with knock down of endogenous OPA1 the isoproterenol-induced glycerol release was increased 2-fold compared to OPA1 knock down cells and adrenergic regulation was partially restored ($p < 0.001$). In contrast, expression of GFP-FL-OPA1[#] AKBmut in OPA1 knock down cells did not restore isoproterenol-induced glycerol release (57.8% reduction compared to cells transfected with scrambled siRNA, $p < 0.001$) and was not significantly different from the level of glycerol release in OPA1 knock down cells. The fact that GFP-FL-OPA1 expression did not completely rescue the effect of OPA1 knock down on isoproterenol-induced lipolysis may be due to transfection efficiency of the mammalian expression vector which was lower than that of the siRNA as observed by GFP fluorescence compared to fluorescence of FITC-labeled siRNA in separate experiments (data not shown).

To examine the role of OPA1 in the control of lipolysis independently of its mitochondrial localization, we next engineered chimeric constructs consisting of the lipid droplet targeting sequence from perilipin (LDTS, amino acids 233 to 366, notably without any of the four PKA phosphorylation sites as Ser 276 inside the sequence was to Ala) (Subramanian et al, 2004) fused to OPA1 deleted of the mitochondrial targeting sequence (Δ MTS-OPA1[#]) that directs the localization of wild type OPA1 to both mitochondria and

lipid droplets (Supplemental Figure S2G-V') with or without the AKB mutated (perilipin-LDTS Δ MTS-OPA1[#] or perilipin-LDTS Δ MTS-OPA1[#] AKBmut) and fused to green fluorescent protein in the N- or C-terminal position (see Figure 6E, top for design of fusion proteins). Expression of the chimeric proteins in the absence of endogenous OPA1 revealed colocalisation with lipid droplets as evident from correlation analysis of GFP-fluorescence with perilipin immunolabeling (correlation coefficients of R=0.63 to 0.66, see Supplemental Figure S3D-F' for analysis of perilipin-LDTS Δ MTS-OPA1[#] AKBmut-GFP and perilipin). In the absence of endogenous OPA1 expression of the two chimeric LDTS- Δ MTS-OPA1 proteins with a functional AKB domain were able to restore isoproterenol-induced glycerol release (both $p < 0.001$) compared to OPA1 knock down cells whereas expression of chimeric constructs with a mutant AKB domain did not (Figure 6E). These data strongly suggest that LD-targeted OPA1 with a functional AKB domain is able to confer adrenergic regulation of perilipin phosphorylation by localizing of PKA independently of the mitochondrial pool of OPA1.

To control for the possibility that changes in glycerol release observed upon manipulations of OPA1 levels were not due merely to changes in the stored amount of TAG available for lipolysis or an effect on lipid droplet integrity, lipid droplets of OPA1 knock down cells were examined by immunostaining with perilipin antibody (Figure 6F and H, green) in combination with Oil Red O staining for TAG (Figure 6G and H, red). Lipid droplets in OPA1 knockdown cells were intact and with normal TAG stores. Furthermore, the level of TAG stored in adipocytes after siRNA-mediated OPA1 knockdown and reconstitution with exogenous OPA1 were quantified by staining cell cultures with Oil Red O, washing, extracting the remaining Oil Red O in isopropanol and measuring optical density (Figure 6I). No significant difference in TAG content was observed in adipocytes after siRNA knock down or OPA1 expression compared to cells transfected with scrambled siRNA. To

ensure that siRNA mediated knock down and reconstitution experiment did not affect the β adrenergic signaling pathway or integrity and function of mitochondria we measured the intracellular cAMP production and the ATP concentration in adipocytes before and after isoproterenol stimulation. Whereas a clear increase of cAMP was observed after stimulation with 10 nM isoproterenol compared to basal levels, no significant difference in intracellular cAMP production was observed after the different transfections (Figure 6J). Moreover, no differences in ATP concentration were observed in cells after siRNA mediated knock down and reconstitution experiments (Figure 6K).

Together our knock down and reconstitution experiments suggest that OPA1 plays a key role in the regulation of lipolysis by coordinating a signal complex on lipid droplets containing both PKA and perilipin where discretely controlled phosphorylation of perilipin to regulate access of lipases to the lipid stores upon adrenergic stimuli.

The dual-specificity AKAP OPA1 permits both type I and type II PKA to regulate lipolysis

A typical feature of AKAP-coordinated phosphorylation events regulating physiological processes is that displacement of the anchored pool of PKA by anchoring disruptor peptides should block regulation by cAMP. The prototypic anchoring disruptor peptide Ht31 derived from AKAP-Lbc (Carr et al, 1992) has been extensively used for this type of experiment by offering soluble binding sites for PKA and was already in Figure 1A shown to disrupt isoproterenol-induced perilipin phosphorylation. Ht31 disrupts anchoring of type II PKA, but at somewhat higher concentrations also competes anchoring of type I PKA (Gold et al, 2006; Herberg et al, 2000; Ruppelt et al, 2007). More recently, the development of the high-affinity, isoform-specific anchoring disruptors RI Anchoring Disruptor (RIAD) and SuperAKAP-*IS*

