ACBP Knockdown Leads to Down-regulation of Genes Encoding Rate-limiting Enzymes in Cholesterol and Fatty Acid Metabolism

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Key Words

RNAi • ACBP • HepG2 cells • Fatty acids • Cholesterol metabolism

Abstract

The human Acyl-CoA binding protein (ACBP) is a structural and functional highly conserved protein. As an intracellular pool former and carrier of acyl-CoAs, ACBP influences overall lipid metabolism. Its nuclear abundance and physical interaction with hepatocyte nuclear factor 4 α suggested a gene regulatory function of ACBP. To identify ACBP target genes we performed genome-wide transcript profiling under siRNA-mediated ACBP knockdown in human liver HepG2 cells. Based on a single sided permutation T-test ($p < 0.05$) we identified 256 down-regulated and 198 up-regulated transcripts with a minimal fold change of 1.32 (log 0.5). Gene annotation enrichment analysis revealed ACBP-mediated down-regulation of 18 genes encoding key enzymes in glycerolipid (i.e. mitochondrial glycerol-3-phosphate acyltransferase), cholesterol (i.e. HMG-CoA synthase and HMG-CoA reductase) and fatty acid (i.e. fatty acid synthase) metabolism. Integration of these genes in common pathways suggested decreased lipid biosynthesis.

Accordingly, saturated (16:0) and monosaturated (16:1, 18:1) fatty acids were significantly reduced to 75% in ACBP-depleted cells. Taken together, we obtained evidence that ACBP functions in lipid metabolism at the level of gene expression. This effect seems to be translated into certain metabolites. The identified 454 ACBP regulated genes present a first reference for further studies to define the ACBP regulon in mammalian cells.

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Introduction

The human Acyl-CoA binding protein (ACBP) is a highly conserved protein, which can be found in nearly all eukaryotes. It is a typical housekeeping gene, although its expression varies between different cells and tissues [1]. ACBP possesses a number of important physiological and biochemical functions, such as inhibition of diazepam binding to benzodiazepine binding site of gamma-aminobutyric acid-ergic (GABA)-receptor [2, 3], regulation of glucose-induced insulin secretion from pancreatic beta-cells [4, 5], release of cholecystokinin (CCK) in intestine [6], stimulation of steroidogenesis

through peripheral-type benzodiazepine receptor (PBR) [7-9] and modulation of cell proliferation [10]. ACBP seems also to be part of the calcium-dependent proteolytic system through the activation of calpains [11]. In this respect ACBP seems to be involved in pro-apoptotic processes and cell death [12].

ACBPs participation in acyl-CoA metabolism is well established and supported by its ability to bind and sequester long chain acyl-CoAs (LCFACoA) with high affinity [13]. This suggests that ACBP functions as intracellular carrier and pool former of LCFACoA. Thereby, ACBP protects membranes and transport functions as well as enzymes against LCFACoAs detergent and inhibitory effects [14-17]. Furthermore, ACBP mediates intermembrane transport of acyl-CoAs by displacing them from membranes and donating them to utilizing enzymes, i.e. β -oxidation in mitochondria, microsomal glycerolipid synthesis and cholesteryl ester acylation [18-20]. Yeast studies applying *Acb1p* (the yeast homologue of ACBP) overexpression or knockout, substantiate the proteins function in overall lipid metabolism. Especially knockout of ACBP evoked profound changes in membrane structure, assembly and organization [21]. Loss of ACBP in mice causes defective cutaneous fatty acid metabolism [22].

ACBP is localized throughout the cytosol and at special cell organelles as Golgi apparatus or endoplasmic reticulum [23], in nucleus and in the perinuclear area [24]. Except for a distinct nuclear localization during adipocyte differentiation [25], a functional relevance for ACBPs nuclear abundance is not known. A direct impact of ACBP on gene regulation was shown by Petrescu et al., which reported physical and functional interaction with the hepatocyte nuclear factor (HNF)-4 α [24]. Expression profiling in *Acb1p* yeast knockout strain revealed transcriptional changes which were however not complementable by an *Acp1p* mutant unable to bind acyl-CoA esters. This supports finally the idea of ligand-mediated impact of ACBP on gene expression [26].

Here, a genome-wide expression profiling approach was applied in HepG2 cells to identify human ACBP target genes and their functional allocation. Therefore we performed siRNA mediated knockdown of ACBP and the resulting changes in gene expression were assessed with Affymetrix microarray technology. Our study shows, that ACBP-depletion in HepG2 cells reduces the transcript levels of central rate-limiting enzymes of cholesterol, fatty acid as well as glycerolipid synthesis. Accordingly, the fatty acid content of ACBP-depleted cells was also reduced.

Materials and Methods

Cell culture

The human hepatocellular carcinoma cell line HepG2 was cultured in RPMI-Medium + 1% Glutamax (Invitrogen, Carlsbad, USA) containing 10% fetal calf serum in a 5% CO₂/95% air atmosphere at 37°C. Media was replaced every two days and cells were passaged at subconfluency with 0.25% Trypsin in PBS to maintain exponential growth.

siRNA design

Two ACBP specific stability enhanced twenty-one-nucleotide short interfering RNAs (siRNAs), were purchased from Dharmacon Research (Lafayette, USA). siRNA1 (5'- GAA AAAATA CGG GAT ATG A -3'), located in exon 4 at 246-264 bp relative to ATG start codon 1A, was designed according to Reynolds design criteria [27] and in consideration of RNA secondary structure. To assess secondary structure of ACBP mRNA (NM_001079862) we used the Mfold web server [28]. siRNA2 (5'- GGA AGA TGC CAT GAA AGC T -3') located in exon 4 at 201-219 bp relative to ATG start codon was according to Faergeman et al. [29]. Sequence information about ACBP was taken from GenBank (NCBI, <http://www.ncbi.nlm.nih.gov>). To avoid off-targeting effects of the designed siRNAs, sequence comparisons of the ACBP protein family and ACBP domain containing proteins were done using web based alignment tools at NCBI and EBI (<http://www.ebi.ac.uk>). Control siRNAs targeted to Lamin A/C, Cyclophilin B and a stabilized scrambled siRNA were also obtained from Dharmacon Research Inc.

Transient Transfection

For transfection experiments 1.1x10⁵ cells were seeded in 24-well plates (Sarstedt, Newton, USA). Cells were transfected at 30-40% confluency in a final siRNA concentration of 100 nmol/L with Dharmafect4 transfection reagent (Dharmacon Research, Lafayette, USA) according to the manufacturer's instructions. 24 h post-transfection medium was changed. Cells treated with transfection reagent were used as negative control (mock control).

Transfection efficiency was initially monitored with fluorescein-labeled siRNA (Qiagen, Hilden, Germany) by microscopy.

