SUPPLEMENTARY INFORMATION TO:

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

by

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Supplementary Methods

Cell culture

Two days after confluence (day 0), differentiation to adipocytes was initiated in DMEM containing high glucose, 10% FCS, insulin (1 µg/ml), dexamethasone (1 µM) and methylisobutylxanthine (0.5 mM). After 3 days, only insulin was maintained for an additional three days. On day 6 and thereafter medium was replaced every two days until mature lipid droplets appeared.

Protein sample preparation and immunoblot analysis

Cell extracts were sobulized in lysis buffer (50 mM Tris-HCl pH 8, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% Triton X-100, 1 mM PMSF, 10 mM sodium pyrophosphate, 50 mM N-octyl-β-d-glucoside, 2 mM sodium orthovanadate and protease inhibitor tablets (Roche)). Protein were submitted to SDS-PAGE and transferred to nitrocellulose sheets. The resulting filters were blocked in 5% non-fat dry milk or 3% BSA for phospho-specific antibodies in Tris-buffered saline pH 7.4 (TBS) with 0.1% Tween 20 (TBS-T) for 45 min at ambient temperature and incubated overnight at 4°C with described antibodies.

Lipid droplet purification

Cells were scraped in cold HLM buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 10 mM NaF and protease inhibitor tablets) and cell lysates loaded at the top of a 35 to 50% sucrose gradient and subjected to 154,000 *g* centrifugation, in a SW55Ti rotor (Beckman) at 4°C for 4 h. Floating lipid droplets were collected from the top of the gradient. Isolated lipid droplets were then submitted to SDS-PAGE analysis.

R-overlay

Purified recombinant RI and RII (4 µg) were radiolabeled with purified catalytic subunit (C) (0.02 µg/µl) and [γ-^{32}P]ATP (1.4 µCi/µl) in a buffer containing 50 mM MOPS pH 6.8 with 50 mM NaCl, 2 mM $MgCl_2$ and 1 mM DTT, and separated from free ^{32}P-ATP by gel filtration in G-50 sepharose. Membranes with immobilized proteins or peptides were blocked in Blotto (5% (w/v) non-fat dry milk and 0.1% BSA in TBS). Membranes were incubated with 1 x 10^6 cpm/ml of ^{32}P-labeled recombinant R in TBS-T. For competition assays, Ht31 peptide (500nM) was added to the radiolabeled R and incubated for 2 h before added to the membranes. The membranes were washed in TBS-T and subjected to autoradiography.

Mass spectrometric (MS) analysis

Molecular weight regions on 1D SDS-PAGE that showed a signal in the R-overlay were cut out and proteins therein were digested with endoproteinase Trypsin according to Shevchenko et al. (Shevchenko et al, 1996). Peptides were extracted and analysed by liquid chromatography (LC) coupled electrospray ionization (ESI) tandem MS on a Q-ToF ultima (Waters) under standard conditions (Bessonov et al, 2008). Product ion spectra were searched against the NCBInr data base using MASCOT as search engine.

Peptide-arrays

Peptide arrays were spot-synthesized on cellulose membranes by using a Multipep-automated peptide synthesizer (Intavis Bioanalytical Instruments AG, Germany) as described (Frank, 2002).

Peptide synthesis

Peptides used for Surface Plasmon Resonance studies, AlphaScreen and peptide loading experiments (OPA1 940-958: KKVREIQEKLDAFIEALHQ; OPA1 940-958-3P: KKPREPQEKPDAFIEALHQ; Ht31: R11-LIEEAASRIVDAVIEQV; RIAD: LEQYANQLADQIIKEATE-R11 ; scrambled RIAD: IEKELAQQYQNADAITLE-R11 ; SuperAKAP-*IS*: R11-QIEYVAKQIVDYAIHQA ; and scrambled SuperAKAP-*IS*: R11-VVHEIQDAAYYQKQIAI) were synthesized on an Intavis MultiPep robot (Intavis Bioanalytical Instruments AG), uncoupled and verified by High Performance Liquid Chromatography (HPLC) and MS. The concentration of the peptides was determined by amino acid analysis using an amino acid analyzer from Applied Biosystems.

Protein expression and purification

PKA RI and RII protein were expressed in *E. coli* BL21 and *E. coli* Rosetta, respectively, using 0.1 – 1.0 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) induction at RT (4 h) and purified on Rp-8-AHA-cAMP agarose beads [8-(6-aminohexyl)aminoadenosine-3′-,5′-cyclic monophosphorothioate, Rp-isomer, immobilized on agarose] (BioLog) and eluted with

cAMP. MBP-OPA1 was expressed in *E. coli* BL21, induced using 0.5 mM IPTG at 30ºC (2 h), and purified on Amylose Resin E8021L (New England BioLabs). The purified recombinant R and OPA1 proteins were dialysed extensively against a buffer containing 20 mM MOPS, pH 7.0 and 150 mM NaCl. Protein concentrations were determined using the Bradford protein assay and SDS-PAGE (10% gels) using BSA as a standard.

Ligand proximity assay

For characterization of biomolecular interactions using AlphaScreen (Amplified Luminescence Proximity Homogenous Assay) biotinylated RI (Stokka et al, 2006) and GST-D-AKAP1 were conjugated to streptavidin-coated donor beads and anti-GST-coated acceptor beads (PerkinElmer), respectively. Ligand proximity assay were performed as previously described (Stokka et al, 2006). Briefly, 20 nM GST-D-AKAP1 was incubated with 20 nM biotinylated RI in the presence of different concentrations of peptide for 15 min on ice in a total of 15 µl assay buffer (25 mM Hepes pH 7.4, 100 mM NaCl and 0.1% BSA). Five µl acceptor beads were added followed by a 30 min incubation on ice. Subsequently, 5 µl donor beads were added and the mixture was incubated at RT for 90 min before collecting interaction data on an EnVision^{TM} multiplate reader (PerkinElmer). Both beads-types were added at 20 µg/ml final concentration. IC_{50}-values were estimated by non-linear regression analysis using SigmaPlot (SPSS Inc).