has provided tools to specifically delineate effects mediated by type I or type II PKA (Carlson et al, 2006; Gold et al, 2006). Here, we used the tool box of anchoring disruptor peptides to further characterize the AKAP coordinating adrenergic control of lipolysis. First, conditions for peptide loading of differentiated adipocytes were established by incubating 3T3-L1 adipocytes with FITC-labeled, poly-arginine-tagged RIAD and SuperAKAP-*IS* peptides at different concentrations and for different time periods. Maximal peptide loading was observed at 120 min (Figure 7A and 7B respectively). Furthermore, an optimal peptide concentration of 30 μ M was determined by toxicity testing (data not shown). The effect of anchoring disruptors on lipolysis was next analyzed by glycerol-release assay. Adipocytes pre-incubated with Ht31 peptide presented significantly decreased glycerol release after stimulation with 30 or 100 nM isoproterenol compared to mock loaded cells (42% reduction, $p < 0.005$; 92% reduction, $p < 0.001$, respectively) (Figure 7C). In contrast, 3T3-L1 adipocytes incubated separately with either RIAD (Figure 7D) or SuperAKAP-*IS* peptide (Figure 7E) did not show any significant differences in lipolysis compared to the effect of the corresponding scrambled peptides. However, incubation with a combination of RIAD and SuperAKAP-*IS* peptides (Figure 7F, 30 μ M each) effectively blocked isoproterenol-induced glycerol release (74 and 97% decrease at 30 and 100 nM isoproterenol compared to scrambled control with both peptides, $p < 0.001$). Our results are compatible with the notion derived from the kinetic data on the PKA-OPA1 association in Figure 2 that OPA1 is a true dual-specificity AKAP that permits both type I and type II PKA to bind and regulate lipolysis (see Figure 7G for depiction of effect of various anchoring disruptors). From this notion it can be derived that if both type I and type II PKA are available for binding to OPA1 (although RI appears to be the preferred binding partner in 3T3-L1 cells), only the combination of RIAD and SuperAKAP-*IS* would compete binding of both forms of PKA and block adrenergic regulation of lipolysis.

This is consistent with our observations of hormonally regulated lipolysis in the presence of various anchoring disruptors (Figure 7F and 7G, bottom right panel).

Discussion

Here we report the identification of OPA1 as an AKAP for perilipin. OPA1, normally found in mitochondria in other cell types, is expressed at increasing levels during adipocyte differentiation and targeted to lipid droplets where it organizes a supramolecular complex with both PKA and perilipin. We show that OPA1 is necessary for discrete control of perilipin phosphorylation and lipolysis.

The observation that adipocytes expressing the Ht31 anchoring disruptor lacked perilipin phosphorylation upon adrenergic stimulation indicated that AKAP-mediated targeting of PKA was necessary for perilipin phosphorylation. This prompted us to search for an AKAP for perilipin. Several AKAPs have already been identified in adipocytes (Bridges et al, 2006; Chaudhry et al, 2002; Nomura et al, 2002; Tao et al, 2003; Zhang et al, 2005). According to Tao *et al* a complex formed of the β -AR, an AKAP and PKA is involved in receptor internalization, resensitization and recycling of the β -AR in adipocytes (Tao et al, 2003). Although some observations suggested that the association between β -AR and PKA in adipocytes is mediated by AKAP150 or gravin (Fraser et al, 2000; Tao et al, 2003), Zhang *et al* proposed the involvement of a yet unidentified AKAP (Zhang et al, 2005). Moreover, the compartmentalization of HSL with AKAP150 and PKA has been described to be required for the induction of lipolysis by controlling HSL phosphorylation (Nomura et al, 2002), but the importance of this observation is challenged by the more recent reports that HSL is dispensable in catecholamine-stimulated lipolysis. A more recent work proposed D-AKAP1 as a major adipocyte PKA binding protein and discussed the possibility of D-AKAP1 as a

lipid droplet AKAP, but failed to convincingly demonstrate an association with lipid droplets and did not show data to support any functional effect of D-AKAP1 (Bridges et al, 2006). Furthermore, Chaudhry et al showed that D-AKAP1 is associated with mitochondria in differentiated adipocytes (Chaudhry et al, 2002). In summary, no AKAP has previously been clearly shown to be associated with lipid droplets and to be involved in the regulation of the lipolytic process.

We report here for the first time that OPA1 is a dual-specificity AKAP targeted to lipid droplets in adipocytes. An amphipathic helix AKB domain localized in the C-terminal part of OPA1 is able to bind both type I and type II PKA with low nanomolar affinities. While most dual-specificity AKAPs appear to have lower affinity for type I than for type II PKA due to a faster off-rate of RI (Herberg et al, 2000), OPA1 appears to display equal affinities for both isozymes or even a slightly higher affinity for type I PKA. Our kinetic analysis demonstrated that this was due to similar on- and off-rates of both isozymes. When the affinity of an AKAP for type I and type II PKA is similar, the availability of the two isozymes would be expected to determine association. Indeed, in 3T3-L1 cells that express both RI α and RII β we observed that PKA type I associated with OPA1 and controlled lipolysis. In contrast, OPA1 was associated with RII β in WAT where it is the predominantly expressed R-subunit and where type II PKA has been reported to control lipolysis (Beebe et al, 1984; Robinson-Steiner et al, 1984). Whether lipolysis is controlled by type I or type II PKA is clearly important as K_{act} for the two isozymes differ (50-100 and 200-400 nM for type I and type II, respectively). This, in turn, affects the sensitivity to catecholamine-stimulated lipolysis as illustrated in the RII β null mutant mice where rescue by upregulation of RI α provides a type I PKA to control lipolysis and results in lean mice (Cummings et al, 1996). Furthermore, regulation of RI α levels may be a means to control adipocyte sensitivity to cAMP-regulated lipolysis. This is illustrated in mice with high levels of expression of FoxC2

that controls RI α expression resulting in mice protected against diet-induced obesity and insulin resistance (Cederberg et al, 2001).

