

RESEARCH ARTICLE

AMP-Activated Protein Kinase Interacts with the Peroxisome Proliferator-Activated Receptor Delta to Induce Genes Affecting Fatty Acid Oxidation in Human Macrophages

Marina Kemmerer¹, Florian Finkernagel², Marcela Frota Cavalcante³, Dulcineia Saes Parra Abdalla³, Rolf Müller², Bernhard Brüne^{1‡}, Dmitry Namgaladze^{1‡*}

1 Institute of Biochemistry I, Faculty of Medicine, Goethe-University Frankfurt, Theodor-Stern-Kai 7, 60590, Frankfurt, Germany, **2** Institute of Molecular Biology and Tumor Research (IMT), Philipps-University Marburg, Marburg, Germany, **3** Department of Clinical and Toxicological Analyses, Faculty of Pharmaceutical Sciences, University of Sao Paulo, Sao Paulo, Brazil

‡ These authors are joint senior authors on this work.

* namgaladze@biochem.uni-frankfurt.de



OPEN ACCESS

Citation: Kemmerer M, Finkernagel F, Cavalcante MF, Abdalla DSP, Müller R, Brüne B, et al. (2015) AMP-Activated Protein Kinase Interacts with the Peroxisome Proliferator-Activated Receptor Delta to Induce Genes Affecting Fatty Acid Oxidation in Human Macrophages. PLoS ONE 10(6): e0130893. doi:10.1371/journal.pone.0130893

Editor: Hervé Guillou, INRA, FRANCE

Received: November 6, 2014

Accepted: May 26, 2015

Published: June 22, 2015

Copyright: © 2015 Kemmerer et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files. Microarray data are available from Array Express database (www.ebi.ac.uk/arrayexpress/), accession number E-MTAB-2524.

Funding: This work was supported by Deutsche Forschungsgemeinschaft (www.dfg.de): Grant NA429/2-1 (DN, BB); Grant SFB 1039-A05 (DN); and Grant SFB 1039-B04 (BB). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

AMP-activated protein kinase (AMPK) maintains energy homeostasis by suppressing cellular ATP-consuming processes and activating catabolic, ATP-producing pathways such as fatty acid oxidation (FAO). The transcription factor peroxisome proliferator-activated receptor δ (PPAR δ) also affects fatty acid metabolism, stimulating the expression of genes involved in FAO. To question the interplay of AMPK and PPAR δ in human macrophages we transduced primary human macrophages with lentiviral particles encoding for the constitutively active AMPK α 1 catalytic subunit, followed by microarray expression analysis after treatment with the PPAR δ agonist GW501516. Microarray analysis showed that co-activation of AMPK and PPAR δ increased expression of FAO genes, which were validated by quantitative PCR. Induction of these FAO-associated genes was also observed upon infecting macrophages with an adenovirus coding for AMPK γ 1 regulatory subunit carrying an activating R70Q mutation. The pharmacological AMPK activator A-769662 increased expression of several FAO genes in a PPAR δ - and AMPK-dependent manner. Although GW501516 significantly increased FAO and reduced the triglyceride amount in very low density lipoproteins (VLDL)-loaded foam cells, AMPK activation failed to potentiate this effect, suggesting that increased expression of fatty acid catabolic genes alone may be not sufficient to prevent macrophage lipid overload.

Introduction

The number of people with diabetes is expected to rise to 366 million in 2030 worldwide [1]. Patients with the metabolic syndrome—symptoms of which include abdominal obesity,

Competing Interests: The authors have declared that no competing interests exist.

dyslipidemia, glucose intolerance, and hypertension—have a five-fold increased risk of developing type 2 diabetes mellitus and usually show a decreased capacity for exercise [2–6]. The connection between metabolism and immune responses is increasingly being appreciated in the context of metabolic diseases, including atherosclerosis and obesity-driven diabetes [7, 8]. Particularly, lipid metabolism in macrophages undergoing foam cell formation is crucial to regulate inflammatory processes in developing atherosclerotic plaques and expanding adipose tissue [9, 10]. During foam cell formation macrophages take up considerable amounts of lipids and adapt to lipid loading by activating transcriptional programmes aimed at preventing excessive lipid overload and limiting inflammation.

Transcription factors of the peroxisome proliferator-activated receptor (PPAR) family (PPAR α , - δ and - γ) are critical for adaptation to lipid overload [11]. PPAR δ acts as a heterodimer with the retinoid X receptor (RXR), binding to PPAR response element (PPRE) DNA sequences [12]. There are three different types of target gene regulation by PPAR δ : agonist-independent repression (type I); agonist-sensitive repression (type II), and agonist-independent activation (type III) [13]. In case of type II regulation, PPAR δ induces a repressive state by executing a transcriptional co-repressor function in the absence of agonists. Once activated by a ligand, the heterodimer PPAR δ -RXR recruits co-activators promoting initiation of gene transcription [14]. Among the different PPARs PPAR δ is most ubiquitously expressed and may be particularly relevant for macrophages handling triglyceride-rich lipoproteins [15]. Recently, we and others found that PPAR δ is activated in triglyceride-rich foam cells following the uptake of phospholipolyzed lipoproteins or very low density lipoproteins (VLDL). Subsequently, activated PPAR δ attenuates inflammatory responses in macrophages [16, 17]. This is consistent with the anti-atherogenic effects of PPAR δ in animal models [18–20]. Transcriptional reprogramming of macrophage lipid metabolism by PPAR δ is primarily characterized by increased mitochondrial and peroxisomal fatty acid oxidation (FAO) [21, 22], similar to the effects of PPAR δ activation in metabolically active tissues such as skeletal muscle [23]. Induction of FAO was linked to anti-obesity and insulin-sensitizing *in vivo* phenotypes following PPAR δ activation [22, 23].

In addition to transcriptional regulators, AMP-activated protein kinase (AMPK) plays a key role to connect metabolism and inflammation [8]. AMPK senses metabolic stresses via its activation by increased AMP/ATP and ADP/ATP ratios. Activated AMPK shuts off energy-consuming processes, while inducing protein, carbohydrate, and fat catabolism. AMPK activates FAO through phosphorylation and inactivation of acetyl-CoA carboxylase (ACC) thus, reducing levels of malonyl-CoA, an allosteric inhibitor of carnitine palmitoyltransferase (CPT1a) [8]. AMPK also inactivates glycerol-3-phosphate acyltransferase, channeling acyl-CoA towards β -oxidation [24]. This may underlie insulin-sensitizing effects of AMPK activation, and contribute to anti-inflammatory functions of AMPK in adipose tissue macrophages [25]. An allosteric AMPK activator A-769662 has been described to act independently of the upstream AMPK kinases, inhibiting AMPK dephosphorylation [26] and to decrease plasma glucose and triglyceride levels in a mouse diabetes model ob/ob mice [27].

As both, AMPK and PPAR δ provide beneficial metabolic effects, at least in part by targeting FAO, it is of interest how these regulators cooperate. Previous studies in skeletal muscle explored the interaction of AMPK and PPAR δ and showed that combined pharmacological activation of AMPK and PPAR δ in mice created a unique phenotype associated with increased running endurance through enhanced muscle fatty acid metabolism [28]. It was proposed that the combined activation of AMPK and PPAR δ may provide additional metabolic benefits compared to single treatments. In our study we explored the transcriptome of human macrophages under conditions of single and combined activation of AMPK and PPAR δ . We found enhanced activation of FAO-associated genes by combined AMPK/PPAR δ agonism.

However, these transcriptional changes were not accompanied by enhanced macrophage FAO or protection against lipid overload.

Materials and Methods

Cell Culture

Human peripheral blood monocytes were isolated from buffy coats provided by anonymous donors (DRK-Blutspendedienst Baden-Württemberg-Hessen, Institut für Transfusionsmedizin und Immunhämatologie, Frankfurt, Germany, URL:<http://www.blutspende.de/en/institutes-affiliates/frankfurt-am-main/frankfurt-am-main.php>) using Ficoll gradient (LSM 1077, GE Healthcare) centrifugation according to the manufacturer's protocol and CD14 microbead selection (Miltenyi Biotec). Monocytes were seeded for differentiation in serum-free medium (Macrophage-SFM, Life Technologies), supplemented with 50 ng/ml human recombinant macrophage colony-stimulating factor (M-CSF, Immunotools) and maintained for 6 days. For treatments, cells were incubated in RPMI 1640 medium (GE Healthcare) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Stimulation with GW501516 (Axxora), salicylate (Sigma-Aldrich) and A-769662 (LC Laboratories) was for 24 or 48 hours. This investigation conforms to the ethical principles outlined in the Declaration of Helsinki and was approved by the university ethics committee (Ethik-Kommission des Fachbereichs Medizin der Goethe-Universität Frankfurt am Main). The ethics committee waived the need for consent when using the blood of anonymous donors.

THP-1 human acute monocytic leukemia cells (ATCC) were maintained in RPMI 1640 medium, supplemented with FCS, glutamine, penicillin, and streptomycin.

Plasmid constructs

Cloning of the human truncated AMPK α 1 subunit was performed using SBI System Biosciences, Clone-it Enzyme free Lentivectors according to the manufacturer's protocol. Lentivector LF521A-1 containing a puromycin resistance was used. Briefly, isolated total DNA of human primary macrophages served as a template in PCR using High Fidelity DNA Polymerase (Roche). The following primers were used: AMPK1-for- 5' -atgCGcagactcagttcctg-3' ; AMPK2-rev- 5' -ggcaactgccaaggatcc-3' ; AMPK2-for- 5' -GAGGCAGCAGAGACCG atgCGcagactcagttcctg-3' ; AMPK1-rev- 5' -CGAACAGAGAGAGA-CCGggcaactgccaaggatcc-3' . PCR products were cleaned by QIAquick PCR Purification Kit (Qiagen) and annealed by heating the mix at 95°C and slowly cooling down. Following transformation resulting clones were verified by sequencing. For lentiviral production the lentiviral packaging vector pCMV-dR8 and the viral envelope plasmid pMD2G were used. Adenoviruses coding for AMPK γ 1 regulatory subunit carrying an activating R70Q substitution were kindly provided by Dr Jason Dyck, Cardiovascular Research Centre, University of Alberta, Canada. Control adenoviruses (AdTrack) were from Addgene.