Surface Plasmon Resonance

Interaction studies between PKA-R and OPA1 were performed on a BIAcore T100 instrument (BIAcore Life Sciences, GE Healthcare Europe) as described previously (Herberg et al, 2000)

to determine the affinity of the interaction between cAMP-free RI and RII subunits and MBP-OPA1. CM-5 chips (Biacore), coated with 8-aminohexylamino-cAMP (BioLog), were used to capture cAMP-free RI and RII subunits at a flowrate of 5 µl/min (surface immobilization level of 150-350 resonance units (RU) for each subunit) in a running buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 50 µM EDTA and 0.1% surfactant P20). The fusion protein MBP-OPA1 was injected at a flow rate of 30 µl/min in a series of dilutions (300 – 0.6 nM; for 180 s) to determine the affinity of the interaction. For analysis of RI interaction 0.5 mM ATP and 10 mM $MgCl_2$ was added to the buffer. All subsequent interaction studies were performed in the same buffers at 25 ºC. After injection of the MBP-OPA1, the dissociation phase was monitored for 300 s. Unspecific binding was subtracted from the response using blank runs performed on a surface immobilized with 8-aminohexylamino-cAMP with no R-subunit captured. Competition experiments were performed on immobilized RI or RII subunits. MBP-OPA1 (75 nM) was injected in the presence or absence of increasing concentrations (10 - 50 µM) of the peptide OPA1 940-958 or the negative control peptide OPA1 940-958-3P at a flow rate of 30 µl/min for 180 s, and the binding level to the R subunit was analysed. OPA1 peptide was also injected in the absence of AKAP. Kinetic analyses were performed using the BIAcore T100 evaluation software.

Immunolocalization studies

Confocal microscopy was performed on 3T3-L1 adipocytes attached to collagen/fibronectin-coated coverslips with Olympus FV1000 and Zeiss LSM 510 Meta confocal microscopes. The relative fluorescence intensities of each signal (green or red) along indicated line from one single plane from a z-stack. For co-localization, the two fluorescent profiles were compared and correlation coefficient-based analysis based on Pearson's coefficient calculated using

Image CorrelationJ (Bolte & Cordelieres, 2006). For Oil Red O staining, cells were fixed as previously described and stained for 30 min with filtered 0.5% Oil Red O in 60% isopropanol. After washing, cells were examined by light microscopy.

SiRNA, mammalian expression vectors and transfection

A 21-nucleotide siRNA duplex targeting the 8 mouse OPA1 mRNA splice variants were designed and synthesized in-house. The sequences of the OPA1 siRNA oligos tested are: mOPA1-1257, 5'-ggaagaucuugcagcauuaag-3'; 5'-uaaugcugcaagaucuuccuc-3'; mOPA1-2153, 5'-caguagacaucaagcuuaaac-3'; 5'-uuaagcuugaugucuacugug-3'. Briefly, 50 nM OPA1 siRNA or scrambled siRNA (Invitrogen) were mixed with the lipofectamine reagent, diluted in Opti-MEM medium (Invitrogen) and incubated with the cells for 24 h at 37°C after which the transfection was repeated by changing the solution and continuing the transfection for another 24 h. Cells were then incubated in media for 24 h and used for analyses 72 h post-transfection when OPA1 levels were below 80% of control.

Mammalian expression vectors containing the adipocyte-specific AP1 promoter and cDNA encoding Ht31 or the proline substituted Ht31-P (Lester et al, 1997) were kind gifts from Drs. Sven Enerbäck, Göteborg University, Sweden and John D. Scott, University of Washington School of Medicine, Seattle WA, respectively.

The OPA1 cDNA was made insensitive to the mOPA1-1257 siRNA by introduction of four nucleotide switches, T1264C, T1267A, A1270C and A1273C without changing the encoded OPA1 amino acid sequence. Mutagenesis was performed using two sets of primers P1(+), 5'-ccaaggaggaagacctagcagcattaagacatg-3'; P1(-), 5'-catgtcttaatgctgctaggtcttcctccttgg-3'; P2(+), 5'-ccaaggaggaagacctagccgccttaagacatg-3'; P2(-), 5'-

catgtcttaaggcggctaggtcttcttccttgg-3' and the QuickChange XL kit (Stratagene) according to the manufacturer's protocol. Sequences (OPA1 wild type, and OPA1 siRNA insensitive, labeled by $^{\#}$) were cloned into pENTR/D-TOPO vector using the gateway cloning technology (Invitrogen) and thereafter transferred into pDEST-EGFP to yield a OPA1-GFP fusion protein. OPA1$^{\#}$ AKBmut was generated from siRNA insensitive OPA1$^{\#}$ in the pENTR vector by introducing three prolines (V942P, I945P, L949P) using three sets of primers P3(+), 5'-gaagatctcaagaaacctagagaaattcaaga-3'; P3(-), 5'-tcttgaatttctctaggtttcttgagatcttc-3'; P4(+), 5'-aagaaacctagagaacctcaagaaaagcttga-3'; P4(-), 5'-tcaagcttttcttgaggttctctaggtttctt-3'; P5(+), 5'-aacctcaagaaaagcctgatgctttcattga-3'; P5(-), 5'-tcaatgaaagcatcaggcttttcttgaggtt-3'. OPA1$^{\#}$ AKBmut was cloned into pENTR/D-TOPO and then transferred into pDEST-EGFP. Constructs with a deletion of the OPA1 mitochondrial targeting sequence (amino acids 1-87, (Kita et al, 2009; Misaka et al, 2002; Olichon et al, 2002; Satoh et al, 2003)) (GFP-ΔMTS-OPA1 and GFP-ΔMTS-OPA1$^{\#}$) were generated using GFP-OPA1 and GFP-OPA1$^{\#}$ as templates and primers P6(+), 5'-caccttggccagcaaggttag-3'; P6(-), 5'-cttctcctagtgaagagctt-3'. Constructs with a deletion of the 30 first or next 57 amino acids of the mitochondrial targeting sequence (GFP-Δ1-30-OPA1, GFP-Δ30-87-OPA1) were generated using GFP-OPA1 as template using primers P7(+), 5'-cacccaaaaactccatctggtttcac-3'; P7(-), 5'-cttctcctagtgaagagctt-3' and P8(+), 5'-gaaacgtaccgctcttttggccagcaag-3'; P8(-), 5'-cttgctggccaaaagagcggtacgtttc-3'. To generate chimeric OPA1 constructs with a different lipid droplet targeting domain, the perilipin LDTS (amino acids 233 to 366), notably without any of the four PKA phosphorylation sites (Ser 276 inside the sequence was mutated to Ala) was amplified using perilipin as template with primers P9(+), 5'-ggggacaagtttgtacaaaaaagcaggctggcacaccatgcaaaccacagca-3', and P9(-), 5'-ggggacaacttttgttatacaaagttgttcccaccgtgcccagcac-3' and cloned into pDONR/D-TOPO. The ΔMTS-OPA1$^{\#}$ or ΔMTS-OPA1$^{\#}$ AKBmut part of the chimeric constructs were cloned into

pDONR/D-TOPO using GFP-OPA1$^{\#}$ and GFP-OPA1$^{\#}$ AKBmut plasmids as template and primers P10(+), 5'-ggggacaactttgtatacaaaagttgtgttttggccagcaaggttag-3'; P10(-), 5'-ggggaccactttgtacaagaaagctgggtactacttctcctggtgaagagcttc-3'. pDEST vectors were generated from the corresponding pDONR constructs for all chimeric constructs.