OPA1 is earlier reported to be expressed in different cell types with phenotypes of mutations in the *opal* gene related to its function in the nervous system. Here we show that OPA1 is expressed in WAT and BAT and upregulated during adipocyte differentiation at a time consistent with its regulation by adipocyte specific transcription factors such as PPAR γ . The dynamin-related GTPase OPA1 is reported to be targeted to the mitochondrial membrane in different cell types including adipocytes (Kita et al, 2009; Misaka et al, 2002; Olichon et al, 2002; Satoh et al, 2003) and involved in mitochondrial fission and fusion and apoptosis through mitochondrial cristae remodeling (Cipolat et al, 2004; Frezza et al, 2006; Ishihara et al, 2006; Olichon et al, 2003). By transfection of COS-7 cells with the 30, 60 or 90 N-terminal amino acids of the OPA1 mitochondrial targeting sequence (MTS) fused to the N-terminal end of EGFP amino acids 30 to 90 of the MTS were shown to be important for the mitochondrial location (Kita et al, 2009; Misaka et al, 2002; Olichon et al, 2002; Satoh et al, 2003). In contrast, our immunolocalization and cell fractionation studies in adipocytes revealed that a substantial fraction of OPA1 is associated with lipid droplets and that amino acids 1 to 30 of the OPA1 MTS appeared to be required for OPA1 to access lipid droplets. Furthermore, deletion of amino acids 30 to 87 also reduced lipid droplet targeting of OPA1, although not to the same extent as its mitochondrial targeting. The differences in subcellular distribution may reflect that the lipophilic nature of the OPA1 membrane targeting domain favor its association with lipid droplets in cell types where these are available. Transport of OPA1 to lipid droplets may occur via a direct route to lipid droplets in parallel with the mitochondrial targeting (dual targeting) or by an indirect route via mitochondria. While the observation that deletion of the full 90 amino acids of the OPA1 MTS lead to loss of both and lipid droplet targeting may point towards the mitochondrial transit route, the fact that lipid

droplet targeting was lost also upon deletion of the first 30 amino acids of the MTS that have been shown to be less important for mitochondrial targeting may indicate distinct targeting mechanisms. However, the targeting mechanism could not be readily defined and requires future investigation in more detail.

Two recent papers indicate that OPA1 may be redistributed from mitochondria to membrane tubulations extending from mitochondria (Ban et al, 2010) or lost during ER stress (Zhang et al, 2008) indicating some mobility of OPA1. It is interesting to speculate that OPA1 localized on both mitochondria and lipid droplets could coordinate the positioning of lipid droplets in close proximity to mitochondria to facilitate the supply of FFA from lipolysis to mitochondria for ATP synthesis. Silencing of mitochondrial fusion proteins such as mitofusin 2 in adipocytes lead to accumulation of lipid droplets, whereas silencing of fission proteins such as Drp1 is responsible of a decrease in TAG content (Kita et al, 2009) indicating interplay between mitochondrial dynamics and lipid storage in adipocytes. Our findings indicate here indicate that the silencing of OPA1 has a distinct effect as it does not impact lipid droplet accumulation or lipid content of adipocytes, but affects the adrenergic regulation of the lipolysis.

In addition to binding PKA, our data also showed that OPA1 bound perilipin, consistent with observations made with other macromolecular complexes organized by AKAPs that include the substrate for PKA as well as other signaling enzymes (Tasken & Aandahl, 2004; Wong & Scott, 2004). While preliminary data indicate a direct interaction between OPA1 and perilipin, the molecular determinants for interaction and possible presence of other signaling enzymes in the OPA1-scaffolded macromolecular complex remain to be investigated. Furthermore, the function of OPA1 in adipocyte mitochondria and the possibility of OPA1 functioning as an AKAP also in mitochondria in different cell types would be interesting to pursue in future studies.

By silencing and reconstitution experiments we demonstrated a central role for OPA1 in catecholamine-stimulated lipolysis. siRNA-mediated knock down of OPA1 induced a decrease in isoproterenol-induced lipolysis, while reconstitution experiments with siRNA-resistant wild type OPA1 rescued this effect. Moreover, reconstitution experiments using a mutated construct substituted in the AKB domain so that it cannot associate with PKA did not rescue isoproterenol-induced lipolysis. **Finally, reconstitution experiments with OPA1 deleted of its mitochondrial and lipid droplet targeting sequences but fused to a lipid droplet targeting domain also reconstituted adrenergic regulation of lipolysis when the AKB domain was present.** Recent work revealed that loss of function of OPA1 increased mitochondrial fragmentation without altering bioenergetics or increasing apoptosis (Spinazzi et al, 2008) which would indicate that the loss of isoproterenol-stimulated lipolysis observed after OPA1 silencing in adipocytes is not due to induction of apoptosis. Nor was this lack in response to adrenergic stimulation due to a deficiency in substrate for lipolysis as we observed no decrease in lipid droplet TAG content. Together with the reconstitution experiments this indicates that it is the loss of PKA from the perilipin/OPA1 macromolecular complex which prevents perilipin phosphorylation. The silencing of OPA1 and reconstitution experiments had no effect on HSL phosphorylation supporting the hypothesis of another AKAP controlling the HSL phosphorylation state (Nomura et al, 2002). The silencing of OPA1 also led to a decrease in perilipin protein levels consistent with the notion that absence of one of the partners of the complex could destabilize the remainder. Indeed, it has been confirmed recently in other models that the knockdown of a protein may affect the stability of other protein partners present in the same supramolecular complex (Oswald et al, 2009). Here, however, perilipin levels appeared to be rescued by an upregulation of ADRP (Supplemental Figure 4), in line with earlier studies (Tansey et al, 2001).