Site-directed mutagenesis

Threonine-172 to aspartic acid mutation (T172A) of AMPK α 1 was performed by Quick-Change II XL Site-directed Mutagenesis Kit (Agilent Technologies) using PfuUltra HF DNA polymerase (Stratagene). Following mutagenesis primers were used: mutAMPK-for- 5' -cagatggtgaatTTTTAAGAGATAGTTGTGGCTCACCCAACATGC-3' ; mutAMPK-rev- 5' -gcatagttgggtgagcc-acaactatctcttaaaaattcaccatctg-3' . The inserts were sequenced in their entirety in order to confirm the authenticity of the mutation and to

ensure no other mutations occurred. Mutation creates a constitutively active AMPK α 1 subunit consisting of 312 amino acids. This truncated form of AMPK α 1 was used in the microarray analysis.

Lentiviral production

2.5×10^6 293T HEK cells were seeded in 10 cm dishes and transfected by pCMV-dR8 and pMD2G plasmids (proportion 5.25:1) together with a control vector pCDH-EF1-T2A-puro or LF521A-1-puro-AMPK α 1 using JetPRIME transfection reagent (Polyplus) followed by medium exchange 24 hours post-transfection. Supernatants containing lentiviral particles were collected 72 hours post-transfection and concentrated using Lenti-X concentrator (Clontech) according to manufacturer's protocol.

Microarray analysis

Primary human macrophages were transduced with control (CV) or lentiviral particles encoding the constitutively active AMPK α 1 (AMPK OE) for 48 hours and treated with 100 nM GW501516 for additional 24 hours. Total RNA isolation was performed by phenol-chloroform extraction. RNA was further purified by RNeasy total RNA Cleanup Kit (Qiagen) and eluted in RNase-free water. RNA quality was analyzed by the Agilent RNA 6000 analyzer. Whole genome microarray analysis was performed using the Illumina Sentrix Human HT-12 v4 chip. Raw microarray data were normalized using the VSN method and assigned to human gene symbols using R/Bioconductor [29] and the bead array package [30]. Triplicates were contrasted using Limma [31] and differentially expressed genes were selected based on a 1.5-fold change and a Benjamini-Hochberg adjusted p-value smaller than 0.1. Functional annotation was performed using Gene Set Enrichment Analysis [32] against gene sets derived from the molecular signature database version 3.1, datasets c2, c3 and c6 [32], from Pathway Commons [33] and from Genome Ontology via Ensembl, revision 70 [34].

RNA extraction, reverse transcription, and real-time quantitative PCR

Total RNA of 1×10^6 cells was isolated using peqGOLD RNAPure (PepLab) according to manufacturer's protocol. 1 μ g of total RNA was reverse transcribed using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific). Real-time quantitative PCR assays were performed with the iQ Custom SYBR Green Supermix (Bio-Rad) using the CFX96 system from Bio-Rad. Each amplification sample contained 20 ng of cDNA, 250 nM each of forward and reverse primers and 5 μ l of 2x iQ SYBR Green Supermix. The mRNA expression was normalized to GAPDH. Following primers were used for quantitative PCR: PDK4-forward—5' -cctttggctggttttgggta-3' ; PDK4-reverse— 5' -cctgcttgggatacaccagt-3' ; CPT1a-forward— 5' -tcgtcacctcttctgccttt-3' ; CPT1a-reverse— 5' -acacaccatagccgtc atca-3' ; PLIN2-forward— 5' -aagaaaaatggcatccggttg-3' ; PLIN2-reverse— 5' -caatttgcggtctagcttc-3' ; PPAR δ -forward— 5' -tcacacagtggcttctgctc-3' ; PPAR δ -reverse— 5' -tctacagggtggttcccatc-3' ; Angptl4-forward— 5' -gcctatagcc tgcagctcac-3' ; Angptl4-reverse— 5' -agtactggccggttgagggttg-3' ; GAPDH-forward— 5' -tgcaccaccaactgcttagc-3' ; GAPDH-reverse— 5' -ggcatggactgtggctc atgag-3' .

Western analysis

Cell pellets were harvested in lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 1 mM Na₂VO₄, 0.5% NP-40, 1 mM PMSF, protease inhibitor cocktail (Roche)).

Nuclei were isolated using nuclear lysis buffer A (20 mM Tris-HCl pH 8.0, 10 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 mM PMSF, protease inhibitor cocktail) followed by centrifugation at 16000g for 20s. Nuclear pellets were sonicated in lysis buffer B (20 mM Tris-HCl pH 8.0, 400 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 mM PMSF, protease inhibitor cocktail). 50–100 µg of protein extracts were separated using 8% polyacrylamide gels, transferred to nitrocellulose membrane, blocked in 5% nonfat milk in TBS-Tween buffer (0.1% Tween 20, pH 7.4) and incubated with a desired primary antibody overnight at 4°C. Primary antibodies directed against PDK4 (Abcam, ab38242 and Proteintech Europe, 12949-1-AP), CPT1a (Proteintech Europe, 15184-1-AP), phospho-AMPK (#2531), AMPK α (#2532), AMPK β (#4150), phospho-ACC (#3661), ACC (#8578), phospho-S6 (#4856), S6 (#2317) (all Cell Signaling Technology), PPAR δ (Santa Cruz Biotechnology, sc-74517 and Abcam, ab58137) and nucleolin (Santa Cruz Biotechnology, sc-13057) were used followed by IRDye 680 or IRDye 800-coupled secondary antibodies (LICOR Biosciences). Nucleolin was used for normalizing the amount of protein loaded onto each lane.

Oxygen consumption measurements

Macrophages were plated in Seahorse cell culture plates and treated as indicated. For oxygen consumption measurements the medium was changed to Krebs-Henseleit buffer (111 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl₂, 2 mM MgSO₄, 1.2 mM NaH₂PO₄) supplemented with 0.5 mM carnitine, 5 mM HEPES and 100 µM palmitate-BSA conjugate, adjusted to pH 7.4 at 37°C, prior to the assay. FAO was measured using Seahorse 96 extracellular flux analyzer (Seahorse Bioscience) as the difference in oxygen consumption rates before and after the addition of 25 µM CPT1a inhibitor etomoxir and was normalized to the protein amounts in the wells.

Triglyceride measurement

Human VLDL was isolated from the plasma samples of healthy volunteers by sequential ultracentrifugation. Primary macrophages were pretreated with 100 nM GW501516 and/or 250 µM A-769662 for 48 hours. After medium changes cells were loaded by 20 µg/ml VLDL for additional 24 hours. Triglyceride (TG) content was determined using TG determination kit (Roche) according to the manufacturer's instructions and normalized to protein content.

siRNA-mediated RNA interference

siRNAs (ON-Target plus SMARTpool, Dharmacon) targeting human PPAR δ , AMPK α 1 or scrambled control RNA oligonucleotides were transfected into primary macrophages at a final concentration of 50 nM for 72 hours using Hiperfect transfection reagent (Qiagen) according to the manufacturer's instructions. Stimulation with 250 µM A-769662 or 100 nM GW501516 for additional 24 hours followed.

Statistical analysis

The significance of the differences in mean values among two groups was evaluated by one-way ANOVA test. Differences were considered statistically significant for $p < 0.05$ (*/#/\$) and $p < 0.01$ (**/##). Data are presented as averages \pm 95% Confidence Interval of at least three independent experiments.

Results

Previous studies revealed interactions of AMPK and PPAR δ in muscle cells, causing a transcriptional reprogramming to increase fatty acid oxidative metabolism [28]. We questioned

functional interactions of AMPK and PPAR δ in primary human macrophages by analyzing alterations of the macrophage transcriptome following single and combined treatments to activate AMPK and PPAR δ . To avoid off-target effects associated with pharmacological AMPK activation we overexpressed a lentiviral construct coding for a truncated, constitutively active human AMPK α 1 subunit (AMPK OE) in primary human macrophages, followed by 24 hour-treatments with 100 nM of the selective PPAR δ agonist GW501516. Genome-wide mRNA expression profiling was then performed using Illumina HT12v4 bead arrays (EMBL-EBI Array Express accession number E-MTAB-2524). As illustrated by the Venn diagram (Fig 1A), 238 genes were regulated by AMPK overexpression (107 up, 131 down, $\log_2(\text{fold change}) \geq 0.58$), while 79 genes changed their expression in response to GW501516 (46 up, 33 down) with an overlap of 8 genes (3 up, 5 down). Combined AMPK/PPAR δ activation altered the expression of 322 genes (128 up, 194 down). Testing the cooperativity of gene regulation by AMPK and PPAR δ we did not find any probe showing >50% difference in intensity after co-activation of AMPK and PPAR δ compared with single stimulations, indicating no synergistic effects.

Gene Set Enrichment Analysis (GSEA) is a method to interpret gene expression data focusing on gene sets [32]. Comparing combined AMPK/PPAR δ activation with untreated samples revealed fatty acid oxidative metabolism dominating the list of 20 mostly enriched pathways (Table 1). Among the 20 strongest induced genes upon GW501516-treatment and AMPK OE shown in Table 2, 7 referred to fatty acid metabolism, including FAO-associated genes pyruvate dehydrogenase kinase 4 (PDK4), CPT1a, acetyl-CoA acyltransferase 2 (ACAA2), and very long chain acyl-CoA dehydrogenase (ACADVL). Perilipin 2 (PLIN2), a common PPAR δ target gene, was also present in this list.

We then confirmed that FAO is the major pathway undergoing transcriptional activation after AMPK and PPAR δ co-activation. Validation by quantitative PCR analysis showed enhanced induction of FAO-associated genes PDK4 and CPT1a, as well as PLIN2 after a single treatment with GW501516 or after AMPK overexpression (Fig 1B). Besides, we observed a significantly increased PDK4, CPT1a, and PLIN2 mRNA expression in PPAR δ /AMPK-coactivated macrophages compared to individual activation.