Real-time RT-PCR analysis

Real-time reverse transcriptase PCR (RT-PCR) was carried out on total RNA from HepG2 cells transfected with ACBP specific or scrambled siRNA after 24 h, 36 h and 48 h on an ABI PRISM 7000 Sequence Detection System instrument (PE Applied Biosystems, Foster City, CA). For RNA isolation RNeasy Kit (Qiagen, Hilden, Germany), for cDNA preparation iScript cDNA synthesis kit (BioRad Laboratories, Hercules, USA) were used. cDNA was diluted 1:5 with RNase-free H₂O before PCR analysis. Differential regulated target genes identified by the expression profiling approach were verified accordingly. Each real-time RT-PCR reaction was done in triplicate according to manufacturer's instructions. Gene expression assays [ACBP (Hs01554584_m1), 3-hydroxy-3-methylglutaryl-CoA synthase (Hs_00266810_m1), 3-hydroxy-

3-methylglutaryl-CoA reductase (Hs_00168352_m1), isopentenyl-diphosphate delta isomerase 1 (Hs_01057440_m1), lanosterol synthase (Hs_01057440_m1), acyl-CoA:cholesterol acyltransferase 2 (Hs_00186048_m1), fatty acid synthase (Hs_00188012_m1), mitochondrial glycerol-3-phosphate acyltransferase (Hs_00326039_m1), peroxisomal trans 2-enoyl CoA reductase (Hs_00218365_m1), early growth response 1 (Hs_00152928_m), 3-hydrox-3-methylglutaryl-CoA lyase (Hs_00609306_m1)] were obtained from ABI. Quantities of target mRNA were determined via standard curves using hypoxanthine phosphoribosyl-transferase 1 (HPRT1) as endogenous control. Two negative controls (-reverse transcriptase/-RNA template) were included to detect possible contaminations.

Cytotoxicity and apoptosis assays

Cytotoxicity was determined 72 h after transfection of HepG2 cells using CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Mannheim, Germany) according to manufacturer's instructions with regard of modified lysis time of 30 min. Luminescence was measured in a GloMax™ microplate luminometer (Promega, Mannheim, Germany) as a signal proportional to the amount of ATP present in metabolically active cells.

Caspase 3 and/or caspase 7 activity was determined 72 h post-transfection using the Caspase-Glo™ 3/7 Assay (Promega, Mannheim, Germany) according to the manufacturer's instructions with prolonged lysis of 30 min. Camptothecin (Sigma Aldrich, Saint Louis, USA) was used as a positive control for apoptosis induction. 16 h before caspase 3/7 measurement cells were incubated with 5 μmol/L Camptothecin. All experiments were performed at least in triplicate.

Western blotting

72-h after transfection of ACBP specific and control siRNAs HepG2 cells were lysed for 10 min at 4°C in 30 μl NET-buffer (50 mM TRIS (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 0.5% NP40 and 3 μl Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany). Homogenates were sonicated for 30 s and incubated for 20 min at 4°C before centrifugation at 14.000 rpm for 20 min. After protein estimation (Bio-Rad Protein Assay, Hercules, USA), samples were suspended in NuPage LDS sample buffer (Invitrogen, Carlsbad, USA) and denaturated at 70°C for 10 min. Samples were loaded on 12% polyacrylamide gel and transferred by electroblotting to a PVDF membrane (Roth, Karlsruhe, Germany).

For immunodetection membranes were probed with specific primary antibodies Ab ACBP_total (1:2000) [30], HMG-CoA synthase 1 (1:1000, Santa Cruz Biotechnology, Santa Cruz, USA), fatty acid synthase (1:1000, Santa Cruz Biotechnology, Santa Cruz, USA), fatty acid binding protein 1 (1:1000, Santa Cruz Biotechnology, Santa Cruz, USA) or actin goat polyclonal antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, USA) followed by horseradish peroxidase-coupled secondary antibody (1:10.000). Immunoreactive bands were visualized using chemiluminescent substrate ChemiGlow and quantified with Alpha Ease FC software (AlphaImager HP, AlphaInnotech, San Leandro, USA).

Gas chromatography

For fatty acid analysis, 4x10⁵ HepG2 cells were plated on 6-well dishes. Transfection of ACBP specific and scrambled control siRNA was performed as described above. Accordingly, 72 h post-transfection cells were trypsinized, pelleted and resuspended in 1 ml 1x PBS. An aliquot was used cell counting and for the determination of protein content as described above (Bio-Rad Protein Assay, Hercules, USA). Until analysis the samples were stored at -80°C. For GC-analysis cells were extracted in dichloromethane:methanol (2:1). Methyl margaric acid (C17:0; 250 μg) was added as internal standard. After incubation for 15 min at 37°C, lipid phase was extracted using a CaCl₂ (0.04%)-solution in HPLC H₂O and dichloromethane/methanol. For preparation of fatty acid methyl esters (FAME) solvents were evaporated under nitrogen, remixed with 1.5 ml methanolic NaOH (0.5 M), and incubated at 105°C for 30 min. After adding 2 ml boron trifluoride, samples were incubated another 35 min at 105°C. 5 ml saturated NaCl-solution and 2 x 1 ml isooctane were added. The upper phase was used for gas chromatography analysis, performed on Hewlett-Packard 6890 gas chromatograph equipped with a J&W 122-2362 DB-23 capillary column (Agilent, Palo Alto, USA) with hydrogen carrier flow rate at 40 ml/min, and air at 450 ml/min. Injection split was 1:20. The following temperature program was used: first temperature was set at 80°C, 25°C/min to 180°C, hold for 2 min, 2°C/min to 182°C, hold for 16 min, 6°C/min to 197°C, hold for 10 min, 40°C/min to 240°C, hold for 2 min, at least 250°C for 10 min. FAMES were detected by a flame ionization detector. Sample peaks were compared with retention times of known standards for specific fatty acid identification.

Microarray experiment

Microarray experiments were performed on the human Genome U133 Plus 2.0 Genechip array (Affymetrix, Santa Clara, USA). For the experiments 4x10⁵ HepG2 cells were seeded in 6-well plates and transfected after 24 h with 100 nmol/L ACBP-specific siRNA1 or scrambled siRNA as control for 72 h. Total RNA was prepared with RNeasy Kit (Qiagen, Hilden, Germany). RNA amount and integrity was assessed spectrophotometrically (BioPhotometer, Eppendorf, Hamburg, Germany) and by using the Bioanalyzer (Agilent, Palo Alto, USA). Experiments were done in triplicate and each approach consisted 3 pooled wells.

Processing and statistical analysis was done by the KFB-Center of Excellence for Fluorescent Bioanalysis (University of Regensburg, Germany) based on Affymetrix protocols (Affymetrix, Santa Clara, USA). The samples were hybridized to the human genome array which contains ~38,500 full-length human genes and EST clusters. Digitized chip image data were processed using Affymetrix GeneChip Analysis Suite software version 5.0 (MAS5.0). Comparison of expression profiles from the scrambled siRNA control (set as baseline) and the ACBP specific siRNA1 transfected sample (designated as the experiment) was done with Genomatix ChipInspector software (Munich, Germany), which includes a single sided permutation T-test. For determination of differentially regulated genes the fold change was set 1.32 and false discovery rate (FDR) rests with 1%. Beside that, only significant detected transcripts

after MAS5.0, as a single-rank call algorithm, were taken for further analysis [31].