The targeting domain of the DEAD-box helicase MDDX28 sequence earlier shown by immunogold electron microscopy to be localized to the inner mitochondrial membrane (Valgardsdottir et al, 2004) was cloned into mCherry plasmid to yield a ΔMDDX28-mCherry construct.

All constructs were verified by sequencing. Briefly 2 μg of plasmids were mixed with the transfection reagent, diluted in Opti-MEM medium (Invitrogen) and incubated with the cells for 24 h at 37°C. See also supplementary information for details.

Measurement of TAG concentration

TAG concentration was examined as described previously using the Oil Red O assay (Ramirez-Zacarias et al, 1992). Briefly, cells were fixated in 5% formaldehyde, Oil Red O was added as described above and incubated for 20 min at RT. After washing with PBS, Oil Red O associated to lipid droplets was extracted with isopropanol and absorbance was measured at 510 nm.

Measurement of intracellular cAMP concentration

Intracellular cAMP concentration was assessed using cAMP assay (R&D system) according to the manufacturer's protocol. Briefly, adipocytes were resuspended in cell lysis buffer to a

final concentration of 10^7 cells/mL sonicated, lyates centrifuged to remove cellular debris and supernates assayed. Intracellular cAMP concentrations were determined at 450 nm (with a λ correction at 540 nm) on a reader plate by comparison to a standard curve. All the data were normalized to protein level expression.

Measurement of ATP concentration

Intracellular ATP concentration was performed using ATP determination kit (Invitrogen) according to the manufacturer's protocol. Briefly cells supernatants were prepared as for the intracellular cAMP concentration measurements above and ATP concentrations were determined at 560 nm by comparison to a standard curve. All the data were normalized to protein level expression.

Statistics and quantification

For correlation analysis of co-localization, fluorescence signal from one plane in a z-stack of differentiated 3T3 cell was acquired by laser confocal microscopy and images treated with ImageJ 1.43. The two fluorescent profiles from the whole picture were compared and correlation coefficient-based analysis based on Pearson's coefficient was calculated using JACoP (Bolte & Cordelieres, 2006). Plot profiles were quantified using ImageJ 1.43 and computed in Microsoft Excel.

Supplementary Figure Legends

Supplemental Figure S1. Correlation analysis between lipid droplet and mitochondrial markers. Correlation analysis for overlap of staining of perilipin versus ΔMDDX28mCherry (A), mitofusin 2 (B) and GFP-OPA1$^{\#}$ (C) were conducted as in Fig. 3 and resulting dot plots with correlation coefficient values are presented.

Supplemental Figure S2. Localization of different OPA1 proteins with truncations in the mitochondrial targeting sequence in adipocytes. (A-V) 3T3-L1 adipocytes were transfected with GFP OPA constructs and GFP excited in the following panels: GFP-FL-OPA1 (A), GFP-FL-OPA1$^{\#}$ AKBmut (D), GFP-ΔMTS-OPA1$^{\#}$ (G, K), GFP-Δ1-30-OPA1 (N, Q) and GFP-Δ30-87-OPA1$^{\#}$ (Q, T). Immunostaining for perilipin was done on the same preparations of cells (red; B, E, H, L and R). Co-transfection with ΔMDDX28mCherry was performed on the same cells (red; O and U). Merged pictures are shown in panel C, F, I, M, P, S, and V, respectively. Correlation analysis of co-localisation of perilipin with the different OPA1 proteins and correlation coefficient values are shown on C', F', I', M', P', S', and V'. Scale bar: 20 μm.

Supplemental Figure S3. OPA1 and perilipin protein levels following OPA1 knockdown.

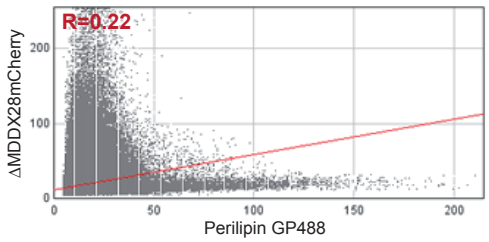
Levels of expression of OPA1 (A) and perilipin (B) were assessed by densitometric scanning of immunoblots from 3T3-L1 adipocytes transfected with OPA1-specific siRNA or scrambled control after 24, 48 or 72 h of culture and normalized to actin levels in the same blots. Results are expressed as the mean ± SEM of n=3 independent experiments (*** $p < 0.001$ and * $p <$

0.05). (C) Levels of perilipin expression normalized to actin levels in 3T3-L1 adipocytes transfected with OPA1-specific siRNA or scrambled control and re-transfected with wild type OPA1-GFP plasmid, siRNA insensitive OPA1-GFP plasmid (GFP-OPA1$^{\#}$) or siRNA insensitive OPA1-GFP 940-958-3P plasmid (GFP-OPA1$^{\#}$ 940-958-3P), after 72 h of culture. Results are expressed as the mean ± SEM of n=3 independent experiments. (D-F) 3T3-L1 adipocytes were transfected with a perilipin LDTS-OPA1 chimeric construct (D; perilipin-LDTS ΔMTS OPA1$^{\#}$ AKBmut-GFP). Immunostaining for perilipin was done on the same preparation (red; E). Merged picture is shown in panel F. Correlation analysis of colocalisation of perilipin with chimera construct and correlation coefficient values are shown in F'. Scale bar: 20 μm. Similar results were obtained for all chimeric constructs with correlation coefficients of R=063-0.66.

Supplemental Figure S4. Expression of ADRP and TIP47. Levels of expression of ADRP (A) and TIP47 were detected by immunoblotting at 24, 48 and 72 h following OPA1 knockdown. Data show relative levels of protein by densitometric scanning of the immunoblots normalized to actin levels. Results are expressed as the mean ± SEM of n=4 independent experiments (*** $p < 0.001$).