The truncated AMPK construct used here lacks the interaction with regulatory subunits, which may be important for AMPK substrate targeting. Indeed, we failed to detect increased phosphorylation of the AMPK substrate ACC in macrophages with AMPK OE (data not shown), similar to previous observations in rat cardiomyocytes [35]. Therefore, to validate our observations we infected macrophages with adenoviruses coding for a regulatory AMPK γ 1 subunit having a R70Q substitution. This mutation was reported to increase the activity of AMPK heterotrimers [36]. Transduction of macrophages with AMPK γ 1 R70Q adenovirus replicated effects of the truncated AMPK OE construct on PPAR δ target mRNA expression with the exception of CPT1a (Fig 1C). Fig 1D shows that this construct also caused modest, but significant elevations of ACC phosphorylation.

Next, we questioned whether pharmacological AMPK activation similarly enhanced PPAR δ target gene mRNA expression as AMPK overexpression. In these experiments we used the allosteric AMPK activator A-769662. Analysis of the phosphorylation status of AMPK, its substrate ACC and ribosomal protein S6, which served as readout of mTOR (mechanistic target of rapamycin) activity, revealed that significant ACC phosphorylation was already observed at 50 μ M A-769662, and continued to increase up to 500 μ M A-769662 (Fig 2A). In contrast, significant down-regulation of phospho-S6 and up-regulation of phospho-AMPK was achieved only at 250 μ M and 500 μ M A-769662, respectively. Therefore, we used 500 μ M of the drug in subsequent experiments. Measuring intracellular ATP did not show changes after exposure to these concentrations of A-769662, ruling out a loss of viability (data not shown). As shown in

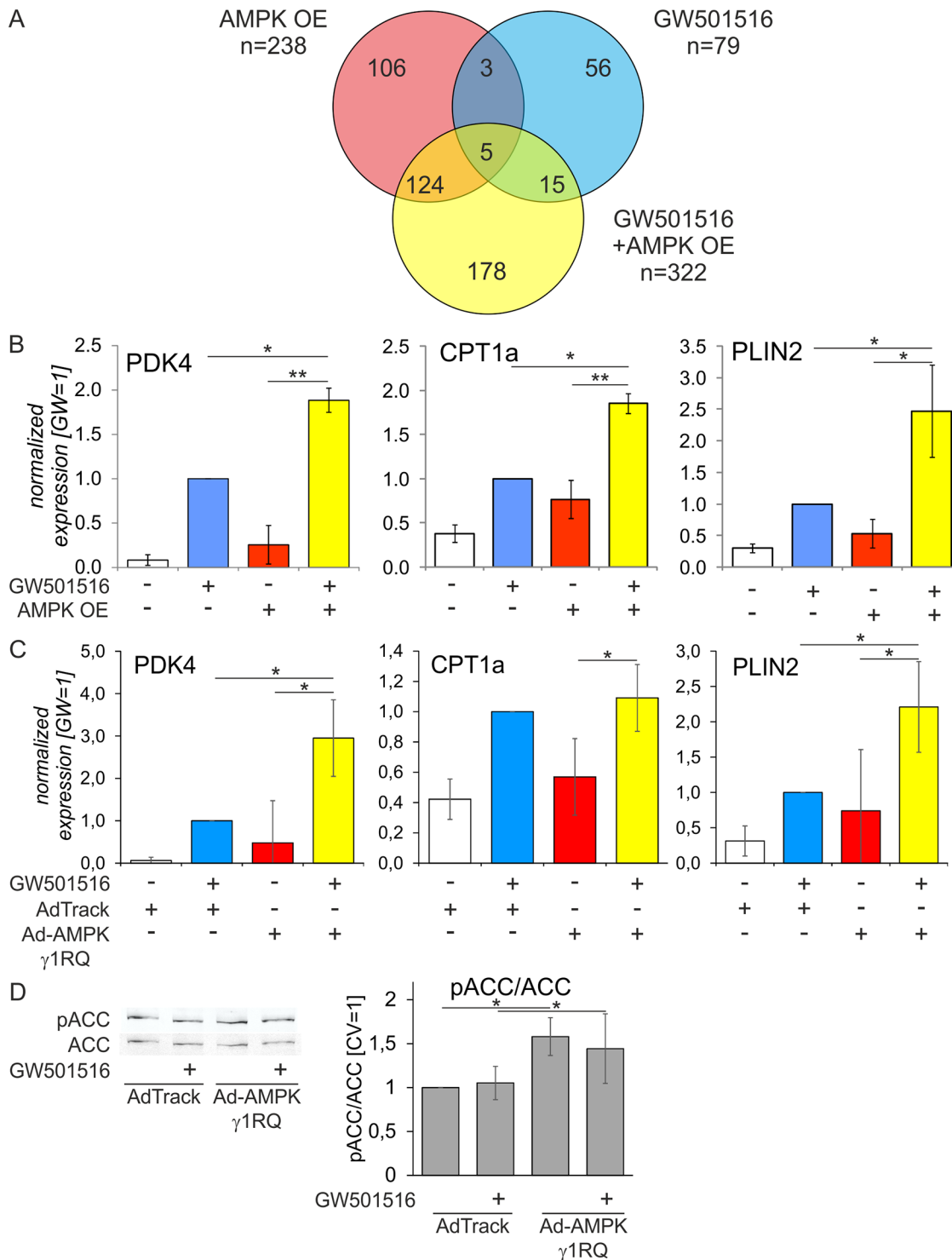


Fig 1. Analysis of AMPK and PPAR δ interactions in primary human macrophages with overexpression of AMPK catalytic or regulatory subunits. Primary human macrophages were transduced with control lentivirus (CV) or lentiviral particles coding for a constitutively active AMPK α 1 (AMPK OE) for 48 hours and treated with 100 nM GW501516 for additional 24 hours. **A** Venn diagram showing numbers of genes regulated by AMPK OE, GW501516, or their combination. **B** Validation of microarray analysis by quantitative PCR. **C** mRNA expression of PDK4, CPT1a, and PLIN2 in macrophages infected with control adenovirus (AdTrack) or AMPK γ 1 R70Q adenovirus for 48 hours prior to 24 hour-treatment with 100 nM GW 501516. **D** Western blot showing ACC phosphorylation and its quantification in macrophages infected with control adenovirus (AdTrack) or AMPK γ 1 R70Q adenovirus for 48 hours. Values represent averages \pm 95% Confidence Interval. *, $p < 0.05$; **, $p < 0.01$ ($n = 3$).

doi:10.1371/journal.pone.0130893.g001

Table 1. Major regulated pathways by Gene Set Enrichment Analysis.

Gene set	Size	NES
Fatty acid beta oxidation	38	2.17
Kinesin binding	20	2.16
ATP biosynthetic process	32	2.15
Pyruvate metabolism and TCA cycle	38	2.13
TCA cycle and respiratory electron transport	110	2.08
Ion transport by P-type ATPases	31	2.07
FFA oxidation	22	2.00
STAT3 targets	46	1.99
TCA cycle	18	1.97
Pyruvate metabolic process	24	1.96
Oxidative stress response	33	1.96
Pyruvate metabolism	17	1.96
Endoplasmic reticulum organization	16	1.95
Cellular aromatic compound metabolic process	7	1.95
PPAR signaling pathway	68	1.93
Hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances	41	1.91
Estrogen metabolic process	12	1.91
Positive regulation of fatty acid beta oxidation	8	1.90

NES, normalized enrichment score

doi:10.1371/journal.pone.0130893.t001

Table 2. 20 strongest induced genes upon combined AMPK/PPARδ activation.

Gene symbol	Name	log ₂ (fold change), GW+AMPK OE vs. CV
PDK4	pyruvate dehydrogenase kinase, isozyme 4	2.11
CPT1A	carnitine palmitoyltransferase 1A (liver)	1.48
FABP4	fatty acid binding protein 4, adipocyte	1.30
PLIN2	perilipin 2	1.08
ACAA2	acetyl-CoA acyltransferase 2	1.03
UCHL1	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	0.96
RNF128	ring finger protein 128, E3 ubiquitin protein ligase	0.96
DHRS9	dehydrogenase/reductase (SDR family) member 9	0.95
ELOVL6	ELOVL fatty acid elongase 6	0.91
FAM160B1	family with sequence similarity 160, member B1	0.91
IMPA2	inositol (myo)-1 (or 4)-monophosphatase 2	0.87
ANKDD1A	ankyrin repeat and death domain containing 1A	0.84
SPINK1	serine peptidase inhibitor, Kazal type 1	0.83
ACADVL	acyl-CoA dehydrogenase, very long chain	0.82
ZNF366	zinc finger protein 366	0.81
HPSE	heparanase	0.81
SEMA3E	semaphorin 3E	0.79
CDH23	cadherin-related 23	0.79
NBL1	neuroblastoma, suppression of tumorigenicity 1	0.79
CD36	CD36 molecule (thrombospondin receptor)	0.76

doi:10.1371/journal.pone.0130893.t002

[Fig 2B](#), treatment of primary human macrophages with A-769662 induced mRNA expression of the PPAR δ target genes PDK4, CPT1a, and PLIN2 to a similar extent as the exposure of cells to the PPAR δ ligand GW501516. Importantly, A-769662 significantly augmented PPAR δ target gene expression in GW501516-treated cells. Similar results were obtained when analyzing mRNA expression of PPAR δ target genes following macrophage treatment with GW501516 and salicylate, which has recently been recognized as another direct allosteric AMPK activator [37] ([S1 Fig](#)). Analysis of PPAR δ target gene expression also revealed cell type-specific differences. Whereas the well-known PPAR δ target gene angiopoietin-like 4 (Angptl4), which is a lipoprotein lipase inhibitor, was robustly induced in the THP-1 macrophage cell line, it was not induced in primary macrophages ([S2 Fig](#)).