We employed a strategy of PKA displacement using anchoring disruptor peptides to confirm the presence and role of a dual-specificity AKAP in regulation of lipolysis. Our results obtained with Ht31 are in agreement with a previous study showing decreased catecholamine-stimulated lipolysis in cells loaded with Ht31 (Nomura et al, 2002; Zhang et al, 2005). Furthermore, we show that RIAD and SuperAKAP-*IS* that selectively displace either type I or type II PKA did not inhibit isoproterenol-stimulated lipolysis when added separately, whereas the combination reduced regulation of lipolysis to the same extent as Ht31. These results allow us to propose a model to explain the adrenergic control of lipolysis (Figure 7G). In 3T3-L1 adipocytes OPA1 is located in a supramolecular complex associated with lipid droplets, which keeps type I PKA preferentially in close proximity to perilipin to control its phosphorylation state during adrenergic stimulation. Phosphorylation of perilipin through the OPA1/PKA complex stimulates lipolysis by allowing access of lipases to TAG in lipid droplets. Interestingly, it is necessary to block the dual-specificity function of OPA1 to abolish stimulation of lipolysis as there is redundancy with respect to use of different PKA isoforms, although the use of PKA type I versus PKA type II affects the sensitivity of the process to catecholamines.

In addition to control the supply of energy in the fed versus the fasting state, control of lipolysis is important to provide FFA for hepatic synthesis of TAG-containing lipoproteins. Furthermore, alterations in lipolysis are frequently observed in obesity, which is increasing rapidly in the industrialized part of the world and constitutes a growing medical problem. Obesity, characterized primarily by an excess of WAT, results in metabolic syndrome which encompasses insulin resistance and hypertension and leads to type-II diabetes, atherosclerosis and cardiovascular disease (Kahn et al, 2000; Unger, 2003). At the cellular level, obesity leads to a pathological accumulation of TAG and an enlargement of adipocyte size, as well as

increased numbers of adipocytes. Consequently, understanding the regulation of lipolysis and its dysregulation are highly interesting in the context of obesity.

In summary, we report a new function for the OPA1 protein as a dual-specificity AKAP associated with lipid droplets in adipocytes whose role is to coordinate a supramolecular signal complex at the surface of lipid droplets. We provide direct evidence that OPA1 mediates the adrenergic control of lipolysis by providing PKA in close proximity of perilipin to regulate its phosphorylation state and thereby control lipolysis.

Materials and methods

Cell culture

3T3-L1 cells (ATCC) were cultured as described previously (Enrique-Tarancon et al, 1998) (see supplementary information).

Protein sample preparation and immunoblot analysis

Total cell lysates were prepared in lysis buffer (see supplementary information) and proteins were resolved by SDS-PAGE and blotted onto nitrocellulose membranes. The resulting filters were immunoblotted with antibodies to OPA1 (0.25 µg/ml, BD Biosciences), perilipin (1:1000, Fitzgerald Industries from both rabbit and guinea pig), HSL (1:1000, Cell signaling), phospho-HSL (1:1000, Cell signaling), PKA RI α (0.25 µg/ml, BD Biosciences), PKA RII β (0.25 µg/ml, BD Biosciences), phospho-PKA substrate (anti-RRXpS/T, 1:2000, Cell signaling), mitofilin (1:2000, AbCam), mitofusin2 (1 µg/ml, AbCam), pyruvate dehydrogenase E1 β subunit (1 µg/ml, AbCam) ADRP and TIP47 (1/1000 each, gift from Dr

KT. Dalen). After incubation with appropriate HRP-conjugated secondary antibody, blots were developed by using Supersignal West Pico substrate (Pierce).

Immunoprecipitation

Protein A/G plus agarose (Santa Cruz Biotechnology) was absorbed with antibody against OPA1 (BD Biosciences), perilipin (Fitzgerald Industries) or PKA RI α or RII β (both BD Biosciences) or left without antibody. Total cell lysates were prepared as above, added to the protein A/G agarose immunocomplex and incubated overnight at 4°C on a rocker platform. Immunocomplexes were washed five times in lysis buffer before SDS-PAGE and immunoblotting with the indicated antibodies.

Lipid droplet purification

Lipid droplets were purified as described previously (Brasaemle et al, 2004) (see supplementary information).

R-overlays

R-overlays were performed as described previously (Hausken et al, 1998) by using ³²P-labeled recombinant RII or RI(A98S), substituted to allow autophosphorylation (Durgerian & Taylor, 1989)(see supplementary information). See supplementary information.

Immunolocalization studies

Immunofluorescence staining was performed on differentiated 3T3-L1. Cells were fixed for 15 min in 3% paraformaldehyde, permeabilized for 15 min with 0.1% saponin and blocked for 20 min in 3% fatty acid-free BSA, 0.2M glycine and 0.1% saponin. Primary antibodies anti-OPA1 (2.5 µg/ml, BD Biosciences), anti-perilipin (1:100, Fitzgerald Industries from guinea pig), anti-HSL (1:100, Cell signaling), anti-PKA RI α (2.5 µg/ml, BD Biosciences), anti-PKA RII β (2.5 µg/ml, BD Biosciences), and mitofusin2 (20 µg/ml, AbCam) were prepared in PBS with 0.01% Tween 20 (PBS-T), 3% BSA, 0.1% saponin and incubated overnight at 4°C. Cells were next incubated with the appropriate fluorochrome-conjugated secondary antibodies (Alexa Fluor 488 and Alexa Fluor 546 (1:1000, Invitrogen)) in PBST, 0.1% saponin for 1 h. Controls without primary antibody or with nonspecific IgG of the same isotype, were all negative (data not shown). See also supplementary information.