Pidoux et al, Supplemental Figure S1

A



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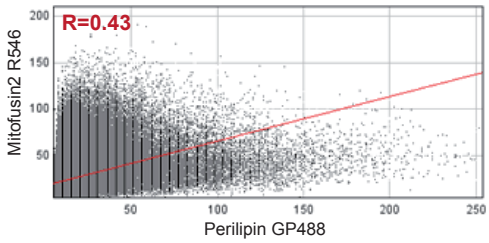

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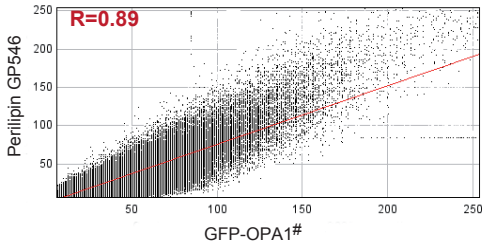
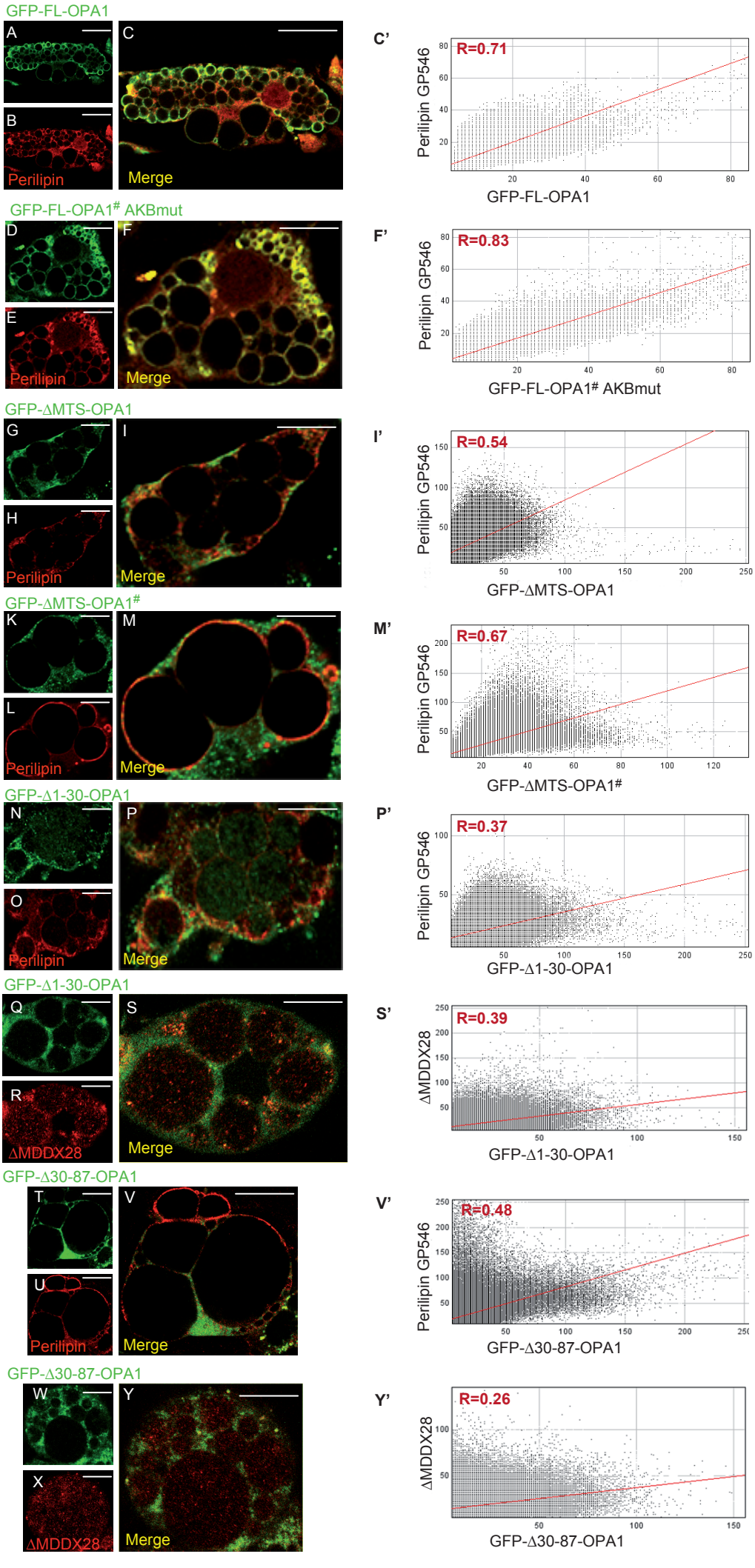
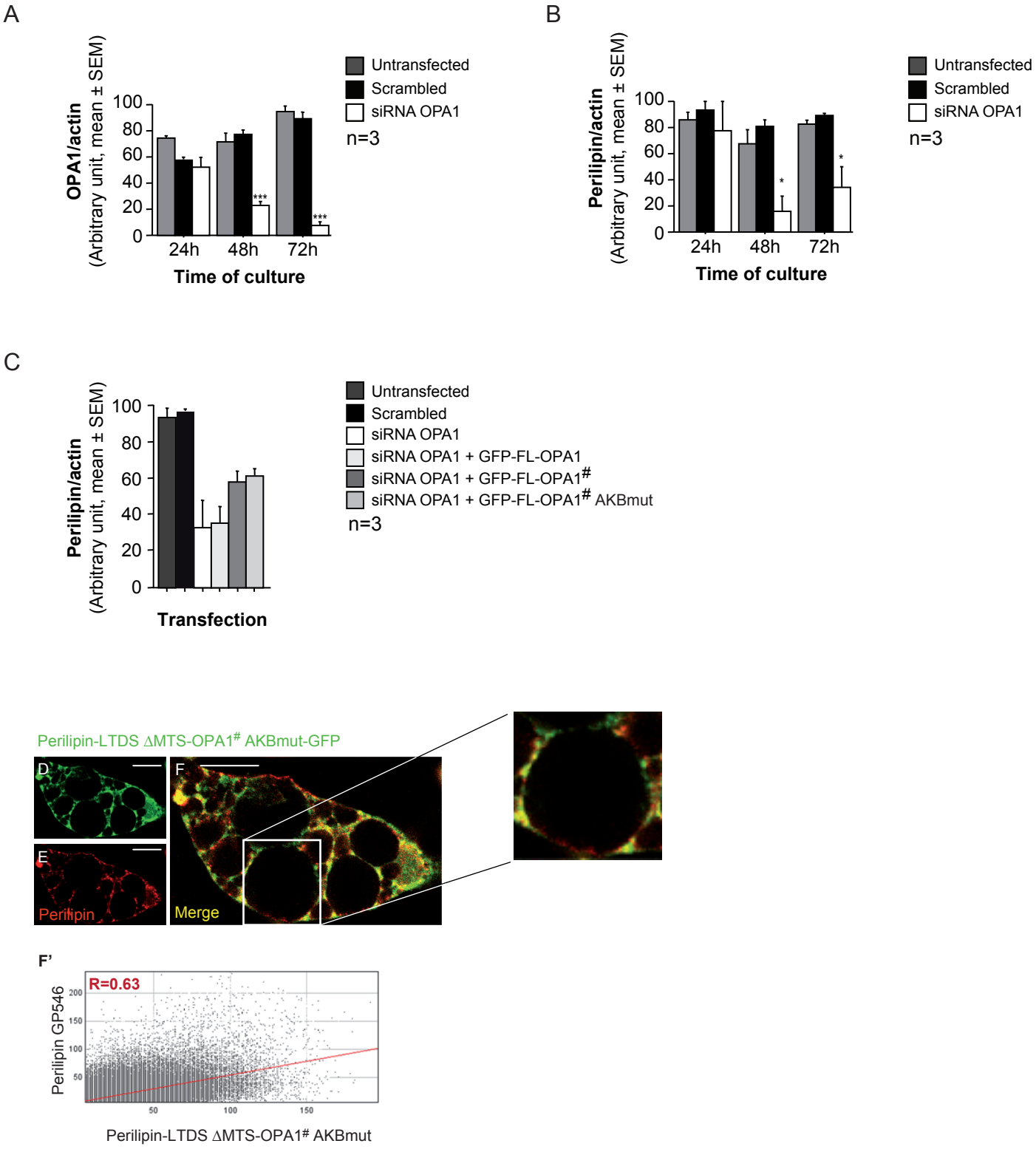