Computational analysis

Gene Ontology (GO) functional classification was performed using Database for Annotation, Visualization and Integrated Discovery (DAVID) (www.david.abcc.ncifcrf.gov/home.jsp) [32]. Classification according biological processes and molecular function was performed on GO term level 2. Statistical significant GO terms with a *p*-value lower than 0.05, based on Benjamini-Hochberg correction for multiple testing, were presented. Gene network analysis was carried out using the database of biological networks in Genomatix Biblosphere Pathway Edition (BSPE) system (www.genomatix.de). This text-mining system should identify the putative functional connection between the 454 regulated target genes. Biblosphere is a data mining tool intended to provide gene relationships from literature databases and genome-wide promoter analysis. Over 12 million Pubmed abstracts were scanned by this strategy using >500,000 gene names, synonyms, and the Genomatix proprietary semantic relation concepts [33]. For each term in a hierarchical filter a statistical analysis is performed, based on the number of observed and expected annotation for terms. The Z-score ranking shows whether a certain annotation, or group of annotations, is over- or under-represented in a set of genes. The z-score was calculated as follows $z = (\text{observed} - \text{expected}) / \text{S.D. (observed)}$. In general, z-score should exceed a value of 2.0. The lowest co-citation filter (GFG level G0) based on abstract level was applied. Subsequent sub-analysis was applied by filtering differential regulated genes according to the GO-term of cellular lipid metabolic process. Promoter sequences of selected target genes were adopted from Genomatix Gene2Promoter. Promoter length was adjusted to 600 bp, 500 bp upstream and 100 bp downstream of transcriptional start site (TSS). Promoter sequences were subjected to in silico promoter analysis using Genomatix MatInspector to search for common transcription factor binding sites (TFBS). TFBS were represented by position weight matrices from the Genomatix Family library (Version 7.0) [34, 35].

Statistical analysis

Statistical analysis was performed with GraphPad Prism4 (Graphpad software, Inc., San Diego, USA) using 2-tailed Student's paired t-test. All values were expressed as mean $\pm$ SD. Significant differences were considered for P values less than 0.05.

Results

siRNA-mediated knockdown of ACBP expression in HepG2 cells

For ACBP knockdown we have designed two specific siRNA sequences and analyzed their silencing effect in HepG2 cells. A non-silencing scrambled siRNA served as a negative control. Based on a fluorescence-

Fig. 1. Repression of ACBP transcript- (A) and protein- (B) levels after siRNA transfection. HepG2 cells were transfected with 100 nmol/L of ACBP specific siRNA1, siRNA2 or scrambled siRNA as negative control. A) For qRT-PCR RNA was isolated after 24, 36 and 48 h of transfection. Expression data are relative to HPRT1 as housekeeping gene and presented as mean $\pm$ SD (* p <0.05; n =2-4). B) Samples for western blot analyses were generated 72 h post-transfection. Beta-actin served as loading control. A representative immunoblot is depicted.

Fig. 2. Effects of ACBP knock-down on cell viability (A) and induction of apoptosis (B). HepG2 cells were transfected with 100 nmol/L of ACBP specific siRNAs1, siRNA2 or control siRNAs against lamin A/C, cyclophilin B and scrambled siRNA for 72 h. Cytotoxicity and induction of apoptosis were determined by chemiluminescent measurement. Camptothecin (5 μ mol/L) was incubated for 16 h as a pro-apoptotic agent. Mock control cells were only incubated with transfection reagent. Data are represented relative to scrambled siRNA as mean $\pm$ SD (* p <0.001; n =4-5).

	Gene count	p-value	Benjamini ¹
<i>GO term biological process</i>			
Anatomical structure development	92	2.5E-6	4.1E-4
Regulation of biological process	169	4.6E-6	3.8E-4
Multicellular organismal development	97	5.2E-5	2.8E-4
Cell cycle	47	1.2E-5	4.7E-4
Cell development	59	1.6E-5	5.1E-4
Regulation of cellular processes	156	2.3E-5	6.2E-4
Cellular developmental process	77	4.2E-5	9.8E-4
Cellular component organization and biogenesis	102	1.6E-4	3.2E-3
Death	41	2.0E-4	3.6E-3
Anatomical structure morphogenesis	49	8.0E-4	1.3E-2
Cell cycle process	36	8.0E-4	1.2E-2
Regulation of molecular function	27	3.4E-3	4.5E-2
<i>GO term molecular function</i>			
Protein binding	256	2.2E-13	2.5E-11
Transcription cofactor activity	19	9.7E-4	5.4E-2
Transmembrane transport	43	1.3E-3	4.7E-2

Table 1. Gene Ontology classification of significant regulated genes in ACBP-depleted HepG2 cells according biological processes and molecular function. ¹p-value after Benjamini-Hochberg correction for multiple comparison.

labeled siRNA, transfection efficiency was between 50-70% (data not shown). Using 100 nmol/L ACBP-siRNA1 or -siRNA2, a reduction of ACBP-mRNA levels to 63% and 73% was observed 24 h after transfection, respectively (Fig. 1A). 36 h post-transfection, a reduction of ACBP-mRNA to 39% (siRNA1) and 48% (siRNA2) could be obtained. No further silencing effect was achieved by increasing transfection time to 48 h where ACBP-mRNA levels were decreased to 48% (siRNA1) or 54% (siRNA2). At protein level, both ACBP-siRNAs caused an approximately 80% reduction of ACBP 72 h post- transfection in HepG2 cells (Fig. 1B). Thus, we were able to establish siRNA-mediated knockdown of ACBP at mRNA and protein level in HepG2 cells.

Cytotoxic or pro-apoptotic events are not evoked by ACBP knockdown

Since ACBP is described as a cell death promoting protein [36] we determined whether ACBP knockdown causes cytotoxicity and/or apoptosis in HepG2 cells. For this purpose, 72 h after transfection of ACBP-siRNAs or control siRNAs, cell viability and apoptosis were determined using ATP and caspase 3/7 measurements. As shown in Fig. 2A, transfection of scrambled siRNA, lamin A/C-siRNA, or cyclophilin B-siRNA is not accompanied by cytotoxic effects. Importantly, the cell viability is not perturbed by ACBP knockdown. For apoptotic measurements, the well known pro-apoptotic agent camptothecin serves as a positive control. As shown in Fig. 2B, camptothecin raised caspase 3/7 activity to about 2042% compared to mock cells. ACBP-siRNA1

did not increase caspase 3/7 activity in comparison to scrambled- or cyclophilin B-siRNA. In contrast, lamin A/C-siRNA and ACBP-siRNA2 increase the caspase 3/7 activity significantly. Therefore, we used the non-cytotoxic and non-apoptotic ACBP-siRNA1 for ongoing experiments.