SiRNA, mammalian expression vectors and transfection

SiRNA transfection was performed using Lipofectamine 2000 CD reagent (Invitrogen) according to the manufacturer's protocol. See also supplementary information.

The mammalian transfection vectors were introduced into 3T3-L1 adipocytes by using Fugene 6 transfection reagent (Roche) according to the manufacturer's protocol. See also supplementary information.

Lipolysis assay

Differentiated 3T3-L1 cells were incubated in DMEM (without phenol red) containing 2% fatty acid-free BSA overnight. Cells were stimulated in fresh media with or without isoproterenol (5 - 100 nM) for lipolysis assays. After 2 h of incubation 50 μ l of medium was withdrawn and used for the assay. Glycerol levels were determined using the Free Glycerol Determination Kit (Sigma-Aldrich) according to the manufacturer's instruction.

Statistics

Quantitative data are presented as mean \pm SEM. Differences were identified by analysis of variance (ANOVA) and considered significant when $p < 0.05$. See also Supplement.

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Conflict of interest

The authors declare that they have no conflict of interest

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Figure legends

Figure 1. OPA1 is an AKAP associated with lipid droplets. (A) Perilipin was immunoprecipitated from extracts of 3T3-L1 fibroblasts differentiated into adipocytes, transfected with a mammalian expression vector encoding Ht31 or Ht31-P (Ct), and stimulated with or without isoproterenol (+, -). Perilipin phosphorylation status was assessed by immunoblotting for PKA substrates (anti-RRXpS/T antibody) and perilipin. (B) Purified lipid droplets and total cell lysates from 3T3-L1 adipocytes were subjected to a solid phase binding assay using ^{32}P -radiolabeled RII (RII-overlay) as a probe in the absence (left panel) or presence (right panel) of the Ht31 anchoring disruptor peptide (500 nM). Arrows indicate regions with putative AKAPs, which were excised from parallel lanes, and analyzed by mass spectrometry. (C) Relevant parts of the sequences of putative AKAPs identified by mass spectrometry were printed on solid phase as overlapping 20-mer peptides (3 amino acid offset) and subjected to RII overlay in absence (upper panel) or presence (lower panel) of Ht31 (500 nM). Putative AKAP sequences as identified by numbers on the array are (Swissprot database entry in parenthesis): 1, Dip2b (Q3UH60); 2, Matrin3 (Q9R0U5); 3, OPA1 (P58281); 4, LONP (Q8CGK3); 5, LETM1 (Q9Z210); 6, Hsp90b1 (P08113); 7, Importin subunit beta1 (P70168); 8, Unknown protein product; 9, Nuclear myosin 1 beta (Q9ERB6); 10, AKAP-KL amphipathic helix (positive control). (D) RI or RII-overlay in absence or presence of Ht31 anchoring disruptor peptide (500 nM) of array of immobilized OPA1 19-mer peptides (1 amino acid offset). Bold sequence: PKA-R binding region in OPA1. (E) α -helical wheel representation of the PKA-R binding sequence contained within amino acid 940 to 958 of OPA1 (left). Dashed line indicates a hydrophobic region. R overlays of the immobilized OPA1 940-958 sequence substituted sequence with three prolines introduced (OPA1 940-958-3P) (right). (F) Concentration-dependent competition of RI α interaction with GST-D-AKAP1 (20 nM each) by OPA1 940-958 (●), HT31 (○) and

OPA1 940-958-3P (■) peptides in a ligand proximity assay (AlphaScreen). Data represent mean \pm SEM of three independent experiments performed in duplicate.

Figure 2. Studies of the OPA1-RI and OPA1-RII interaction. (A and C) Surface Plasmon Resonance studies of the MBP-OPA1 binding to immobilized PKA-RI (A) or PKA-RII (C) on a sensor chip coated with 8-aminohexylamino-cAMP. MBP-OPA1 at concentrations indicated was injected for 180 s and the dissociation phase was monitored for 300 s. The graphs are representative of three independent experiments performed on different sensor surfaces. (B and D) Steady state binding of the increasing MBP-OPA1 concentrations obtained from A and C. The affinity constant was derived assuming a 1:1 binding in a Langmuir model using a global fit analyses algorithm provided in the BIAcore T100 evaluation software. (E and F) Qualitative surface competition experiments with the OPA1 940-958 peptide. 75 nM MBP-OPA1 was injected on a surface with captured PKA-RI (E) or PKA-RII (F) in the presence or absence of increasing concentrations (10 - 50 μ M) of the OPA1 940-958 or the OPA1 940-958-3P peptide. Both peptides were also injected without AKAP present and the binding response obtained in the absence of an AKAP present were subtracted in the graph shown. The graphs show one representative experiment of three independent experiments performed on different sensor surfaces.

Figure 3. OPA1 is expressed in adipocytes and co-localizes with RI α on lipid droplets.