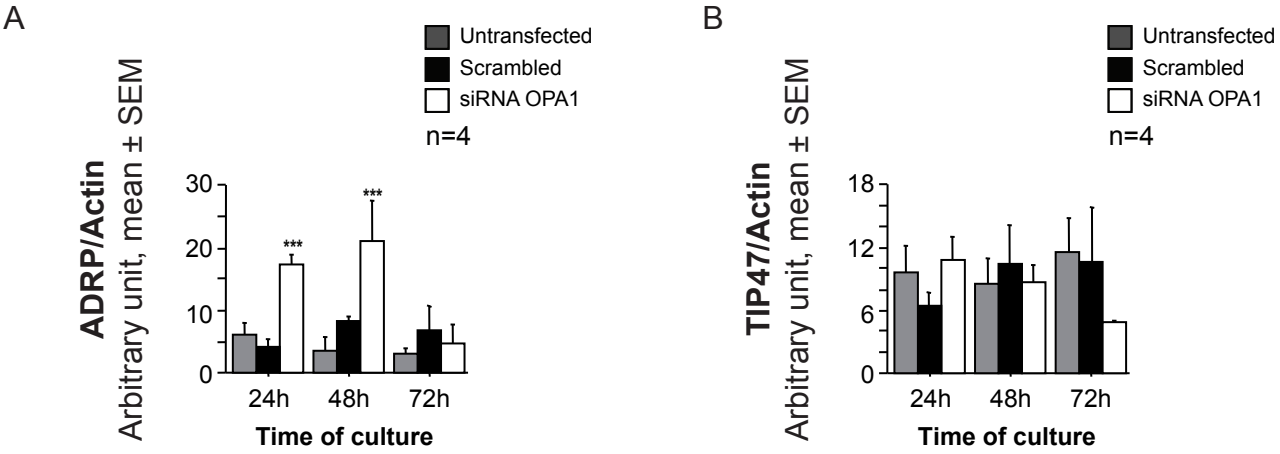
Pidoux et al, Supplemental Figure S2



Pidoux et al, Supplemental Figure S3



Pidoux et al, Supplemental Figure S4



Supplementary Table I. Proteins from lipid droplet fractions identified by LC-MS/MS analysis of tryptic digests of bands excised from SDS-PAGE gels representing regions of approximate mobility corresponding to bands detected by RII overlay.

Band no / band size	Protein name	Accession / gi no.	Protein Score*	Sequence coverage %	Total numbers of seq. peptides	Peptide score# of highest scoring peptide	Peptide sequence of highest scoring peptide§
1 ~200kDa	pyruvate carboxylase; pyruvate decarboxylase	NP_032823 gi|6679237	1279	32	43	104	**AEAEAQAEELSFPR**
"	leucine-rich PPR motif-containing protein; leucine rich protein LRP130	NP_082509 gi|21389320	230	7	11	70	**MVAGLDTIGLSK**
"	procollagen, type VI, alpha 1	NP_034063 gi|6753484	194	6	6	44	**IALVITDGR**
"	plasma membrane associated protein, S3-12; S3-12 protein	NP_065593 gi|10181204	111	6	9	48	**GTVQTGLDTSQR**
"	membrane bound C2 domain containing protein	NP_035973 gi|33859650	90	2	3	51	**QLLDDEER**
"	cis-Golgi matrix protein GM130 [Rattus norvegicus]	NP_072118 gi|12018260	68	1	2	68	**QELQEAQER**
"	tumor rejection antigen gp96; tumor rejection antigen (gp96) 1	NP_035761 gi|6755863	64	3	2	47	**GVVDSDDLPLNVSR**
"	oxoglutarate dehydrogenase (lipoamide); alpha-ketoglutarate dehydrogenase	NP_035086 gi|33563270	45	0	1	45	**LSGQDVER**
"	nicastrin	AAG11413 gi|9992880	37	1	1	37	**ALANVATVLAR**
"	lysosomal membrane glycoprotein LAMP-1 - mouse	A28067 gi|91055	37	5	2	26	**ALQATVGNSYK**
"	similar to hypothetical protein	XP_355145 gi|38084849	36	1	1	36	**DLLVQQASQCLSK + Carbamidomethyl (C)**
"	integrin alpha 6 subunit precursor	CAA49527 gi|408128	22	0	1	22	**YTQELTLNR**

2 ~140kDa	pyruvate carboxylase; pyruvate decarboxylase	NP_032823 gi|6679237	1927	41	64	83	**AEAEAQAEELSFPR**
"	tumor rejection antigen gp96; tumor rejection antigen (gp96) 1	NP_035761 gi|6755863	116	3	3	69	**GVVDSDDLPLNVSR**
"	membrane bound C2 domain containing protein	NP_035973 gi|33859650	80	2	3	46	**VGTQTFCSR + Carbamidomethyl (C)**
"	Long-chain-fatty-acid--CoA ligase 2; Long-chain acyl-CoA synthetase 2 (LACS 2)	P41216 gi|729927	66	2	2	53	**IGFFQGDIR**
"	oxoglutarate dehydrogenase (lipoamide) (EC 1.2.4.2) precursor - human	A38234 gi|283950	60	1	2	45	**LSGQDVER**
"	similar to hypothetical protein	XP_355145 gi|38084849	44	1	1	44	**ISKESLADR**
"	Lon protease-like protein [Homo sapiens]	CAA52291 gi|414046	40	4	3	17	**FSVGGMTDVAEIK + Oxidation (M)**
"	hexokinase 2	NP_038848 gi|7305143	39	0	1	39	**EVCTVVAR + Carbamidomethyl (C)**
"	heterogenous nuclear ribonucleoprotein U; nuclear matrix protein sp120	NP_058085 gi|7949051	36	2	2	24	**VTEKIPVR**
3 ~110kDa	protease, serine, 15	XP_128721 gi|38082728	567	16	21	87	**AGVTCIILPAENR + Carbamidomethyl (C)**
"	tumor rejection antigen gp96; tumor rejection antigen (gp96) 1	NP_035761 gi|6755863	377	12	11	119	**GVVDSDDLPLNVSR**
"	hexokinase 2	NP_038848 gi|7305143	372	12	14	78	**LSDETLLEISR**
"	oxoglutarate dehydrogenase (lipoamide); alpha-ketoglutarate dehydrogenase	NP_035086 gi|33563270	370	9	13	49	**DPAAAPATGNK**