To confirm the mRNA results of additively regulated genes affecting β -oxidation we performed Western analysis of FAO-associated targets PDK4 and CPT1a to test the effects of AMPK and/or PPAR δ activation. [Fig 2C](#) shows that both PDK4 and CPT1a were elevated in response to GW501516, A-769662, or their combination as compared to untreated cells. However, the magnitude of the responses was less pronounced compared to mRNA expression changes. Assessment of FAO, measured as etomoxir-sensitive oxygen consumption in the presence of palmitate, revealed that although GW501516 induced moderate increases of FAO, A-769662 at concentration of 100 μ M failed to do so ([Fig 2D](#)). Higher concentrations of A-769662 inhibited respiration (data not shown).

It has been shown that macrophage triglyceride (TG) accumulation induced by VLDL is significantly reduced by PPAR δ activation [16]. To evaluate the effect of AMPK/PPAR δ co-activation on VLDL-triggered foam cell formation, we treated primary macrophages with 100 nM GW501516 or 250 μ M A-769662 for 48 hours and then stimulated cells with VLDL (20 μ g/ml) for additional 24 hours ([Fig 2E](#)). VLDL-stimulation increased triglyceride accumulation in macrophages. Pre-treatment with A-769662 did not reduce the triglyceride amount, whereas the PPAR δ agonist GW501516 significantly decreased triglycerides. No evidence for a stronger reduction in combined stimulation was observed.

To further dissect the roles of AMPK and PPAR δ in regulating FAO-related gene expression, we silenced AMPK α 1 catalytic subunit (the predominant isoform in human macrophages) and PPAR δ and followed mRNA and protein expression of PPAR δ target genes in macrophages treated with A-769662, GW501516 and their combination. Silencing of PPAR δ achieved over 90% knockdown (KD) at the mRNA level ([Fig 3A](#)) and diminished the expression of PPAR δ protein ([Fig 3C](#)). It also increased the basal expression of PPAR δ target genes PDK4, CPT1a, but not PLIN2, consistent with the known repressor function of ligand-free PPAR δ ([Fig 3B](#)) [38]. Cells with a PPAR δ KD had also a blunted response to A-769662 and GW501516; PLIN2 mRNA expression was unaltered whereas PDK4 was significantly increased only in response to A-769662 and CPT1a was significantly increased only after co-treatment with A-769662 and GW501516. AMPK α 1 KD achieved more than 90% reduction of AMPK α 1 mRNA levels and over 65% reduction of AMPK α 1 protein ([Fig 3A](#) and [3C](#)). Accordingly, basal and A-769662-stimulated phosphorylation of the AMPK substrate ACC was significantly attenuated in AMPK α 1-silenced cells ([Fig 3C](#)). Interestingly, AMPK α 1 KD also reduced mRNA and protein levels of PPAR δ and increased mRNA expression of PPAR δ target genes thus, mimicking the PPAR δ target gene mRNA expression changes in PPAR δ KD cells ([Fig 3A–3C](#)). Similarly increased mRNA expression of PPAR δ target genes after AMPK α 1 knock-down was observed using unrelated siControl siRNA as well as in THP-1 macrophages stably transduced with unrelated AMPK α 1 shRNA lentivirus (data not shown). AMPK α 1 KD macrophages also did not show significantly increased mRNA expression of PPAR δ target genes after A-769662 treatment. Still, AMPK α 1 KD cells responded to PPAR δ activation by GW501516 or combined GW501516/A-769662 treatment with increased expression of PLIN2,

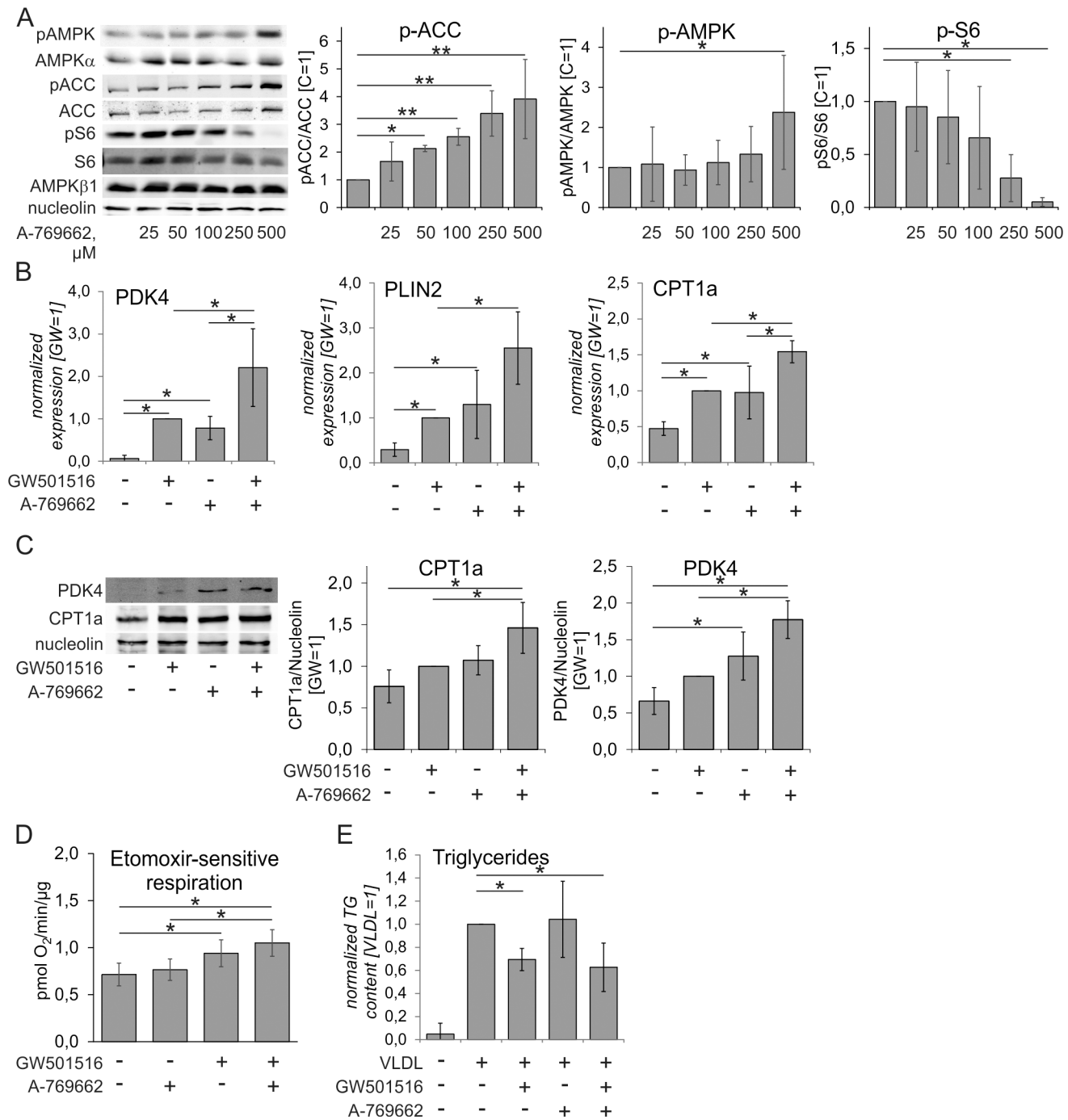


Fig 2. Pharmacological AMPK and PPAR δ activation affects expression of FAO genes, FAO and VLDL-induced lipid accumulation in primary human macrophages. **A** Western analysis of macrophages treated with indicated concentrations of A-769662 for 1 hour (n = 4). **C**, untreated cells. **B**, **C** mRNA (**B**) and protein (**C**) expression of PDK4, CPT1a, and PLIN2 in macrophages treated with 500 μ M A-769662 and/or 100 nM GW501516 for 24 hours (mRNA) or 48 hours (protein) (n = 3). **D** Etomoxir-sensitive respiration in human macrophages following 48 hour-treatment with 100 nM GW501516 and 100 μ M A-769662. **E** Triglyceride content of primary macrophages treated with 100 nM GW501516 and/or 250 μ M A-769662 for 48 hours prior to VLDL (20 μ g/ml) stimulation for 24 hours (n = 4). Values represent averages \pm 95% Confidence Interval. *, p<0.05; **, p<0.01.