(A) Distribution of OPA1 and perilipin in indicated mouse tissues and 3T3-L1 adipocytes by immunoblotting. Dotted lines indicate that an area between the displayed lanes was removed. Shown lanes are from a single gel and exposure. (B) Expression of OPA1, RI α , RII β , perilipin, mitofilin and mitofusin 2 during the differentiation of 3T3-L1 into adipocytes by immunoblotting. (C-N') 3T3-L1 adipocytes were immunostained for perilipin (green; C, F, I,

L) in combination with OPA1 (red; D), RI α (red; G), RII β (red; J) and HSL (red; M). Merged pictures are shown in panel E, H, K and N, respectively. Line plot profile and correlation analysis of co-localization of perilipin with OPA1 (E'), RI α (H'), RII β (K') and HSL (N'). Plot profiles (middle) show for both channels the variation in fluorescence intensity along line indicated in white in detailed area. Scatter plots (right) show pixel distribution whereby the intensities of a given pixel in the green and red images correspond to the x- and y- coordinate, respectively. Correlation coefficients (R) of overlaps are indicated. Scale bar: 20 μ m.

Figure 4. OPA1 organizes a supramolecular complex containing PKA and perilipin. (A-C) Lysates from 3T3-L1 adipocytes were subjected to immunoprecipitation with OPA1 antibody (A, D and E), perilipin (B), and RI α (C). Immunoprecipitates and corresponding lysates were analyzed by immunoblotting for the presence of the indicated proteins. (F, G, H) Lysates from mouse white adipose tissue were subjected to immunoprecipitation with OPA1 (F), RII β (G) or perilipin (H) antibodies and analyzed for the presence of OPA1, RII β and perilipin by immunoblotting. Dotted lines indicate lanes combined from a single gel and exposure.

Figure 5. OPA1 distribution between lipid droplets and mitochondria. (A-I) 3T3-L1 adipocytes were untransfected (D-F) or transfected with mitochondrial marker Δ MDDX28mCherry (red; B) or GFP-OPA1 (green; G) and subsequently immunostained for perilipin (green; A, D and red; H) alone or in combination with mitofusin2 antibody (red; E). Corresponding merged pictures are shown in C, F and I. Scale bar: 20 μ m. (J) Lipid droplets were purified from 3T3-L1 adipocytes by sucrose gradient centrifugation. Fractions were examined by immunoblotting for distribution of perilipin, OPA1, mitofilin (mitochondrial

inner membrane marker), mitofusin 2 (mitochondrial outer membrane marker), or pyruvate dehydrogenase E1 β subunit (mitochondrial matrix marker). Arrow indicates the mobility of the lipid droplet fraction in the sucrose gradient. One representative experiment of three is shown. (K) Relative distribution of the perilipin, OPA1, mitofilin, mitofusin2 and pyruvate dehydrogenase E1 β subunit in fraction 1 (lipid droplet fraction) versus fractions 9 to 11 (mitochondria). Results are expressed as the mean \pm SEM of n = 3 independent experiments (***) p < 0.001).

Figure 6. OPA1 is involved in the adrenergic control of lipolysis. (A) Immunoblot analysis of OPA1, perilipin, RI α , RII β , HSL and actin levels in 3T3-L1 adipocytes transfected with OPA1 siRNA or scrambled control after 24, 48 or 72 h of culture. (B) Glycerol release from 3T3-L1 adipocytes after siRNA transfection and stimulation with isoproterenol (5, 10 and 100 nM) for 2h. (C) Detection by immunoblot of OPA1, GFP-FL-OPA1, perilipin, RI α , RII β , HSL and actin in 3T3-L1 adipocytes transfected with OPA1-specific siRNA or scrambled control and re-transfected with wild type OPA1-FL-GFP, siRNA insensitive OPA1-FL-GFP (GFP-FL-OPA1[#]) or siRNA insensitive OPA1-GFP 940-958-3P (GFP-FL-OPA1[#] AKBmut) plasmids, after 72 h of culture. (D; left panel) Lysates from 3T3-L1 adipocytes incubated with OPA1 siRNA and GFP-FL-OPA1[#] plasmid or GFP-FL-OPA1[#] AKBmut were subjected to immunoprecipitation with RI α , HSL or GFP antibodies. Lysate and precipitate were analyzed for the presence of OPA1, RI α , perilipin, phospho-perilipin (PKA substrate antibody (anti-RRXpS/T) and phospho-HSL. Dotted lines indicate lanes combined from a single gel and exposure. (D; right panel) The histogram shows levels of phosphorylated-perilipin quantified by densitometry relative to total perilipin levels.

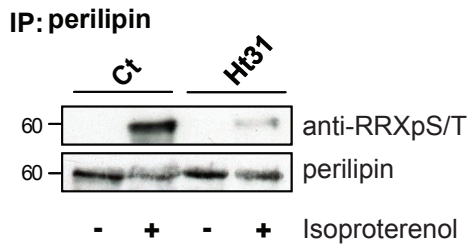
(E) Amount of glycerol released in the media (percent of maximal) of 3T3-L1 adipocytes during 2 h of stimulation by 10 nM isoproterenol after incubation with OPA1 siRNA and

GFP-FL-OPA1[#], GFP-FL-OPA1[#] AKBmut or LDTS-ΔMTS-OPA1 chimeric expression vectors for 72 h. Top: Schematic depiction of constructs. Open box: OPA1 full-length (FL-OPA1[#]) or deleted of the mitochondrial targeting sequence (ΔMTS OPA1[#]); Light grey/dotted box: functional or mutated AKB domain (940-958-3P; AKBmut); Dark grey box: perilipin lipid droplet targeting sequence (perilipin-LTDS). (F-H) 3T3-L1 adipocytes were immunostained for perilipin after OPA1 siRNA transfection (green; F) in combination with Oil Red O (marker of TAG; red; G). (H) Represents merged picture. Scale bar: 20 μm. (I) Intracellular TAG content, (J) intracellular cAMP content and (K) intracellular ATP concentration of 3T3-L1 adipocytes three days after incubation with OPA1 siRNA and GFP-FL-OPA1[#] plasmid or GFP-FL-OPA1[#] AKBmut plasmid. Results represent mean ± SEM of n=3 independent experiments except for (E) where n=5 (*** p < 0.001; ** p < 0.01; * p < 0.05 and n.s. not significant).