"	staphylococcal nuclease domain containing 1; p100 co-activator	NP_062750 gi|9790067	313	7	9	60	**SEAVVEYVFSGSR VITEYLNAQESAK**
"	pyruvate carboxylase; pyruvate decarboxylase	NP_032823 gi|6679237	271	6	8	92	**AEAEAQAEELSFPR**
"	unnamed protein product	BAC29337 gi|26331214	247	7	8	67	**DDISKLR**
"	alpha glucosidase 2, alpha neutral subunit	NP_032086 gi|6679891	209	6	8	47	**TCDESSFCKR + 2 Carbamidomethyl (C)**
"	Long-chain-fatty-acid--CoA ligase 2 (Long-chain acyl-CoA synthetase 2) (LACS 2)	P41216 gi|729927	156	4	4	57	**IGFFQGDIR**
"	aconitase 2, mitochondrial	NP_542364 gi|18079339	78	2	2	50	**VDVSPTSQR**
"	Fthfsdc1 protein	AAH30437 gi|20987924	62	2	2	44	**DFTLPISDVR**
"	adaptor protein complex AP-2, alpha 1 subunit; adaptor-related protein complex AP-2, alpha 1 subunit	NP_031484 gi|6671561	50	3	4	35	**NADVELQQR**
"	Nucleolin	AAH05460 gi|13529464	48	2	2	29	**QGAEIDGR**
"	aconitase 2, mitochondrial precursor	NP_542364 gi|18079339	662	22	18	74	**LTGSLSGWTSPK**
"	pyruvate carboxylase; pyruvate decarboxylase	NP_032823 gi|6679237	275	10	13	52	**SSSAPVASPNVR**
"	Long-chain-fatty-acid--CoA ligase 2 (Long-chain acyl-CoA synthetase 2) (LACS 2)	P41216 gi|729927	164	6	4	50	**GLQGSFEELCR + Carbamidomethyl (C) GIQVSNNGPCLGSR + Carbamidomethyl (C)**
"	unnamed protein product	BAC28163 gi|26328849	143	9	7	43	**EIAGATPHITAAEGR**

"	tumor rejection antigen gp96; tumor rejection antigen (gp96) 1	NP_035761 gi|6755863	112	4	3	40	**SILFVPTSAPR**
"	**optic atrophy 1 homolog precursor**	NP_598513 gi|19526960	98	3	3	56	**VVVVGDQSAGK**
"	dnaK-type molecular chaperone precursor, mitochondrial	A48127 gi|1072476	90	3	2	53	**VLENAEGAR**
"	dnaK-type molecular chaperone grp78 precursor	A37048 gi|109893	87	4	3	36	**LTPEEIER**
"	Platelet glycoprotein 4; PAS IV; PAS-4; CD36 antigen	Q08857 gi|729081	79	3	2	55	**FFSSDICR + Carbamidomethyl (C)**
"	perilipin	NP_783571 gi|28316726	72	4	2	51	**ETAEYAANTR**
"	oxoglutarate dehydrogenase (lipoamide); alpha-ketoglutarate dehydrogenase	NP_035086 gi|33563270	0	0	1	69	**SSPYPTDVAR**
"	mitochondrial trifunctional protein, alpha subunit precursor	NP_849209 gi|33859811	58	4	3	21	**ILQEGVDPK**
"	leucine zipper-EF-hand containing transmembrane protein 1 precursor	NP_062668 gi|9789997	41	2	2	34	**ELQAACR + Carbamidomethyl (C)**
"	Hormone-sensitive lipase (HSL)	P54310 gi|1708847	40	1	1	40	**LSNVFAGVR**
"	NADH dehydrogenase (ubiquinone) Fe-S protein 1	NP_663493 gi|21704020	40	1	1	40	**ILQDIASGR**
"	mitochondrial ATP-dependent protease Lon	AAN85210 gi|26984237	28	0	1	28	**YLVPQAR**
"	leucyl-tRNA synthetase, mitochondrial precursor	NP_694808 gi|23346617	28	1	1	28	**VYTLSDTIAR**
"	Karyopherin (importin) beta 1	AAH52711 gi|30931411	20	1	1	20	**VLANPGNSQVAR**
"	HIV TAT specific factor 1	NP_082518 gi|23956212	20	1	1	20	**VLDEEGSER**

4 ~90kDa	heat shock protein gp96 precursor [Homo sapiens]	AAK74072 gi|15010550	851	24	27	85	**GVVDSDDLPLNVSR**
"	pyruvate carboxylase; pyruvate decarboxylase	NP_032823 gi|6679237	506	12	14	112	**AEAEAQAEELSFPR**
"	protease, serine, 15	XP_128721 gi|38082728	439	12	16	105	**QSDENLDLAR**
"	Long-chain-fatty-acid--CoA ligase 2 (Long-chain acyl-CoA synthetase 2) (LACS 2)	P41216 gi|729927	171	8	6	66	**IGFFQGDIR**
"	splicing factor proline/glutamine rich (polypyrimidine tract binding protein associated)	NP_076092 gi|23956214	142	6	4	59	**GIVEFASKPAAR**
"	alanyl-tRNA synthetase like; Unknown (protein for MGC:69820)	NP_941010 gi|38348462	139	7	3	66	**LLAITGEQAQQAR**
"	RIKEN cDNA D330038I09	NP_705771 gi|23956316	85	7	7	30	**LSQHPDIR**
"	perilipin	NP_783571 gi|28316726	82	4	2	46	**VSTLANTLSR**
"	aconitase 2, mitochondrial	NP_542364 gi|18079339	72	4	4	45	**VDVSPTSQR**
"	lysosomal membrane glycoprotein LAMP-1	A28067 gi|91055	71	6	2	46	**ALQATVGNSYK**
"	Ap2a2 protein	AAH10597 gi|14714884	61	3	3	35	**NADVELQQR**
"	alpha glucosidase 2, alpha neutral subunit	NP_032086 gi|6679891	55	2	2	28	**VTEGGEPYR**
"	Ogdh protein	AAH13670 gi|15489120	45	0	1	45	**LSGQDVER**
"	Lysosome-associated membrane glycoprotein 2 precursor (LAMP-2) (Lysosomal membrane glycoprotein-type B) (LGP-B) (CD107B)	P17047 gi|126381	37	2	1	37	**AFQINTFNLK**