Transcript profiling under ACBP knockdown showed 454 differential regulated genes in HepG2 cells

A profound depletion of ACBP protein to 20% compared to scrambled control siRNA was observed 72 h after siRNA transfection of HepG2 cells. This experimental set-up was used in order to identify putative ACBP-regulated genes. We performed microarray analyses applying human Genome U133 Plus 2.0 Genechip arrays (Affymetrix) in three independent experiments and compared the mRNA profile of control cells (scrambled siRNA) with ACBP-depleted cells. Based on a single sided permutation T-test and “present” designated probe sets with a minimal fold change of 1.32 (log 0.5) we identified 454 transcripts which were consistently regulated under ACBP-depletion. 256 transcripts were down- and 198 were up-regulated. As a mandatory control of our whole set-up, we found a down-regulation of the ACBP-mRNA by a factor of 2.35 ± 0.19 (mean $\pm$ SD). As a further control, 10 transcripts were selected for verification by TaqMan-based qRT-PCR. As shown in Table 2, we verified all selected genes. The consistency between array and qRT-PCR data is mentionable. For example, array data revealed a down-regulation of the

Gene ID	Gene symbol	Gene description	Fold change	
			microarray	Real-time RT-PCR
8435	ACAT2 ^a	Acyl-CoA:cholesterol acyltransferase 2	+1.35±0.08	+1.31±0.30
51	ACOX1 ^{b,i}	acyl-Coenzyme A oxidase 1, palmitoyl	-1.35±0.09	
972	CD74 ^{b,f}	CD74 molecule, major histocompatibility complex, class II invariant chain	+1.41±0.05	
55907	CMAS ^{f,g}	cytidine monophosphate N-acetylneuraminic acid synthetase	-1.33±0.12	
4051	CYP4F3 ^b	cytochrome P450, family 4, subfamily F, polypeptide 3	+1.32±0.08	
1958	EGR1 ^k	early growth response 1	-1.67±0.33	-1.62±0.09
79071	ELOVL6 ^{b,f}	ELOVL family member 6, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)	-1.34±0.02	
2168	FABP1 ^b	fatty acid binding protein 1, liver	-1.39±0.03	
2194	FASN ^{b,f}	fatty acid synthase	-1.40±0.01	-1.31±0.21
57678	GPAM ^{e,f}	glycerol-3-phosphate acyltransferase, mitochondrial	-1.45±0.13	-1.37±0.13
3155	HMGCL ^k	3-hydroxy-3-methylglutaryl-Coenzyme A lyase	-1.33±0.03	-1.27±0.51
3156	HMGCR ^{a,e,f}	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	-1.36±0.09	-1.39±0.25
3157	HMGCS1 ^{a,e,f}	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	-1.60±0.10	-1.56±0.14
3422	IDI1 ^{a,e,f}	isopentenyl-diphosphate delta isomerase 1	-1.32±0.07	-1.23±0.20
3612	IMPA1 ^{e,f}	inositol(myo)-1(or 4)-monophosphatase 1	-1.50±0.16	
4047	LSS ^{a,f}	lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)	-1.37±0.11	-1.11±0.33
55825	PECR ^{b,f}	peroxisomal trans-2-enoyl-CoA reductase	-1.53±0.01	-1.49±0.09
80339	PNPLA3 ^{d,i,j}	patatin-like phospholipase domain containing 3	-1.36±0.21	
5565	PRKAB2 ^{b,f}	protein kinase, AMP-activated, beta 2 non-catalytic subunit	-1.48±0.08	
51109	RDH11 ^e	retinol dehydrogenase 11 (all-trans/9-cis/11-cis)	-1.35±0.03	
81846	SBF2 ^h	SET binding factor 2	+1.33±0.14	
10402	ST3GAL6 ^c	ST3 beta-galactoside alpha-2,3-sialyltransferase 6	-1.39±0.08	

Table 2. ACBP regulated genes allocating to the GO term cellular lipid metabolic process and verification by qRT-PCR. Gene expression profiling was performed 72 h after ACBP siRNA1 transfection in HepG2 cells. Data comprise 3 independent experiments. Selected target genes were verified with real-time RT-PCR. Data are present mean fold changes ± SD. GeneID is based on NCBI GenBank database. Sub-analysis of GO-term *cellular lipid metabolic process*. ^asteroid metabolic process; ^bfatty acid metabolic process; ^cMembrane metabolic process; ^dneutral lipid metabolic process; ^eisoprenoid metabolic process; ^flipid biosynthetic process; ^glipopolysaccharide metabolic process; ^hlipid modification; ⁱcellular lipid catabolic process ; ^jglycerolipid metabolic process, ^kliterature-based allocation.

PECR-gene with a fold change of -1.53±0.01 (mean±SD) whereas qRT-PCR shows a factor of -1.49±0.09. Taken together, we identified 454 ACBP-regulated genes with high accuracy and assurance by using an RNAi-approach and whole genome expression profiling in HepG2 cells.

In addition, western blotting of selected genes was performed in order to confirm the mRNA levels established by whole genome gene expression (Fig. 3). Thus, immunodetection was done for fatty acid synthase (FASN), HMG-CoA synthase 1 (HMGCS1) and for the liver-specific fatty acid binding protein (FABP1). In accordance to the reduced transcript levels, HMGCS1 protein was diminished to 70% in ACBP-depleted HepG2 cells in comparison to scrambled siRNA treated cells. No changes were however observed for FASN and FABP1 at protein level.

Fig. 3. Western blot analysis of HMGCS1, FASN and FABP1. Detection of HMGCS1, FASN and FABP1 was done by western blotting in three independent experiments. HepG2 cells were transfected with 100 nmol/L of ACBP specific siRNA (A) and a scrambled siRNA (S). Whole cellular protein was isolated 72 h post-transfection and analyzed by immunodetection. Beta-actin served as loading control.

Gene annotation enrichment analysis revealed that ACBP-regulated genes are involved in fatty acid, glycerolipid and cholesterol metabolism

To allocate the corresponding genes of regulated transcripts with regard to biological processes and

molecular function the functional annotation tool DAVID was applied. We used Gene Ontology (GO) classification of level 2 which maintains good term coverage and provides meaningful term specificity [32]. Table 1 shows the number of ACBP-regulated genes which are

Fig. 4. Catalyzed reactions of regulated target genes allocated to cellular lipid metabolism and the involvement of human ACBP. Human ACBP is involved in overall acyl-CoA metabolism. RNAi-mediated ACBP depletion resulted in down-regulation of rate-limiting enzymes of fatty acid, glycerolipid as well as cholesterol metabolism. ACBPs impact on gene regulation is assumed based on its nuclear localization. Attention was particularly given to regulated genes connected to acyl-CoA metabolism. G3P, glycerol-3-phosphate; LA, lysophosphatic acid; CE, cholesterol ester.

significantly overrepresented in certain GO-terms. For example, 169 genes allocate to the term regulation of biological processes, 156 genes assign to regulation of cellular processes and 102 allocate to cellular component organization and biogenesis. According to molecular function half of the ACBP-regulated genes allocate to protein binding. As a second approach to allocate identified genes we used the text-mining system BiospherePathwayEdition (BSPE) which allows a more comprehensive sub-analysis. Since ACBP has a designated function in fatty acid and steroid metabolism, we subsequently filtered 223 ACBP-regulated genes according to the GO-classification cellular metabolic process and cellular lipid metabolic process. Based on this, we identified 16 down-regulated and 4 up-regulated genes (Table 2) allocated to *cellular lipid metabolic process*. For the term cellular metabolic process and cellular lipid metabolic process the statistical analysis, based on the number of observed and expected annotations, reached a z-score of 2.13. In detail, 14.99 genes were expected for this annotation and 23 were finally observed in the set of 454 regulated genes. Independent from GO-based gene selection we identified the 3-hydroxy-3-methylglutaryl-Coenzyme A lyase (HMGCL; +1.33) and the early growth response 1 gene (EGR1; -1.67) as regulated by ACBP. Thus, we subjected 22 ACBP regulated to further sub-analysis. A consistent down-regulation of four genes involved in cholesterol biosynthesis was observed: 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (HMGCS1; -1.60), 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGCR; -1.36), isopentenyl-diphosphate delta isomerase 1 (IDI1; -1.32) and lanosterol synthase (LSS; -1.37). Acyl-

Fig. 5. Effect of ACBP depletion in HepG2 cells on fatty acid content. HepG2 cells were transfected with 100 nmol/L ACBP specific siRNA1 and scrambled control siRNA. Fatty acid content was assessed 72 h post-transfection by gas chromatography as described in Material & Methods. Data are presented as mean $\pm$ SD (n=3; *p<0.05).