doi:10.1371/journal.pone.0130893.g002

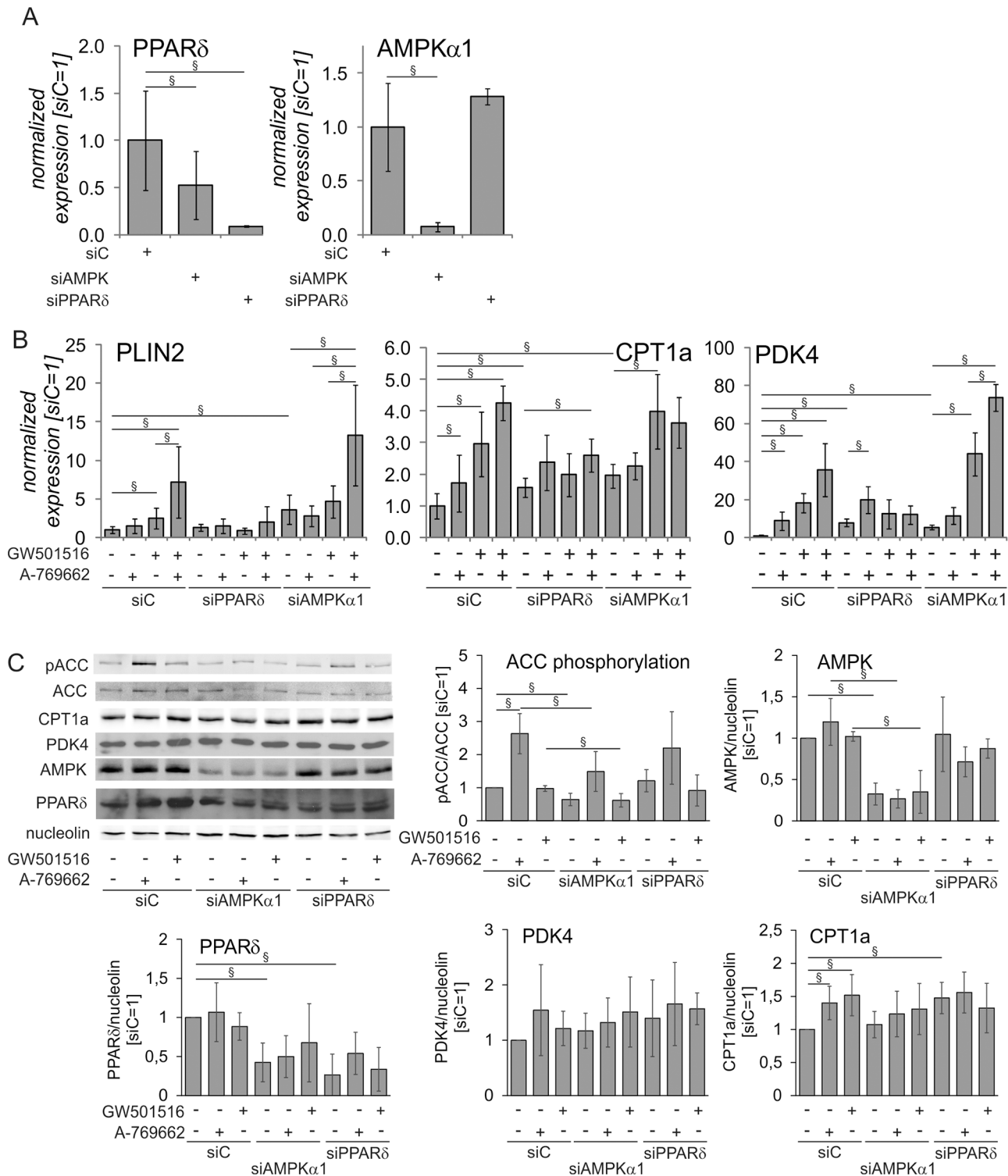


Fig 3. AMPK increased the expression of PPAR δ target genes through PPAR δ . Primary macrophages were transfected with 50 nM siControl (siC), AMPK α 1 or PPAR δ siRNA for 72 hours and treated with 250 μ M A-769662 or 100 nM GW501516 for additional 24 hours. **A** mRNA expression of PPAR δ and AMPK α 1. **B** mRNA expression of PDK4, CPT1a, and PLIN2 in macrophages treated as indicated above. **C** Protein expression of phospho-ACC, ACC, PDK4, CPT1a, PPAR δ , and AMPK α 1 in macrophages treated as indicated above. Values represent averages \pm 95% Confidence Interval. \S , $p < 0.05$ ($n = 5-8$).

doi:10.1371/journal.pone.0130893.g003

PDK4 or CPT1a. However, we did not observe any effect of A-769662 on PPAR δ mRNA (data not shown). We also did not notice an effect of A-769662 treatment on nuclear PPAR δ levels (S3 Fig).

Interestingly, Western analysis revealed only small changes of CPT1a or PDK4 protein expression under these experimental settings. Whereas we observed small, but significant increases of CPT1a protein after A-769 or GW501516-treatment in siControl-transfected macrophages, no differences were observed in AMPK α 1 or PPAR δ knockdown cells (Fig 3C), and siPPAR δ -transfected cells also showed increased basal expression of CPT1a protein, reflecting, at least in part, mRNA data. For PDK4 similar tendencies were observed, but the changes did not reach statistical significance.

Collectively, these observations indicate that PPAR δ mediates the effect of A-769662 and FAO-related target gene expression. Furthermore, AMPK may be involved in maintaining PPAR δ expression through regulation of PPAR δ mRNA. Surprisingly, changes in mRNA expression accompanying AMPK α or PPAR δ knock-down are much less pronounced at the protein level for the PPAR δ targets CPT1a and PDK4, indicating the discordance of mRNA and protein regulation of FAO genes in our experimental system.

Discussion

Studies in mice have shown that simultaneous AMPK and PPAR δ activation increased running endurance due to cooperative induction of oxidative and fatty acid metabolism in skeletal muscle [28]. As AMPK senses energy levels of the whole body we asked, if AMPK overexpression or activation can cooperate with the lipid metabolism regulator PPAR δ and alter the expression of genes associated with fatty acid metabolism in human macrophages. Increased fatty acid catabolism might be beneficial under conditions of fatty acid oversupply associated with the metabolic syndrome [39]. Our results indicate that AMPK and PPAR δ additively induce mRNA expression of a subset of fatty acid metabolism-related genes in primary human macrophages. We also show that the effect of AMPK on these genes is PPAR δ -dependent. Despite robust changes of mRNA expression, the expression of proteins associated with FAO was only modestly enhanced by PPAR δ /AMPK co-activation, and AMPK activation failed to further increase FAO or to prevent VLDL-induced TG accumulation in macrophages with activated PPAR δ . Apparently, changes of the transcriptome alone may not be sufficient or are not pronounced enough to alter macrophage metabolic phenotype towards enhanced lipid catabolism.

For an unbiased approach to search for AMPK- and PPAR δ -dependent genes, we performed microarray and quantitative PCR analyses of human primary macrophages. Cells were lentivirally transduced with a constitutively active construct coding for a truncated human AMPK α 1 carrying mutation of Thr172 to an aspartic acid residue (T172D), treated with the PPAR δ agonist GW501516, or the combination of both stimuli. Previous studies have shown that the T172D substitution in AMPK α resulted in about 50% increased activity of the kinase complex in comparison with the wild-type enzyme [40].

Our microarray analysis revealed FAO, pyruvate metabolism, TCA cycle, processes regulating kinesin binding, oxidative stress response, and ATP biosynthesis as the most affected pathways after combined PPAR δ and AMPK activation. Addressing genes showing highest regulation in the FAO pathway we confirmed increased expression of PDK4, CPT1a, and PLIN2 mRNAs. Similar results were obtained when overexpressing the regulatory AMPK γ 1 subunit with activating R70Q mutation. Furthermore, we observed that the same transcripts were up-regulated after pharmacological activation of AMPK using the direct allosteric activators A-769662 or salicylate [26, 37]. PDK4 is a mitochondrial kinase, which phosphorylates and inactivates pyruvate dehydrogenase, inhibiting the formation of glucose-derived acetyl-

CoA. In tissues with high metabolic flexibility, such as skeletal muscle, increased PDK4 expression is a major regulatory switch, reducing glucose oxidation in favor of increased β -oxidation [41, 42]. CPT1a is a rate-limiting enzyme of FAO transferring long-chain acyl-CoA into mitochondria and serves as a key regulatory enzyme of β -oxidation [39]. In addition, PLIN2, a typical PPAR δ target protein associated with cytosolic lipid droplet stabilization [43], was additively induced by AMPK and PPAR δ .

Although we observed robustly increased mRNA levels of PDK4 and CPT1a (Figs 1B and 2B), only small differences appeared at the protein level (Figs 2C and 3C). This may reflect the known ability of AMPK to generally suppress protein synthesis by interfering with the mTOR pathway, which we confirmed in our system (Fig 2A) [8]. Furthermore, while PPAR δ activation induced a moderate increase of FAO, this was not seen with AMPK activation (Fig 2D). Similarly, suppressing triglyceride accumulation by GW501516 is not significantly enhanced by AMPK activation (Fig 2E). Apparently, the small increase of CPT1a and PDK4 protein expression observed after combined AMPK/PPAR δ activation does not translate to the enhancement of FAO or triglyceride lowering achieved by activation of PPAR δ alone. It should be noted that FAO is governed not only by amounts of the corresponding enzymes or substrates, but also to a major extent by the ATP demand. This may explain our observations that dramatic increases of CPT1a protein expression were accompanied by only a 2-fold increase in FAO in CPT1a-overexpressing THP-1 macrophages [39]. Since the major outcome of pharmacological AMPK activation in the absence of falling ATP levels is the suppression of ATP-consuming processes, this may decrease ATP demand and negatively affect ATP generation, including that provided by FAO. The complexity of FAO regulation has been recently illustrated by a study of mutated ACC double knock-in mice where changes of a major allosteric FAO modulator malonyl-CoA had no impact on cardiac FAO rate [44]. The insensitivity of FAO to malonyl-CoA levels may also apply to our system. While observing robust changes of phospho-ACC after AMPK activation, we fail to observe a significant impact on FAO. It can be envisioned that strategies aiming at increasing ATP demand, such as increasing energy dissipation through mitochondrial uncoupling [45], may be needed to translate enhanced expression of fatty acid catabolic genes into increased FAO in this setting. Mechanistically, suppression of VLDL-induced TG accumulation in macrophages treated with PPAR δ ligands was attributed to both, enhanced FAO and increased expression of the lipoprotein lipase inhibitor Angptl4 [16, 46]. Although we observed a robust induction of Angptl4 mRNA by GW501516 in THP-1 cells, the same PPAR δ ligand failed to induce Angptl4 in primary human macrophages (S2 Fig), indicating that FAO is the primary pathway for TG reduction in the human system.

FAO has been suggested to promote an anti-inflammatory phenotype of macrophages polarized by IL-4 [47, 48]. Both PPAR δ and AMPK can contribute to anti-inflammatory macrophage polarization [49, 50], however whether increased FAO is critical for anti-inflammatory effects of activated PPAR δ or AMPK is unclear. Our recent observations indicate that FAO is dispensable for IL-4-induced human macrophage polarization [51], suggesting important differences between human and mouse macrophages regarding the impact of metabolism on macrophage phenotype. Thus, FAO may play more important roles to specifically attenuate saturated fatty acid-induced inflammation [25, 39].