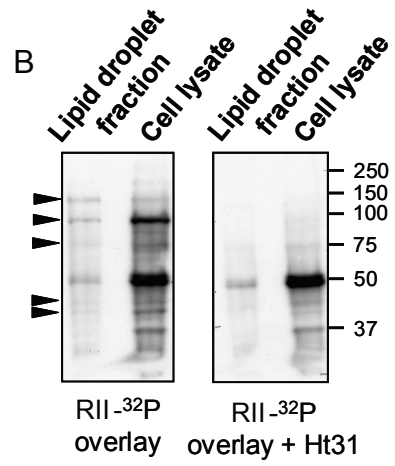
Figure 7. The dual-specificity function of OPA1 allows redundance in the anchored PKA isozyme regulating lipolysis. (A, B) Time-dependent (30, 60 or 120 min) peptide loading of 3T3L1 adipocytes with RIAD-FITC (A, green; upper row) or SuperAKAP-*IS*-FITC (B, green; upper row) at 15 μM each. Subsequently, 3T3-L1 adipocytes were immunostained for perilipin (red; A and B, lower rows). Scale bar; 20 μm. (C-F) Effect of anchoring disruptor peptides in isoproterenol-regulated lipolysis were studied by measuring the amount of glycerol released into media of 3T3-L1 adipocytes after incubation with Ht31 (C), RIAD (D), SuperAKAP-*IS* (E) or RIAD and SuperAKAP-*IS* peptides together (F). Stimulation was accomplished by incubation with 30 nM or 100 nM isoproterenol for 2 h at 72 h of culture. Results are expressed as the mean ± SEM of n=6 independent experiments (*** p < 0.001, and ** p < 0.01). (G) Schematic illustration of the effect of specific anchoring disruptor peptides on lipolysis. Left: model of OPA1 as an AKAP organizing activation of lipolysis

upon adrenergic stimulation. Middle row: Model for effect of RIAD or SuperAKAP-*IS* on lipolysis. Right row: Effect of Ht31 or RIAD and SuperAKAP-*IS* together on adrenergic regulation of lipolysis.