CoA:cholesterol acyltransferase 2 (ACAT2; +1.35) was up-regulated. Eight differential expressed genes were involved in *fatty acid metabolic processes*, including acyl-CoA oxidase (ACOX1; -1.35), CD74 molecule (CD74; +1.39); cytochrome P450, family 4, subfamily F, polypeptide 3 (CYP4F3; +1.32); ELOVL family member 6 (ELOVL6; -1.34); liver fatty acid binding protein (L-FABP 1; -1.39), fatty acid synthase (FASN; -1.40), peroxisomal trans 2-enoyl CoA reductase (PECR; -1.53) and protein kinase, AMP-activated, beta 2 non-catalytic subunit (PRKAB2; -1.49). Three regulated genes encoded enzymes involved in *membrane lipid metabolic processes*: mitochondrial glycerol-3-phosphate acyltransferase (GPAM; -1.45); inositol (myo)-1(or 4)-monophosphatase 1 (IMPA1; -1.49) and ST3 beta-galactoside alpha-2,3-sialyltransferase 6 (ST3GAL6; -1.39).

Cell type/organism	Essentiality	Transcriptional and/or metabolic effects
Yeast		
Schjerling et al. 1996	no	growth rate↓; C18:0-CoA↑; Δ9-desaturase (OLE-1) expression↑; FA-composition ↔; FA-content ↓
Gaigg et al. 2001	no	C18:0-CoA ↑; phospholipids ↔; C26:0-CoA ↓; sphingolipids ↓; perturbed plasma membrane structure; vesicle accumulation; Elo1/2/3p expression ↔, OLE-1 expression ↑
Faergeman et al. 2004	no	ceramide ↓; altered vacuole morphology & assembly; phospholipid composition altered
Feddersen et al. 2007	no	134 differential expressed genes (44↓, 90↓) induction of FA-; phospholipid-; glycolysis-synthesis and stress response related genes; phospholipid composition altered
Cell culture		
this study	no	no morphological changes; no apoptosis & cytotoxicity; 454 differential expressed genes (256 ↓, 196↑), repression of rate-limiting genes of FA - synthesis, glycerolipid and cholesterol synthesis, FA-content ↓
HepG2 cells		
Boujrad et al. 1993	no	hormone-stimulated steroid hormone synthesis ↓
MA-10 Leydig cells		
Mandrup et al. 1998	no	lipid accumulation ↓; adipocyte differentiation ↓; PPARγ, C/EBPα expression ↓; expression of adipogenic genes ↓
3T3L1 fibroblasts		
Faergeman et al. 2002	yes	growth arrest, detachment of cells, early apoptosis induction, cell death
HeLa, HepG2, Chang-cells		
Shulga et al. 2003	no	prevention of apoptosis, t-Bid-induced activation of mitochondrial μ-calpain ↓
HeLa		
other		
Milne et al. 2001	yes	cell death
Trypanosoma brucei		
Lee et al. 2007	no	sebocyte hyperplasia, sparse, matted hair, abnormal hair lipid profile, liver lipids ↔
mouse		

Table 3. Metabolic and transcriptional profiling studies conducted in ACBP-depleted cells and organisms.

ACBP knockdown reduces the fatty acid content in HepG2 cells

ACBP-regulated genes involved in lipid metabolism (Table 2, n=22) were allocated into common metabolic pathways based on literature and database searches. As shown in Fig. 4, 10 regulated genes encode key enzymes which could be integrated into fatty acid, glycerolipid and cholesterol metabolism. As described, the regulation of these genes was verified by qRT-PCR (Table 2). A close inspection of the depicted metabolism suggested a down-regulation of lipid biosynthetic pathways. Consequently, we determined fatty acid content in HepG2 cells after ACBP knockdown. As shown in Fig. 5, saturated (16:0) and monosaturated (16:1, 18:1) fatty acid were significantly reduced to nearly 75% in ACBP-depleted cells. Thus, the ACBP-siRNA mediated down-regulation of the lipid metabolism at mRNA-level seems to be translated into the relevant metabolites.

In silico promoter analysis of ACBP target genes

We performed in silico promoter analysis to identify common regulatory motifs in the 20 ACBP regulated

genes allocating the GO-term cellular lipid metabolic process (Table 2). Promoter sequences were extracted and analyzed according common transcription factor binding sites (TFBS). Comparative analysis resulted in the significant identification of TFs belonging to EGR/ nerve growth factor induced protein C & related factor (EGFR) (p=4.33E-6), zinc factor binding protein factors (ZFBP) (p=8.75E-4) and GC-Box factors SP1/GC (SP1) (p=1.2 E-3) family. Based on the observed transcriptional repression of EGR1 we concentrated on EGR-family. Sub-analysis allocated putative binding sites for EGR1 in 16 of total 20 differential expressed genes: ACOX1, CMAS, ELOVL6, FASN, GPAM, HMGCR, HMGCS1, IDI1, IMPA1, LSS, PECR, PNPLA3, PRKAB2, RDH11, SBF2, ST3GAL6.

Four identified target genes seem not be regulated by EGR1, possess however putative TFBS for other EGR-family members. CD74 might be regulated by Wilms tumor 1 (WT1) and nerve growth factor-induced protein C (NGFIC). WT 1 and EGR2 seem to bind to CYP4F3 promoter, whereas EGR2 possesses a target site in FABP1-promoter and collagen krox protein (CKROX) in ACAT2-promoter.

Discussion

The ubiquitous expressed multifunctional protein ACBP is a well known intracellular transporter of acyl-CoAs thereby determining profoundly their intracellular fate. Through binding and directing acyl-CoAs towards nucleus and nuclear factors ACBP may influence gene expression [37]. Supportively, ACBP is located in the nucleus [38] and interacts with HNF-4 α [24]. Here we aimed to unravel the impact of human ACBP in gene expression by applying RNAi-mediated silencing of ACBP in HepG2 cells in combination with microarray gene chip technology. We achieved a down-regulation of the ACBP protein of about 80%. Similar reductions were also reported in other siRNA-mediated knockdown studies [39–41]. Our microarray approach revealed high accuracy because the measured transcript levels by microarray were very consistent to qRT-PCR verification data. In comparison to complex tissues available from ACBP overexpressing [42, 43] and knockout mice [22], our approach employed a homogenous cell population cultured under highly standardized conditions. Therefore, the identified 454 ACBP regulated transcripts in liver HepG2 cells is an appropriate collection to unravel the cellular function of ACBP in gene expression.