Previous data in skeletal muscle suggested that AMPK may directly activate PPAR δ transcriptional activity [28], although mechanistic details remain obscure. Our approach to silence PPAR δ confirmed that the increased mRNA expression of PPAR δ target genes in cells co-activated with AMPK is indeed PPAR δ -mediated (Fig 3). Consistent with previous observations PDK4, CPT1a, and PLIN2 are actively repressed by ligand-free PPAR δ in human macrophages, reflected by their mRNA increase in PPAR δ -depleted cells [38, 52, 53]. Accordingly, AMPK or PPAR δ activation does not influence the expression of these genes after a KD of

PPAR δ , indicating an AMPK impact on FAO-associated genes through PPAR δ . In accordance to our data, recent study showed that reduction of ER stress in vascular cells by AMPK activator metformin was dependent on PPAR δ activity [54]. Interestingly, AMPK α 1 KD data revealed a role for AMPK in control of PPAR δ mRNA and protein expression, although AMPK activation did not affect PPAR δ mRNA, suggesting that the impact of AMPK on PPAR δ expression may be independent of AMPK catalytic activity. However, we do not see the reduction of PPAR δ target gene induction by GW501516 in AMPK α 1 knockdown cells, and we noticed no activation of AMPK substrate phosphorylation by GW501516, indicating that AMPK is not downstream of PPAR δ in our system. Although AMPK-mediated reduction of ER stress in muscle cells by GW501516 has been recently reported [55], this study used high concentration of GW501516 (10 μ M), which changed the cellular AMP/ATP ratio. We also noticed that expression of some PPAR δ target genes, such as ACAA2, ACADVL or FABP4, was not affected by AMPK activation (data not shown), suggesting that modulation of PPAR δ activity may be gene-specific and not through direct PPAR δ activation. Accordingly, no changes of PPAR δ nuclear levels were noticed by us. Similarly, no evidence of direct post-translational modification of PPAR δ by AMPK was found in a previous study investigating the interaction of these proteins in muscle cells [28]. Instead, several alternative mechanisms linking AMPK to altered genes expression may be envisioned, such as modification of histone deacetylases [56].

In summary, our data indicate that activated AMPK increases PPAR δ -dependent expression of a subset of genes involved in fatty acid metabolism, which requires the transcriptional activity of PPAR δ . Potentiation of gene expression on its own is unable to increase FAO and prevent VLDL-induced lipid accumulation, suggesting that additional interventions to increase fatty acid catabolism are needed to therapeutically exploit AMPK/PPAR δ interaction in the macrophages.

Supporting Information

S1 Fig. Effects of salicylate on mRNA expression of PPAR δ target genes. Primary macrophages were stimulated by 100 nM GW501516 and 3 mM salicylate for 24 hours. mRNA expression was analyzed by quantitative PCR. Values represent averages \pm 95% Confidence Interval. *, $p < 0.05$ (n = 5).

(TIF)

S2 Fig. Gene expression of Angptl4 in human macrophages. THP-1 (A) or primary macrophages (B) were stimulated by 100 nM GW501516 for 24 hours. mRNA expression of Angptl4 was analyzed by quantitative PCR. Values represent averages \pm 95% Confidence Interval. *, $p < 0.05$ (n = 4).

(TIF)

S3 Fig. Effect of A-769662 on nuclear PPAR δ levels. Western blotting of PPAR δ in nuclear extracts of primary macrophages stimulated with 500 μ M A-769662 for 24 hours. Values represent averages \pm 95% Confidence Interval.

(TIF)

Acknowledgments

We would like to thank Marcel Boss, Dr. Ryan Snodgrass and Isabelle C. Elschner for experimental support.

Author Contributions

Conceived and designed the experiments: MK BB DN. Performed the experiments: MK FF MFC DN. Analyzed the data: MK FF RM BB DN. Wrote the paper: MK DSPA RM BB DN.

References

1. Nathan DM, Buse JB, Davidson MB, Ferrannini E, Holman RR, Sherwin R, et al. Medical management of hyperglycemia in type 2 diabetes: a consensus algorithm for the initiation and adjustment of therapy: a consensus statement of the American Diabetes Association and the European Association for the Study of Diabetes. *Diabetes care*. 2009; 32(1):193–203. Epub 2008/10/24. doi: [10.2337/dc08-9025](https://doi.org/10.2337/dc08-9025) PMID: [18945920](https://pubmed.ncbi.nlm.nih.gov/18945920/); PubMed Central PMCID: PMC2606813.
2. Ruderman N, Chisholm D, Pi-Sunyer X, Schneider S. The metabolically obese, normal-weight individual revisited. *Diabetes*. 1998; 47(5):699–713. Epub 1998/05/20. PMID: [9588440](https://pubmed.ncbi.nlm.nih.gov/9588440/).
3. Pickup JC. Inflammation and activated innate immunity in the pathogenesis of type 2 diabetes. *Diabetes care*. 2004; 27(3):813–23. Epub 2004/02/28. PMID: [14988310](https://pubmed.ncbi.nlm.nih.gov/14988310/).
4. Eckel RH, Grundy SM, Zimmet PZ. The metabolic syndrome. *Lancet*. 2005; 365(9468):1415–28. Epub 2005/04/20. doi: [10.1016/S0140-6736\(05\)66378-7](https://doi.org/10.1016/S0140-6736(05)66378-7) PMID: [15836891](https://pubmed.ncbi.nlm.nih.gov/15836891/).
5. Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA, et al. Diagnosis and management of the metabolic syndrome. An American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. Executive summary. *Cardiology in review*. 2005; 13(6):322–7. Epub 2006/05/20. PMID: [16708441](https://pubmed.ncbi.nlm.nih.gov/16708441/).
6. Stern MP, Williams K, Gonzalez-Villalpando C, Hunt KJ, Haffner SM. Does the metabolic syndrome improve identification of individuals at risk of type 2 diabetes and/or cardiovascular disease? *Diabetes care*. 2004; 27(11):2676–81. Epub 2004/10/27. PMID: [15505004](https://pubmed.ncbi.nlm.nih.gov/15505004/).
7. Odegaard JI, Chawla A. The immune system as a sensor of the metabolic state. *Immunity*. 2013; 38(4):644–54. Epub 2013/04/23. doi: [10.1016/j.immuni.2013.04.001](https://doi.org/10.1016/j.immuni.2013.04.001) PMID: [23601683](https://pubmed.ncbi.nlm.nih.gov/23601683/); PubMed Central PMCID: PMC3663597.
8. O'Neill LA, Hardie DG. Metabolism of inflammation limited by AMPK and pseudo-starvation. *Nature*. 2013; 493(7432):346–55. Epub 2013/01/18. doi: [10.1038/nature11862](https://doi.org/10.1038/nature11862) PMID: [23325217](https://pubmed.ncbi.nlm.nih.gov/23325217/).
9. Moore KJ, Tabas I. Macrophages in the pathogenesis of atherosclerosis. *Cell*. 2011; 145(3):341–55. Epub 2011/05/03. doi: [10.1016/j.cell.2011.04.005](https://doi.org/10.1016/j.cell.2011.04.005) PMID: [21529710](https://pubmed.ncbi.nlm.nih.gov/21529710/); PubMed Central PMCID: PMC3111065.
10. Odegaard JI, Chawla A. Alternative macrophage activation and metabolism. *Annual review of pathology*. 2011; 6:275–97. Epub 2010/11/03. doi: [10.1146/annurev-pathol-011110-130138](https://doi.org/10.1146/annurev-pathol-011110-130138) PMID: [21034223](https://pubmed.ncbi.nlm.nih.gov/21034223/); PubMed Central PMCID: PMC3381938.
11. Chawla A. Control of macrophage activation and function by PPARs. *Circulation research*. 2010; 106(10):1559–69. Epub 2010/05/29. doi: [10.1161/CIRCRESAHA.110.216523](https://doi.org/10.1161/CIRCRESAHA.110.216523) PMID: [20508200](https://pubmed.ncbi.nlm.nih.gov/20508200/); PubMed Central PMCID: PMC2897247.
12. Peters JM, Shah YM, Gonzalez FJ. The role of peroxisome proliferator-activated receptors in carcinogenesis and chemoprevention. *Nature reviews Cancer*. 2012; 12(3):181–95. Epub 2012/02/10. doi: [10.1038/nrc3214](https://doi.org/10.1038/nrc3214) PMID: [22318237](https://pubmed.ncbi.nlm.nih.gov/22318237/); PubMed Central PMCID: PMC3322353.
13. Adhikary T, Brandt DT, Kaddatz K, Stockert J, Naruhn S, Meissner W, et al. Inverse PPARbeta/delta agonists suppress oncogenic signaling to the ANGPTL4 gene and inhibit cancer cell invasion. *Oncogene*. 2013; 32(44):5241–52. Epub 2012/12/05. doi: [10.1038/onc.2012.549](https://doi.org/10.1038/onc.2012.549) PMID: [23208498](https://pubmed.ncbi.nlm.nih.gov/23208498/); PubMed Central PMCID: PMC3938163.
14. Coll T, Rodriguez-Calvo R, Barroso E, Serrano L, Eyre E, Palomer X, et al. Peroxisome proliferator-activated receptor (PPAR) beta/delta: a new potential therapeutic target for the treatment of metabolic syndrome. *Current molecular pharmacology*. 2009; 2(1):46–55. Epub 2009/12/22. PMID: [20021445](https://pubmed.ncbi.nlm.nih.gov/20021445/).
15. Chawla A, Lee CH, Barak Y, He W, Rosenfeld J, Liao D, et al. PPARdelta is a very low-density lipoprotein sensor in macrophages. *Proceedings of the National Academy of Sciences of the United States of America*. 2003; 100(3):1268–73. Epub 2003/01/24. doi: [10.1073/pnas.0337331100](https://doi.org/10.1073/pnas.0337331100) PMID: [12540828](https://pubmed.ncbi.nlm.nih.gov/12540828/); PubMed Central PMCID: PMC298762.
16. Bojic LA, Sawyez CG, Telford DE, Edwards JY, Hegele RA, Huff MW. Activation of peroxisome proliferator-activated receptor delta inhibits human macrophage foam cell formation and the inflammatory response induced by very low-density lipoprotein. *Arterioscler Thromb Vasc Biol*. 2012; 32(12):2919–28. Epub 2012/10/02. doi: [10.1161/ATVBAHA.112.255208](https://doi.org/10.1161/ATVBAHA.112.255208) PMID: [23023367](https://pubmed.ncbi.nlm.nih.gov/23023367/).
17. Namgaladze D, Morbitzer D, von Knethen A, Brune B. Phospholipase A2-modified low-density lipoprotein activates macrophage peroxisome proliferator-activated receptors. *Arterioscler Thromb Vasc Biol*. 2010; 30(2):313–20. Epub 2009/12/02. doi: [10.1161/ATVBAHA.109.199232](https://doi.org/10.1161/ATVBAHA.109.199232) PMID: [19948841](https://pubmed.ncbi.nlm.nih.gov/19948841/).