"	Fthfsdc1 protein	AAH30437 gi|20987924	31	1	1	31	**DFTLPISDVR**
"	hypothetical protein XP_126921	XP_126921 gi|38050506	27	1	2	17	**SLKEAMASR**
"	cell death-inducing DNA fragmentation factor, alpha subunit-like effector B	NP_034024 gi|6753422	22	3	1	22	**VLRELLR**
"	eukaryotic translation elongation factor 2	NP_031933 gi|33859482	22	1	1	22	**GGGQIIPTAR**
"	similar to myeloid/lymphoid or mixed-lineage leukemia 2; ALL1-related gene [Rattus norvegicus]	XP_343327 gi|34868150	22	0	1	22	**KDGDLDTEELLK**
"	similar to 60S ribosomal protein L23a	XP_357733 gi|38086277	21	7	1	21	**TNQHQMEESVK + Oxidation (M)**
"	pyruvate carboxylase; pyruvate decarboxylase	NP_032823 gi|6679237	309	10	12	63	**AEAEAQAEELSFPR**
"	tumor rejection antigen gp96; tumor rejection antigen (gp96) 1	NP_035761 gi|6755863	183	6	5	89	**GVVDSDDLPLNVSR**
"	mitochondrial ATP-dependent protease Lon	AAN85210 gi|26984237	169	6	7	83	**QSDENLDLAR**
"	heterogeneous nuclear ribonucleoprotein U	NP_058085 gi|7949051	113	5	5	43	**LNTLLQR**
"	oxoglutarate dehydrogenase (lipoamide); alpha-ketoglutarate dehydrogenase	NP_035086 gi|33563270	105	2	3	55	**NMEEEVAITR + Oxidation (M)**
"	nuclear myosin I beta	AAG02570 gi|11067002	92	3	4	79	**LGTEEISPR**
"	aconitase 2, mitochondrial precursor	NP_542364 gi|18079339	89	3	2	45	**VDVSPTSQR**
"	perilipin	NP_783571 gi|28316726	83	5	3	40	**ETAEYAANTR**
"	similar to hypothetical protein	XP_355145 gi|38084849	81	4	3	45	**ISKESLADR**

"	integrin alpha 6 subunit precursor	CAA49527 gi|408128	80	1	2	52	**AEALPLQR**
"	matrin 3	NP_034901 gi|25141233	43	1	2	43	**SFQQSSLGR**
"	hexokinase 2	NP_038848 gi|7305143	36	1	1	36	**NVELVDGEEGR**
"	p53-related protein kinase; Nori-2	Q99PW4 gi|26398355	33	4	1	33	**DPQCLLDLAR**
"	60 kDa heat shock protein, mitochondrial precursor (Hsp60); Mitochondrial matrix protein P1	P19226 gi|3219998	29	1	1	29	**VTDALNATR**
"	neutral alpha-glucosidase AB	NP_032086 gi|6679891	22	2	3	6	**SLLLSVNAR**
"	lysosomal membrane glycoprotein LAMP-1	A28067 gi|91055	20	2	1	20	**ALQATVGNSYK**
"	mKIAA1463 protein	BAC41479 gi|26006273	18	1	1	18	**MDGLLMVSGR**
5 ~75kDa	hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), alpha subunit	NP_849209 gi|33859811	687	22	20	81	**LPAKPEVSSDEDVQYR**
"	Long-chain-fatty-acid--CoA ligase 2 (Long-chain acyl-CoA synthetase 2) (LACS 2)	P41216 gi|729927	600	20	18	75	**GIQVSNNGPCLGSR + Carbamidomethyl (C)**
"	dnaK-type molecular chaperone grp78 precursor	A37048 gi|109893	538	22	13	96	**NQLTSNPENTVFDAK**
"	mortalin mot-1=hsp70 homolog cytosolic form [CD1-ICR embryonic fibroblasts, MEF, Peptide, 679 aa]	AAB28640 gi|435838	381	16	10	78	**QAASSLQQASLK**

"	P450 (cytochrome) oxidoreductase; NADH cytochrome P450 oxydoreductase	NP_032924 gi|6679421	372	16	13	66	**YYSIASSSK**
"	Protein disulfide isomerase A4 precursor (Protein ERp-72) (ERp72)	P08003 gi|119531	241	13	9	56	**VDATEQTDLAK**
"	Bcl-2-like protein 13 (Mil1 protein) (Bcl-rambo)	P59017 gi|23396700	165	9	4	83	**AEGAAQLSEER**
"	glycerol-3-phosphate dehydrogenase	BAA08926 gi|1339938	158	4	4	61	**KQEELETATR**
"	lanosterol synthase	NP_666118 gi|22122469	145	5	4	52	**ILGIGPDDPDLVR**
"	3-methylcrotonyl-CoA carboxylase alpha subunit	AAG50244 gi|12276064	137	6	5	54	**SNVDFLLR**
"	methylmalonyl-CoA mutase	AAA99226 gi|896283	119	4	4	38	**GYDSDNPR**
"	NADH dehydrogenase (ubiquinone) Fe-S protein 1	NP_663493 gi|21704020	114	3	3	60	**ILQDIASGR**
"	tumor rejection antigen gp96; tumor rejection antigen (gp96) 1	NP_035761 gi|6755863	88	3	2	64	**GVVDSDDLPLNVSR**
"	programmed cell death 8; programmed cell death 8 (apoptosis inducing factor); apoptosis-inducing factor; harlequin	NP_036149 gi|6755004	54	3	2	42	**SATEQSGTGIR**
"	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial precursor (Fp)	P31040 gi|1169337	50	1	1	50	**ISKLYGDLK**
"	serum deprivation response	NP_620080 gi|20270267	0	2	1	43	**YQASTSNTVSK**
"	Dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex)	AAH31495 gi|21594641	0	1	1	40	**ILVPEGTR**