The broad abundance of the multifunctional ACBP in the eukaryotic kingdom could be a hint for its essential function. Indeed, target disruption of ACBP in the parasite *Trypanosoma brucei* leads to lethality [44]. In ACBP-depleted yeast a retarded cell growth was described [45]. A mutation within the ACBP locus in mice resulted in sparse hair, male infertility, failure to thrive, hydrocephaly and anemia [46]. Furthermore, Faergeman et al. [29] showed growth arrest, cell detachment and apoptosis-induction in ACBP-depleted HepG2, HeLa and Chang cells. In contrast but in accordance to our study in HepG2 cells, ACBP knockdown in murine 3T3-L1 fibroblasts [47] and HeLa cells [12] did not point out impairment of cell viability. The differences in the degree of ACBP knockdown may partly explain conflicting results in cell culture experiments. We obtained a 80% reduction of the ACBP protein, whereas other studies showed almost complete ACBP depletion. However, to investigate the putative gene regulatory function of ACBP it seems to be desirable to avoid negative pleiotrophic effects such as cytotoxicity and/or apoptosis by ACBP knockdown. This was achieved in our study. Also expression data excluded the ACBP mediated induction of apoptosis-related genes since those were not over-estimated according to GO classification of

biological processes. Thus, siRNA-mediated depletion of ACBP in HepG2 cells is a suitable tool to study its gene regulatory function. Recent studies revealed even a function of ACBP in promoting and transmitting apoptosis at the level of protein-protein interaction. In this respect, suppression of ACBP ameliorated BH3 interacting domain death agonist (Bid)-induced mitochondrial damage and therefore prevented cell death [12]. Solstad et al. [48] uncovered ACBP recently as phosphorylation target in the initial phase of apoptosis.

Metabolic as well as transcriptional profiling data in ACBP-depleted cells and organisms are summarized in Table 3. Comprehensive studies were raised in yeast and comprised mainly parameters of lipid composition [21, 45, 49] and some gene expression data [26]. Human cell culture studies focused on ACBPs role in adipocyte differentiation [47] and apoptosis induction [12]. In this study, we identified 454 genes to be differential expressed in ACBP-depleted HepG2 cells. Based on gene annotation enrichment analysis using DAVID and BSPE 20 ACBP-regulated genes were allocated towards cellular lipid metabolic processes. Remarkably, the vast amount of these lipogenic genes were transcriptionally repressed in ACBP-depleted HepG2 cells, whereas 6 of these down-regulated genes were classified to fatty acid metabolic processes. In accordance, we observed a significant reduced content of the fatty acids C16:0, C16:1 and C18:1n9 in ACBP exhausted HepG2 cells. Studies in yeast [45] and mice [42] confirmed the role of ACBP in fatty acid and acyl-CoA metabolism. *Acb1p* knockout yeast strain showed reduced total fatty acid content, especially of the very long chain FA C26:0 [21].

The genes encoding FASN, ELOVL6 and PECR were transcriptional repressed in ACBP-exhausted HepG2 cells. This may explain the reduced fatty acid content in ACBP depleted cells, since all three genes encode key enzymes of fatty acid synthesis and elongation. FASN is a multifunctional protein, which has all enzymatic activities required for each cycle of fatty acid synthesis towards palmitic acid. ELOVL6 encodes for a protein catalyzing the elongation of palmitic acid towards long chain fatty acid. ELOVL is predicted to be important for tissue fatty acid composition and is also involved in obesity-induced insulin resistance [50, 51]. PECR functions in fatty acid elongation, possesses high affinity to fatty acids with chain lengths up to 16 carbons and shows marked expression in liver and kidney [52]. Other genes that were down-regulated following ACBP-depletion were FABP1 and ACOX1. FABP1 encodes for a liver-specific lipid binding protein,

sequestering hydrophobic molecules including fatty acids, acyl-CoAs and cholesterol [53]. ACOX1 encodes for a peroxisomal fatty-acyl-CoA oxidase involved in β -oxidation of long and very long chain fatty acid. Interestingly, ACOX1 was documented to interact with ACBP in a yeast-two-hybrid map of *Drosophila melanogaster* [54]. Taken together, the obtained data suggest transcriptional repression of general fatty biosynthesis and elongation. This is supported by fatty acid profiles assessed in our ACBP knockdown HepG2 cells as well in Acb1p knockout yeast [45]. In another point of view, knockdown of ACBP itself directly causes changes in fatty acid metabolism that in turn could impact expression of the described target genes. This question was however not experimentally addressed.

The GPAM gene was also repressed in ACBP knockdown cells. GPAM encodes a key enzyme in phospholipid synthesis catalyzing the acylation of glycerol-3-phosphate to lysophosphatic acid. This suggested a pertubated function of phospholipid metabolism under ACBP knockdown. Supportively, the relative phospholipid content and its composition was shown to be reduced in Acb1p exhausted yeast [26]. Another interesting finding of our study is that ACBP-depletion changes expression of five genes allocating to steroid metabolic processes. Four of these cholesterogenic genes were commonly repressed. These included the two rate-limiting enzymes HMGCS1 and HMGCR as well as the more down-stream acting IDI1 and LSS. ACAT2 is involved in cholesterol esterification and was up-regulated. The fact, that not all genes of cholesterol metabolism were differential regulated was also observed by others [55]. Former studies in steroidogenic cells and tissues uncovered the involvement of ACBP in mitochondrial cholesterol up-take and conversion towards pregnenolone via the interaction with the peripheral-type benzodiazepine receptor (PBR) [7, 8]. In consideration of current data, depletion of ACBP may lead to low cholesterol level thereby interfering also with steroid hormone synthesis. Low cholesterol synthesis might also contribute to disturbed membrane assembly and organization [21]. Recent studies have already shown fragmented vacuoles in yeast lacking the cholesterol equivalent ergosterol [56]. Moreover, a disturbed vesicular trafficking was observed in Acb1p-exhausted yeast [21, 49].

Gene expression profiling in this yeast cells showed a repression the cytochrome P450 lanosterol 14a-demethylase which participates in the ergosterol biosynthesis pathway [26]. Together, ACBP may have a conserved gene regulatory function in cholesterol/ergosterol biosynthesis.

A number of transcription factors are known to substantially regulate key genes of the lipid metabolism. In the context of our study, the sterol regulatory element-binding proteins 1a and 2 (SREBP) are of special interest [57-59] because they regulate most of the genes depicted in Fig. 4. Thus, an interaction between ACBP and SREBP is considerable but has to be tested in future experiments by using promoter-reporter assays, for example. *In silico* promoter analysis of the identified 20 ACBP target genes allocating to cellular lipid metabolic processes revealed that 16 genes possess a putative binding site for EGR1. Interestingly, the EGR1 gene itself was transcriptional repressed in ACBP exhausted HepG2 cells. The precise role of EGR1 in ACBP-mediated suppression of target genes remains open but seems to be a promising approach to unravel the gene regulatory function of ACBP. However, these data are preliminary and need further evaluation.