18. Barish GD, Atkins AR, Downes M, Olson P, Chong LW, Nelson M, et al. PPARdelta regulates multiple proinflammatory pathways to suppress atherosclerosis. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105(11):4271–6. Epub 2008/03/14. doi: [10.1073/pnas.0711875105](https://doi.org/10.1073/pnas.0711875105) PMID: [18337509](https://pubmed.ncbi.nlm.nih.gov/18337509/); PubMed Central PMCID: PMC2393796.
19. Graham TL, Mookherjee C, Suckling KE, Palmer CN, Patel L. The PPARdelta agonist GW0742X reduces atherosclerosis in LDLR(-/-) mice. *Atherosclerosis*. 2005; 181(1):29–37. Epub 2005/06/09. doi: [10.1016/j.atherosclerosis.2004.12.028](https://doi.org/10.1016/j.atherosclerosis.2004.12.028) PMID: [15939051](https://pubmed.ncbi.nlm.nih.gov/15939051/).
20. Bojic LA, Burke AC, Chhoker SS, Telford DE, Sutherland BG, Edwards JY, et al. Peroxisome Proliferator-Activated Receptor delta Agonist GW1516 Attenuates Diet-Induced Aortic Inflammation, Insulin Resistance, and Atherosclerosis in Low-Density Lipoprotein Receptor Knockout Mice. *Arterioscler Thromb Vasc Biol*. 2014; 34(1):52–60. Epub 2013/10/26. doi: [10.1161/ATVBAHA.113.301830](https://doi.org/10.1161/ATVBAHA.113.301830) PMID: [24158519](https://pubmed.ncbi.nlm.nih.gov/24158519/).
21. Lee CH, Kang K, Mehl IR, Nofsinger R, Alaynick WA, Chong LW, et al. Peroxisome proliferator-activated receptor delta promotes very low-density lipoprotein-derived fatty acid catabolism in the macrophage. *Proc Natl Acad Sci U S A*. 2006; 103(7):2434–9. Epub 2006/02/10. 0510815103 [pii] doi: [10.1073/pnas.0510815103](https://doi.org/10.1073/pnas.0510815103) PMID: [16467150](https://pubmed.ncbi.nlm.nih.gov/16467150/); PubMed Central PMCID: PMC1413732.
22. Wang YX, Lee CH, Tiep S, Yu RT, Ham J, Kang H, et al. Peroxisome-proliferator-activated receptor delta activates fat metabolism to prevent obesity. *Cell*. 2003; 113(2):159–70. Epub 2003/04/23. doi: [10.1016/j.cell.2003.02.011](https://doi.org/10.1016/j.cell.2003.02.011) PMID: [12705865](https://pubmed.ncbi.nlm.nih.gov/12705865/)
23. Tanaka T, Yamamoto J, Iwasaki S, Asaba H, Hamura H, Ikeda Y, et al. Activation of peroxisome proliferator-activated receptor delta induces fatty acid beta-oxidation in skeletal muscle and attenuates metabolic syndrome. *Proceedings of the National Academy of Sciences of the United States of America*. 2003; 100(26):15924–9. Epub 2003/12/17. doi: [10.1073/pnas.0306981100](https://doi.org/10.1073/pnas.0306981100) PMID: [14676330](https://pubmed.ncbi.nlm.nih.gov/14676330/); PubMed Central PMCID: PMC307669.
24. Muoio DM, Seefeld K, Witters LA, Coleman RA. AMP-activated kinase reciprocally regulates triacylglycerol synthesis and fatty acid oxidation in liver and muscle: evidence that sn-glycerol-3-phosphate acyltransferase is a novel target. *The Biochemical journal*. 1999; 338 (Pt 3):783–91. Epub 1999/03/03. PMID: [10051453](https://pubmed.ncbi.nlm.nih.gov/10051453/); PubMed Central PMCID: PMC1220117.
25. Galic S, Fullerton MD, Schertzer JD, Sikkema S, Marcinko K, Walkley CR, et al. Hematopoietic AMPK beta1 reduces mouse adipose tissue macrophage inflammation and insulin resistance in obesity. *The Journal of clinical investigation*. 2011; 121(12):4903–15. Epub 2011/11/15. doi: [10.1172/JCI58577](https://doi.org/10.1172/JCI58577) PMID: [22080866](https://pubmed.ncbi.nlm.nih.gov/22080866/); PubMed Central PMCID: PMC3226000.
26. Goransson O, McBride A, Hawley SA, Ross FA, Shpiro N, Foretz M, et al. Mechanism of action of A-769662, a valuable tool for activation of AMP-activated protein kinase. *The Journal of biological chemistry*. 2007; 282(45):32549–60. Epub 2007/09/15. doi: [10.1074/jbc.M706536200](https://doi.org/10.1074/jbc.M706536200) PMID: [17855357](https://pubmed.ncbi.nlm.nih.gov/17855357/); PubMed Central PMCID: PMC2156105.
27. Cool B, Zinker B, Chiou W, Kifle L, Cao N, Perham M, et al. Identification and characterization of a small molecule AMPK activator that treats key components of type 2 diabetes and the metabolic syndrome. *Cell metabolism*. 2006; 3(6):403–16. Epub 2006/06/07. doi: [10.1016/j.cmet.2006.05.005](https://doi.org/10.1016/j.cmet.2006.05.005) PMID: [16753576](https://pubmed.ncbi.nlm.nih.gov/16753576/).
28. Narkar VA, Downes M, Yu RT, Emblar E, Wang YX, Banayo E, et al. AMPK and PPARdelta agonists are exercise mimetics. *Cell*. 2008; 134(3):405–15. Epub 2008/08/05. doi: [10.1016/j.cell.2008.06.051](https://doi.org/10.1016/j.cell.2008.06.051) PMID: [18674809](https://pubmed.ncbi.nlm.nih.gov/18674809/); PubMed Central PMCID: PMC2706130.
29. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, et al. Bioconductor: open software development for computational biology and bioinformatics. *Genome biology*. 2004; 5(10):R80. Epub 2004/10/06. doi: [10.1186/gb-2004-5-10-r80](https://doi.org/10.1186/gb-2004-5-10-r80) PMID: [15461798](https://pubmed.ncbi.nlm.nih.gov/15461798/); PubMed Central PMCID: PMC545600.
30. Dunning MJ, Smith ML, Ritchie ME, Tavare S. beadarray: R classes and methods for Illumina bead-based data. *Bioinformatics*. 2007; 23(16):2183–4. Epub 2007/06/26. doi: [10.1093/bioinformatics/btm311](https://doi.org/10.1093/bioinformatics/btm311) PMID: [17586828](https://pubmed.ncbi.nlm.nih.gov/17586828/).
31. Smyth GK, Michaud J, Scott HS. Use of within-array replicate spots for assessing differential expression in microarray experiments. *Bioinformatics*. 2005; 21(9):2067–75. Epub 2005/01/20. doi: [10.1093/bioinformatics/bti270](https://doi.org/10.1093/bioinformatics/bti270) PMID: [15657102](https://pubmed.ncbi.nlm.nih.gov/15657102/).
32. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences of the United States of America*. 2005; 102(43):15545–50. Epub 2005/10/04. doi: [10.1073/pnas.0506580102](https://doi.org/10.1073/pnas.0506580102) PMID: [16199517](https://pubmed.ncbi.nlm.nih.gov/16199517/); PubMed Central PMCID: PMC1239896.
33. Cerami EG, Gross BE, Demir E, Rodchenkov I, Babur O, Anwar N, et al. Pathway Commons, a web resource for biological pathway data. *Nucleic acids research*. 2011; 39(Database issue):D685–90.