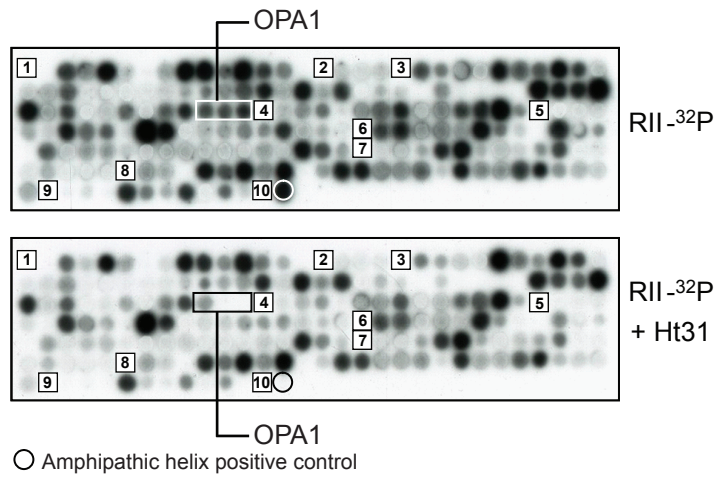
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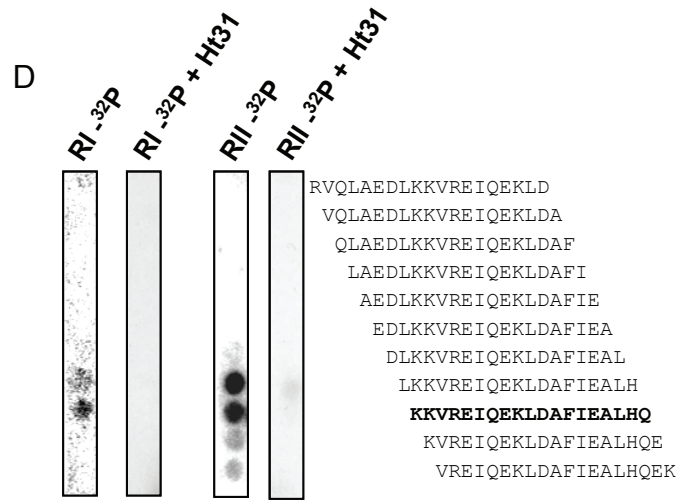
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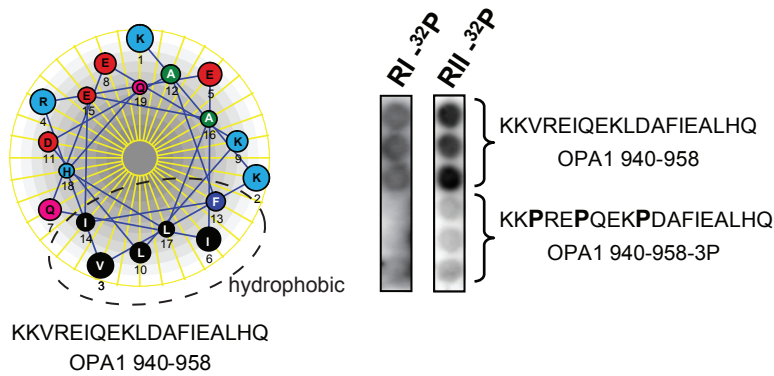
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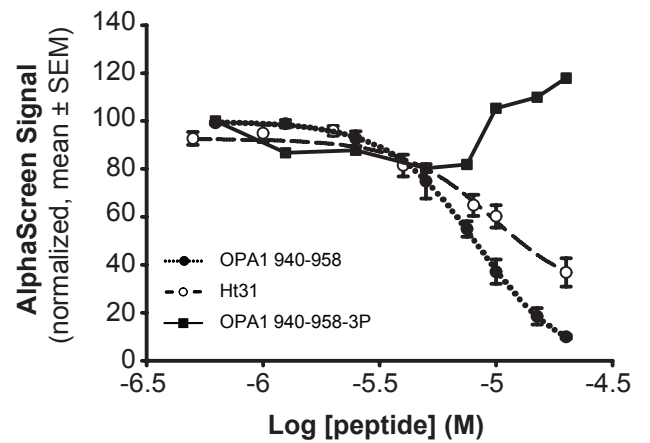
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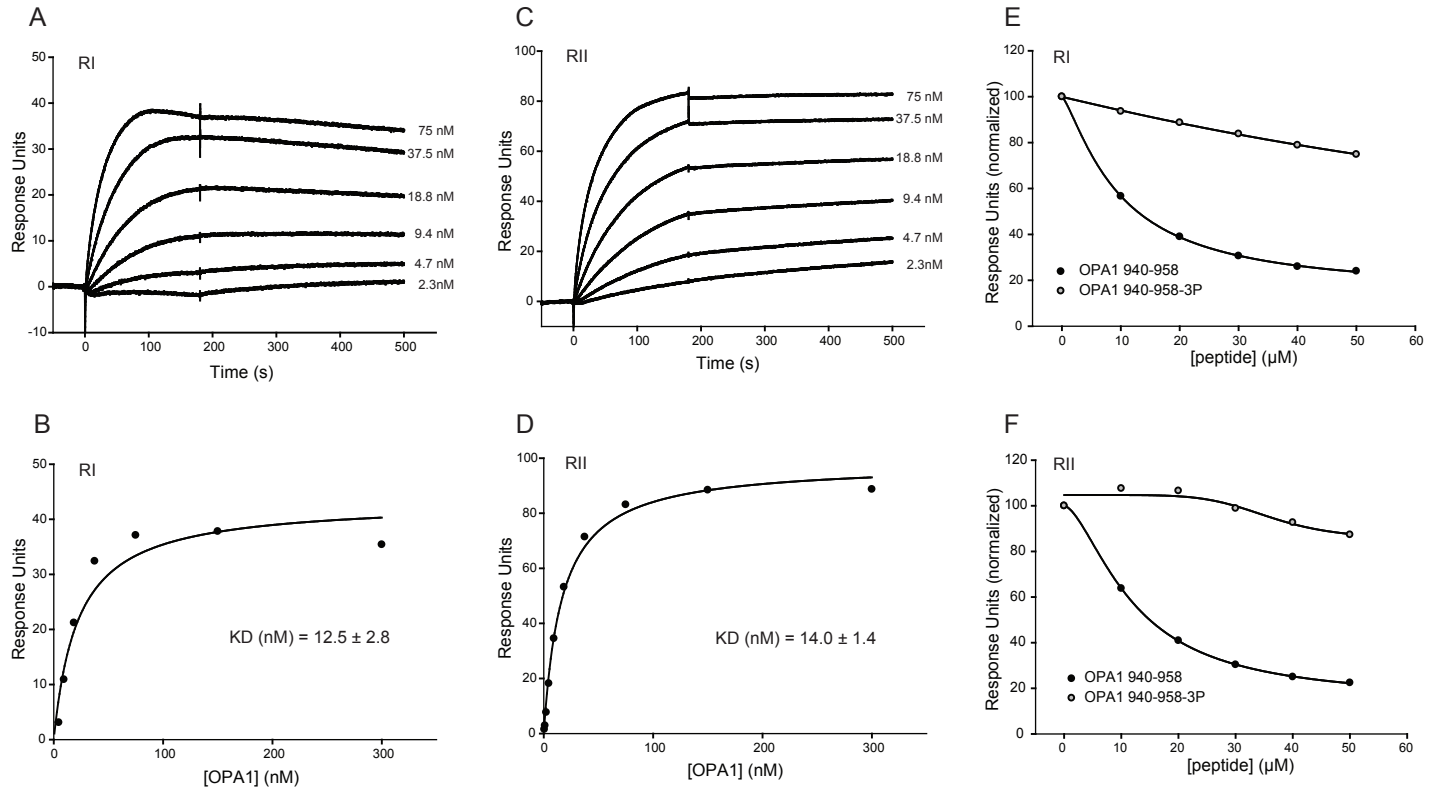


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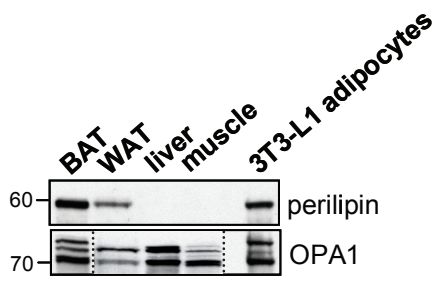


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