In conclusion, genome-wide expression profiling in HepG2 cells under ACBP knockdown revealed a gene regulatory function of human ACBP in cholesterol and fatty acid biosynthesis. This effect seems to be translated into relevant metabolites. Moreover, the identified 454 ACBP regulated transcripts may present a first reference for further studies to define the ACBP regulon in mammalian cells.

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References

- Mandrup S, Hummel R, Ravn S, Jensen G, Andreasen PH, Gregersen N, Knudsen J, Kristiansen K: Acyl-CoA-binding protein/diazepam-binding inhibitor gene and pseudogenes. A typical housekeeping gene family. *J Mol Biol* 1992;228:1011-1022.
- Gray PW, Glaister D, Seeburg PH, Guidotti A, Costa E: Cloning and expression of cDNA for human diazepam binding inhibitor, a natural ligand of an allosteric regulatory site of the gamma-aminobutyric acid type A receptor. *Proc Natl Acad Sci USA* 1986;83:7547-7551.
- Guidotti A, Forchetti CM, Corda MG, Konkelt D, Bennett CD, Costa E: Isolation, characterization, and purification to homogeneity of an endogenous polypeptide with agonistic action on benzodiazepine receptors. *Proc Natl Acad Sci USA* 1983;80:3531-3535.
- Borboni P, Condorelli L, De Stefanis P, Sesti G, Lauro R: Modulation of insulin secretion by diazepam binding inhibitor and its processing products. *Neuropharmacology* 1991;30:1399-1403.
- Chen ZW, Agerberth B, Gell K, Andersson M, Mutt V, Ostenson CG, Efendic S, Barossoderling J, Persson B, Jornvall H: Isolation and characterization of porcine diazepam-binding inhibitor, apolypeptide not only of cerebral occurrence but also common in intestinal tissues and with effects on regulation of insulin resistance. *Eur J Biochem* 1988;174:239-245.
- Herzig KH, Schon I, Tatemoto K, Ohe Y, Li Y, Folsch UR, Owyang C: Diazepam binding inhibitor is a potent cholecystokinin releasing peptide in the intestine (vol 93, pg 7927, 1996). *Proc Natl Acad Sci USA* 1996;93:14214-14214.
- Besman MJ, Yanagibashi K, Lee TD, Kawamura M, Hall PF, Shively JE: Identification of des-(Gly-Ile)-endoneurine as an effector of corticotropin-dependent adrenal steroidogenesis: stimulation of cholesterol delivery is mediated by the peripheral benzodiazepine receptor. *Proc Natl Acad Sci U S A* 1989;86:4897-4901.
- Boujrad N, Hudson JR, Jr., Papadopoulos V: Inhibition of hormone-stimulated steroidogenesis in cultured Leydig tumor cells by a cholesterol-linked phosphorothioate oligodeoxynucleotide antisense to diazepam-binding inhibitor. *Proc Natl Acad Sci U S A* 1993;90:5728-5731.
- Papadopoulos V, Brown AS: Role of the peripheral-type benzodiazepine receptor and the polypeptide diazepam binding inhibitor in steroidogenesis. *J Steroid Biochem Mol Biol* 1995;53:103-110.
- Garnier M, Boujrad N, Oke BO, Brown AS, Riond J, Ferrara P, Shoyab M, Suarez-Quian CA, Papadopoulos V: Diazepam binding inhibitor is a paracrine/autocrine regulator of Leydig cell proliferation and steroidogenesis: action via peripheral-type benzodiazepine receptor and independent mechanisms. *Endocrinology* 1993;132:444-458.
- Melloni E, Aversa M, Salamino F, Sparatore B, Minafra R, Pontremoli S: Acyl-CoA-binding protein is a potent m-calpain activator. *J Biol Chem* 2000;275:82-86.
- Shulga N, Pastorino JG: Acyl coenzyme A-binding protein augments bid-induced mitochondrial damage and cell death by activating mu-calpain. *J Biol Chem* 2006;281:30824-30833.
- Faergeman NJ, Sigurskjold BW, Kragelund BB, Andersen KV, Knudsen J: Thermodynamics of ligand binding to acyl-coenzyme A binding protein studied by titration calorimetry. *Biochemistry* 1996;35:14118-14126.
- Wadum MCT, Villadsen JK, Feddersen S, Moller RS, Neergaard TBF, Kragelund BB, Hojrup P, Faergeman NJ, Knudsen J: Fluorescently labelled bovine acyl-CoA-binding protein acting as an acyl-CoA sensor: Interaction with CoA and acyl-CoA esters and its use in measuring free acyl-CoA esters and non-esterified fatty acids. *Biochem J* 2002;365:165-172.
- Rosendal J, Ertbjerg P, Knudsen J: Characterization of ligand binding to acyl-CoA-binding protein. *Biochem J* 1993;290:321-326.
- Knudsen J, Faergeman NJ, Skott H, Hummel R, Borsting C, Rose TM, Andersen JS, Hojrup P, Roepstorff P, Kristiansen K: Yeast acyl-CoA-binding protein: acyl-CoA-binding affinity and effect on intracellular acyl-CoA pool size. *Biochem J* 1994;302:479-485.
- Mandrup S, Jepsen R, Skott H, Rosendal J, Hojrup P, Kristiansen K, Knudsen J: Effect of heterologous expression of acyl-CoA-binding protein on acyl-CoA level and composition in yeast. *Biochem J* 1993;290:369-374.
- Rasmussen JT, Faergeman NJ, Kristiansen K, Knudsen J: Acyl-CoA binding protein (ACBP) can mediate intermembrane acyl-CoA transport and donate acyl-CoA for beta-oxidation and glycerolipid synthesis. *Biochem J* 1994;299:165-170.
- Rasmussen JT, Rosendal J, Knudsen J: Interaction of acyl-CoA-binding protein (ACBP) on processes for which acyl-CoA is a substrate, product or inhibitor. *Biochem J* 1993;292:907-913.
- Cohen Simonsen A, Bernchou Jensen U, Faergeman NJ, Knudsen J, Mouritsen OG: Acyl-coenzyme A organizes laterally in membranes and is recognized specifically by acyl-coenzyme A binding protein. *FEBS Lett* 2003;552:253-258.
- Gaigg B, Neergaard TBF, Schneider R, Hansen JK, Faergeman NJ, Jensen NA, Andersen JR, Friis J, Sandhoff R, Schroder HD, Knudsen J: Depletion of acyl-coenzyme A-binding protein affects sphingolipid synthesis and causes vesicle accumulation and membrane defects in *Saccharomyces cerevisiae*. *Mol Biol Cell* 2001;12:1147-1160.
- Lee L, DeBono CA, Campagna DR, Young DC, Moody DB, Fleming MD: Loss of the acyl-CoA binding protein (Acbp) results in fatty acid metabolism abnormalities in mouse hair and skin. *J Invest Dermatol* 2007;127:16-23.
- Hansen JS, Faergeman NJ, Kragelund BB, Knudsen J: Acyl-CoA binding protein (ACBP) localizes to ER and Golgi in a ligand dependent manner in mammalian cells. *Biochem J* 2008;410 (3):463-472.
- Petrescu AD, Payne HR, Boedecker A, Chao H, Hertz R, Bar-Tana J, Schroeder F, Kier AB: Physical and functional interaction of acyl-CoA-binding protein with hepatocyte nuclear factor-4 alpha. *J Biol Chem* 2003;278:51813-51824.
- Helledie T, Antonius M, Sorensen RV, Hertz AV, Bernlohr DA, Kolvaar S, Kristiansen K, Mandrup S: Lipid-binding proteins modulate ligand-dependent trans-activation by peroxisome proliferator-activated receptors and localize to the nucleus as well as the cytoplasm. *J Lipid Res* 2000;41:1740-1751.
- Feddersen S, Neergaard TB, Knudsen J, Faergeman NJ: Transcriptional regulation of phospholipid biosynthesis is linked to fatty acid metabolism by an acyl-CoA binding protein dependent mechanism in *Saccharomyces cerevisiae*. *Biochem J* 2007;407:219-230.
- Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorovova A: Rational siRNA design for RNA interference. *Nature Biotechnology* 2004;22:326-330.
- Zuker M: Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* 2003;31:3406-3415.
- Faergeman NJ, Knudsen J: Acyl-CoA binding protein is an essential protein in mammalian cell lines. *Biochem J* 2002;368:679-682.
- Vock C, Doring F, Nitz I: Transcriptional regulation of HMG-CoA synthase and HMG-CoA reductase genes by human ACBP. *Cell Physiol Biochem* 2008;22:515-524.