- Epub 2010/11/13. doi: [10.1093/nar/gkq1039](https://doi.org/10.1093/nar/gkq1039) PMID: [21071392](https://pubmed.ncbi.nlm.nih.gov/21071392/); PubMed Central PMCID: PMC3013659.
34. Flicek P, Ahmed I, Amode MR, Barrell D, Beal K, Brent S, et al. Ensembl 2013. *Nucleic acids research*. 2013; 41(Database issue):D48–55. Epub 2012/12/04. doi: [10.1093/nar/gks1236](https://doi.org/10.1093/nar/gks1236) PMID: [23203987](https://pubmed.ncbi.nlm.nih.gov/23203987/); PubMed Central PMCID: PMC3531136.
 35. Folmes KD, Witters LA, Allard MF, Young ME, Dyck JR. The AMPK gamma1 R70Q mutant regulates multiple metabolic and growth pathways in neonatal cardiac myocytes. *American journal of physiology Heart and circulatory physiology*. 2007; 293(6):H3456–64. Epub 2007/10/02. doi: [10.1152/ajpheart.00936.2007](https://doi.org/10.1152/ajpheart.00936.2007) PMID: [17906100](https://pubmed.ncbi.nlm.nih.gov/17906100/).
 36. Hamilton SR, Stapleton D, O'Donnell JB Jr., Kung JT, Dalal SR, Kemp BE, et al. An activating mutation in the gamma1 subunit of the AMP-activated protein kinase. *FEBS letters*. 2001; 500(3):163–8. Epub 2001/07/11. PMID: [11445078](https://pubmed.ncbi.nlm.nih.gov/11445078/).
 37. Hawley SA, Fullerton MD, Ross FA, Schertzer JD, Chevzoff C, Walker KJ, et al. The ancient drug salicylate directly activates AMP-activated protein kinase. *Science*. 2012; 336(6083):918–22. Epub 2012/04/21. doi: [10.1126/science.1215327](https://doi.org/10.1126/science.1215327) PMID: [22517326](https://pubmed.ncbi.nlm.nih.gov/22517326/); PubMed Central PMCID: PMC3399766.
 38. Lee CH, Chawla A, Urbiztondo N, Liao D, Boisvert WA, Evans RM, et al. Transcriptional repression of atherogenic inflammation: modulation by PPARdelta. *Science*. 2003; 302(5644):453–7. Epub 2003/09/13. doi: [10.1126/science.1087344](https://doi.org/10.1126/science.1087344) PMID: [12970571](https://pubmed.ncbi.nlm.nih.gov/12970571/).
 39. Namgaladze D, Lips S, Leiker TJ, Murphy RC, Ekroos K, Ferreiros N, et al. Inhibition of macrophage fatty acid beta-oxidation exacerbates palmitate-induced inflammatory and endoplasmic reticulum stress responses. *Diabetologia*. 2014. Epub 2014/02/04. doi: [10.1007/s00125-014-3173-4](https://doi.org/10.1007/s00125-014-3173-4) PMID: [24488024](https://pubmed.ncbi.nlm.nih.gov/24488024/).
 40. Stein SC, Woods A, Jones NA, Davison MD, Carling D. The regulation of AMP-activated protein kinase by phosphorylation. *The Biochemical journal*. 2000; 345 Pt 3:437–43. Epub 2000/01/22. PMID: [10642499](https://pubmed.ncbi.nlm.nih.gov/10642499/); PubMed Central PMCID: PMC1220775.
 41. Zhang S, Hulver MW, McMillan RP, Cline MA, Gilbert ER. The pivotal role of pyruvate dehydrogenase kinases in metabolic flexibility. *Nutrition & metabolism*. 2014; 11(1):10. Epub 2014/02/14. doi: [10.1186/1743-7075-11-10](https://doi.org/10.1186/1743-7075-11-10) PMID: [24520982](https://pubmed.ncbi.nlm.nih.gov/24520982/); PubMed Central PMCID: PMC3925357.
 42. Nakamura MT, Yudell BE, Loor JJ. Regulation of energy metabolism by long-chain fatty acids. *Progress in lipid research*. 2014; 53:124–44. Epub 2013/12/24. doi: [10.1016/j.plipres.2013.12.001](https://doi.org/10.1016/j.plipres.2013.12.001) PMID: [24362249](https://pubmed.ncbi.nlm.nih.gov/24362249/).
 43. Brasaemle DL, Barber T, Wolins NE, Serrero G, Blanchette-Mackie EJ, Londos C. Adipose differentiation-related protein is an ubiquitously expressed lipid storage droplet-associated protein. *Journal of lipid research*. 1997; 38(11):2249–63. Epub 1997/12/10. PMID: [9392423](https://pubmed.ncbi.nlm.nih.gov/9392423/).
 44. Zordoky BN, Nagendran J, Pulinilkunnil T, Kienesberger PC, Masson G, Waller TJ, et al. AMPK-Dependent Inhibitory Phosphorylation of ACC Is Not Essential for Maintaining Myocardial Fatty Acid Oxidation. *Circulation research*. 2014; 115(5):518–24. Epub 2014/07/09. doi: [10.1161/CIRCRESAHA.115.304538](https://doi.org/10.1161/CIRCRESAHA.115.304538) PMID: [25001074](https://pubmed.ncbi.nlm.nih.gov/25001074/).
 45. Li B, Nolte LA, Ju JS, Han DH, Coleman T, Holloszy JO, et al. Skeletal muscle respiratory uncoupling prevents diet-induced obesity and insulin resistance in mice. *Nat Med*. 2000; 6(10):1115–20. Epub 2000/10/04. doi: [10.1038/80450](https://doi.org/10.1038/80450) PMID: [11017142](https://pubmed.ncbi.nlm.nih.gov/11017142/).
 46. Makoveichuk E, Sukonina V, Kroupa O, Thulin P, Ehrenborg E, Olivecrona T, et al. Inactivation of lipoprotein lipase occurs on the surface of THP-1 macrophages where oligomers of angiopoietin-like protein 4 are formed. *Biochemical and biophysical research communications*. 2012; 425(2):138–43. Epub 2012/07/24. doi: [10.1016/j.bbrc.2012.07.048](https://doi.org/10.1016/j.bbrc.2012.07.048) PMID: [22820186](https://pubmed.ncbi.nlm.nih.gov/22820186/).
 47. Vats D, Mukundan L, Odegaard JI, Zhang L, Smith KL, Morel CR, et al. Oxidative metabolism and PGC-1beta attenuate macrophage-mediated inflammation. *Cell metabolism*. 2006; 4(1):13–24. Epub 2006/07/04. doi: [10.1016/j.cmet.2006.05.011](https://doi.org/10.1016/j.cmet.2006.05.011) PMID: [16814729](https://pubmed.ncbi.nlm.nih.gov/16814729/); PubMed Central PMCID: PMC1904486.
 48. Huang SC, Everts B, Ivanova Y, O'Sullivan D, Nascimento M, Smith AM, et al. Cell-intrinsic lysosomal lipolysis is essential for alternative activation of macrophages. *Nat Immunol*. 2014; 15(9):846–55. Epub 2014/08/05. doi: [10.1038/ni.2956](https://doi.org/10.1038/ni.2956) PMID: [25086775](https://pubmed.ncbi.nlm.nih.gov/25086775/); PubMed Central PMCID: PMC4139419.
 49. Kang K, Reilly SM, Karabacak V, Gangl MR, Fitzgerald K, Hatano B, et al. Adipocyte-derived Th2 cytokines and myeloid PPARdelta regulate macrophage polarization and insulin sensitivity. *Cell metabolism*. 2008; 7(6):485–95. Epub 2008/06/05. doi: [10.1016/j.cmet.2008.04.002](https://doi.org/10.1016/j.cmet.2008.04.002) PMID: [18522830](https://pubmed.ncbi.nlm.nih.gov/18522830/); PubMed Central PMCID: PMC2586840.
 50. Mounier R, Theret M, Arnold L, Cuvellier S, Bultot L, Goransson O, et al. AMPKalpha1 regulates macrophage skewing at the time of resolution of inflammation during skeletal muscle regeneration. *Cell metabolism*. 2013; 18(2):251–64. Epub 2013/08/13. doi: [10.1016/j.cmet.2013.06.017](https://doi.org/10.1016/j.cmet.2013.06.017) PMID: [23931756](https://pubmed.ncbi.nlm.nih.gov/23931756/).

51. Namgaladze D, Brune B. Fatty acid oxidation is dispensable for human macrophage IL-4-induced polarization. *Biochim Biophys Acta*. 2014; 1841(9):1329–35. Epub 2014/06/25. doi: [10.1016/j.bbaliip.2014.06.007](https://doi.org/10.1016/j.bbaliip.2014.06.007) PMID: [24960101](https://pubmed.ncbi.nlm.nih.gov/24960101/).
52. Shi Y, Hon M, Evans RM. The peroxisome proliferator-activated receptor delta, an integrator of transcriptional repression and nuclear receptor signaling. *Proceedings of the National Academy of Sciences of the United States of America*. 2002; 99(5):2613–8. Epub 2002/02/28. doi: [10.1073/pnas.052707099](https://doi.org/10.1073/pnas.052707099) PMID: [11867749](https://pubmed.ncbi.nlm.nih.gov/11867749/); PubMed Central PMCID: PMC122396.
53. Lee CH, Kang K, Mehl IR, Nofsinger R, Alaynick WA, Chong LW, et al. Peroxisome proliferator-activated receptor delta promotes very low-density lipoprotein-derived fatty acid catabolism in the macrophage. *Proceedings of the National Academy of Sciences of the United States of America*. 2006; 103(7):2434–9. Epub 2006/02/10. doi: [10.1073/pnas.0510815103](https://doi.org/10.1073/pnas.0510815103) PMID: [16467150](https://pubmed.ncbi.nlm.nih.gov/16467150/); PubMed Central PMCID: PMC1413732.
54. Cheang WS, Tian XY, Wong WT, Lau CW, Lee SS, Chen ZY, et al. Metformin protects endothelial function in diet-induced obese mice by inhibition of endoplasmic reticulum stress through 5' adenosine monophosphate-activated protein kinase-peroxisome proliferator-activated receptor delta pathway. *Arterioscler Thromb Vasc Biol*. 2014; 34(4):830–6. Epub 2014/02/01. doi: [10.1161/ATVBAHA.113.301938](https://doi.org/10.1161/ATVBAHA.113.301938) PMID: [24482374](https://pubmed.ncbi.nlm.nih.gov/24482374/).
55. Salvado L, Barroso E, Gomez-Foix AM, Palomer X, Michalik L, Wahli W, et al. PPARbeta/delta prevents endoplasmic reticulum stress-associated inflammation and insulin resistance in skeletal muscle cells through an AMPK-dependent mechanism. *Diabetologia*. 2014; 57(10):2126–35. Epub 2014/07/27. doi: [10.1007/s00125-014-3331-8](https://doi.org/10.1007/s00125-014-3331-8) PMID: [25063273](https://pubmed.ncbi.nlm.nih.gov/25063273/).
56. Mihaylova MM, Vasquez DS, Ravnskjaer K, Denechaud PD, Yu RT, Alvarez JG, et al. Class IIa histone deacetylases are hormone-activated regulators of FOXO and mammalian glucose homeostasis. *Cell*. 2011; 145(4):607–21. Epub 2011/05/14. doi: [10.1016/j.cell.2011.03.043](https://doi.org/10.1016/j.cell.2011.03.043) PMID: [21565617](https://pubmed.ncbi.nlm.nih.gov/21565617/); PubMed Central PMCID: PMC3117637.