"	Nln protein	AAH25520 gi|19343748	34	2	1	34	**LVNTGLLTLR**
6 ~70kDa	perilipin	NP_783571 gi|28316726	472	19	11	103	**LASGGADLALGSIE K**
"	Long-chain-fatty-acid--CoA ligase 2 (Long-chain acyl-CoA synthetase 2) (LACS 2)	P41216 gi|729927	376	13	12	63	**CGVEIISLK + Carbamidomethy l (C)**
"	carnitine palmitoyltransfera se 2; CPT II	NP_034079 gi|6753514	338	14	11	67	**TLTIDAIQFQR**
"	Sdha protein	AAH11301 gi|15030102	337	12	10	69	**TLNEADCATVPPAI R + Carbamidomethy l (C)**
"	MTHSP75	AAA67526 gi|292059	302	13	9	69	**DAGQISGLNVLR**
"	RIKEN cDNA 9130022B02 Phosphoenolpyru vate carboxykinase 2 (mitochondrial)	AAH10318 gi|16307539	277	12	8	49	**GYLTEQVNQDLPK**
"	ribophorin I [Rattus norvegicus]	NP_037199 gi|6981486	266	10	7	80	**AVTSEIAVLQSR**
"	heat shock protein hsp60, hsp60=chaperoni n	AAB21806 gi|247242	263	9	6	76	**NAGVEGSLIVEK**
"	programmed cell death 8; programmed cell death 8 (apoptosis inducing factor); apoptosis-inducing factor; harlequin	NP_036149 gi|6755004	160	5	3	75	**SATEQSGTGIR**
"	glycerol-3-phosphate dehydrogenase	BAA08926 gi|1339938	142	3	3	63	**SADTQSISR**
"	hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein)	NP_849209 gi|33859811	142	4	3	69	**LPAKPEVSSDEDVQ YR**
"	Ddx5 protein	AAH62916 gi|38571767	140	4	3	59	**QVSDLISVLR**

"	70kD peroxisomal integral membrane protein [Homo sapiens]	CAA58470 gi|825711	140	5	5	52	**MGNLDNR**
"	carnitine acetyltransferase	NP_031786 gi|6681009	130	7	5	48	**SASIDSLAFVK**
"	lamin A	BAA02476 gi|220474	113	4	2	84	**LADALQELR**
"	dnaK-type molecular chaperone grp78 precursor	A37048 gi|109893	108	6	4	49	**NELESYAYSLK**
"	pyruvate carboxylase; pyruvate decarboxylase	NP_032823 gi|6679237	89	1	1	89	**AEAEAQAEELSFPR**
"	unnamed protein product	BAB27647 gi|26374514	68	2	1	68	**IVNDNATYCR + Carbamidomethyl (C)**
"	serum deprivation response	NP_620080 gi|20270267	10	4	2	55	**YQASTSNTVSK**
"	P450 (cytochrome) oxidoreductase; NADH cytochrome P450 oxydoreductase	NP_032924 gi|6679421	63	1	1	63	**NPFLAAVTTNR**
"	transketolase	NP_033414 gi|6678359	62	2	2	52	**LAVSQVPR**
"	serum albumin precursor [validated] – bovine	ABBOS gi|418694	60	3	2	30	**LVNELTEFAK YICDNQDTISSK + Carbamidomethyl (C)**
"	lamin B1	NP_034851 gi|6754556	53	1	1	53	**NSQGEEVAQR**
"	unnamed protein product	BAC28163 gi|26328849	49	2	2	42	**GVYSEETLR**
"	Anxa6 protein	AAH05595 gi|13542782	46	4	3	33	**STPEYFAER**
"	aconitase 2, mitochondrial	NP_542364 gi|18079339	46	1	1	46	**VDVSPTSQR**
"	EH-domain containing 2	NP_694708 gi|23346469	35	5	3	33	**GYDFPAVLR**
"	glyceronephosphate O-acyltransferase	NP_034452 gi|31981734	29	1	1	44	**NTYNLVPR**

"	Lanosterol synthase (Oxidosqualene-lanosterol cyclase) (2,3-epoxysqualene--lanosterol cyclase) (OSC)	P48450 gi|1352388	24	1	1	-	−

*Protein score as determined by MASCOT

#Peptide score of the highest scoring peptide (note: different from the protein score).

§Peptide sequence of the highest scoring peptide

Supplement References

Bessonov S, Anokhina M, Will CL, Urlaub H, Luhrmann R (2008) Isolation of an active step I spliceosome and composition of its RNP core. *Nature* **452:** 846-850

Bolte S, Cordelieres FP (2006) A guided tour into subcellular colocalization analysis in light microscopy. *J Microsc* **224:** 213-232

Frank R (2002) The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports--principles and applications. *J Immunol Methods* **267:** 13-26

Herberg FW, Maleszka A, Eide T, Vossebein L, Tasken K (2000) Analysis of A-kinase anchoring protein (AKAP) interaction with protein kinase A (PKA) regulatory subunits: PKA isoform specificity in AKAP binding. *J Mol Biol* **298:** 329-339

Kita T, Nishida H, Shibata H, Niimi S, Higuti T, Arakaki N (2009) Possible role of mitochondrial remodelling on cellular triacylglycerol accumulation. *J Biochem* **146:** 787-796

Lester LB, Langeberg LK, Scott JD (1997) Anchoring of protein kinase A facilitates hormone-mediated insulin secretion. *Proc Natl Acad Sci U S A* **94:** 14942-14947

Misaka T, Miyashita T, Kubo Y (2002) Primary structure of a dynamin-related mouse mitochondrial GTPase and its distribution in brain, subcellular localization, and effect on mitochondrial morphology. *J Biol Chem* **277:** 15834-15842

Olichon A, Emorine LJ, Descoins E, Pelloquin L, Brichese L, Gas N, Guillou E, Delettre C, Valette A, Hamel CP, Ducommun B, Lenaers G, Belenguer P (2002) The human dynamin-related protein OPA1 is anchored to the mitochondrial inner membrane facing the inter-membrane space. *FEBS Lett* **523:** 171-176

Ramirez-Zacarias JL, Castro-Munozledo F, Kuri-Harcuch W (1992) Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O. *Histochemistry* **97:** 493-497

Satoh M, Hamamoto T, Seo N, Kagawa Y, Endo H (2003) Differential sublocalization of the dynamin-related protein OPA1 isoforms in mitochondria. *Biochem Biophys Res Commun* **300:** 482-493

Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem* **68:** 850-858

Stokka AJ, Gesellchen F, Carlson CR, Scott JD, Herberg FW, Tasken K (2006) Characterization of A-kinase-anchoring disruptors using a solution-based assay. *Biochem J* **400:** 493-499

Valgardsdottir R, Ottersen OP, Prydz H (2004) Regulated compartmentalization of the putative DEAD-box helicase MDDX28 within the mitochondria in COS-1 cells. *Exp Cell Res* **299:** 294-302