- 31 Liu WM, Mei R, Di X, Ryder TB, Hubbell E, Dee S, Webster TA, Harrington CA, Ho MH, Baid J, Smeekens SP: Analysis of high density expression microarrays with signed-rank call algorithms. *Bioinformatics* 2002;18:1593-1599.
- 32 Dennis G, Jr., Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA: DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol* 2003;4:P3.
- 33 Scherf M, Epple A, Werner T: The next generation of literature analysis: integration of genomic analysis into text mining. *Brief Bioinform* 2005;6:287-297.
- 34 Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M, Werner T: MatInspector and beyond: promoter analysis based on transcription factor binding sites. *Bioinformatics* 2005;21:2933-2942.
- 35 Klingenhoff A, Frech K, Quandt K, Werner T: Functional promoter modules can be detected by formal models independent of overall nucleotide sequence similarity. *Bioinformatics* 1999;15:180-186.
- 36 Solstad T, Fismen L, Garberg H, Fladmark KE: Identification of a novel phosphorylation site of acyl-CoA binding protein (ACBP) in nodularin-induced apoptotic hepatocytes. *Exp Cell Res* 2008;314:2141-2149.
- 37 Helledie T, Jorgensen C, Antonius M, Krogsdam AM, Kratchmarova I, Kristiansen K, Mandrup S: Role of adipocyte lipid-binding protein (ALBP) and acyl-coA binding protein (ACBP) in PPAR-mediated transactivation. *Mol Cell Biochem* 2002;239:157-164.
- 38 Elholm M, Garras A, Neve S, Tornehave D, Lund TB, Skorge J, Flatmark T, Kristiansen K, Berge RK: Long-chain acyl-CoA esters and acyl-CoA binding protein are present in the nucleus of rat liver cells. *J Lipid Res* 2000;41:538-545.
- 39 Weisschuh N, Alavi MV, Bonin M, Wissinger B: Identification of genes that are linked with optineurin expression using a combined RNAi-microarray approach. *Exp Eye Res* 2007;85:450-461.
- 40 Shoda J, Inada Y, Tsuji A, Kusama H, Ueda T, Ikegami T, Suzuki H, Sugiyama Y, Cohen DE, Tanaka N: Bezafibrate stimulates canalicular localization of NBD-labeled PC in HepG2 cells by PPARalpha-mediated redistribution of ABCB4. *J Lipid Res* 2004;45:1813-1825.
- 41 Evans JR, Kelly DL, Morris KJ, Arvide EM, Harris A: RNA interference-mediated inhibition of hepatocyte nuclear factor 1alpha identifies target genes. *Biochim Biophys Acta* 2008;1779:341-346.
- 42 Huang H, Atshaves BP, Frolov A, Kier AB, Schroeder F: Acyl-coenzyme A binding protein expression alters liver fatty acyl-coenzyme A metabolism. *Biochemistry* 2005;44:10282-10297.
- 43 Oikari S, Ahtialansaari T, Heinonen MV, Mauriala T, Auriola S, Kiehne K, Folsch UR, Janne J, Alhonen L, Herzig KH: Downregulation of PPARs and SREBP by acyl-CoA-binding protein over-expression in transgenic rats. *Pflugers Arch* 2008;456:369-77.
- 44 Milne KG, Guthrie ML, Ferguson MA: Acyl-CoA binding protein is essential in bloodstream form *Trypanosoma brucei*. *Mol Biochem Parasitol* 2001;112:301-304.
- 45 Schjerling CK, Hummel R, Hansen JR, Borsting C, Mikkelsen JM, Kristiansen K, Knudsen J: Disruption of the gene encoding the acyl-CoA-binding protein (ACB1) perturbs acyl-CoA metabolism in *Saccharomyces cerevisiae*. *J Biol Chem* 1996;271:22514-22521.
- 46 Ohgami RS, Campagna DR, Antiochos B, Wood EB, Sharp JJ, Barker JE, Fleming MD: nm1054: a spontaneous, recessive, hypochromic, microcytic anemia mutation in the mouse. *Blood* 2005;106:3625-3631.
- 47 Mandrup S, Sorensen RV, Helledie T, Nohr J, Baldursson T, Gram C, Knudsen J, Kristiansen K: Inhibition of 3T3-L1 adipocyte differentiation by expression of acyl-CoA-binding protein antisense RNA. *J Biol Chem* 1998;273:23897-23903.
- 48 Solstad T, Fismen L, Garberg H, Fladmark KE: Identification of a novel phosphorylation site of acyl-CoA binding protein (ACBP) in nodularin-induced apoptotic hepatocytes. *Exp Cell Res* 2008;314:2141-2149.
- 49 Faergeman NJ, Feddersen S, Christiansen JK, Larsen MK, Schneider R, Ungermann C, Mutenda K, Roepstorff P, Knudsen J: Acyl-CoA-binding protein, Acblp, is required for normal vacuole function and ceramide synthesis in *Saccharomyces cerevisiae*. *Biochem J* 2004;380:907-918.
- 50 Moon YA, Shah NA, Mohapatra S, Warrington JA, Horton JD: Identification of a mammalian long chain fatty acyl elongase regulated by sterol regulatory element-binding proteins. *J Biol Chem* 2001;276:45358-45366.
- 51 Matsuzaka T, Shimano H, Yahagi N, Kato T, Atsumi A, Yamamoto T, Inoue N, Ishikawa M, Okada S, Ishigaki N, Iwasaki H, Iwasaki Y, Karasawa T, Kumadaki S, Matsui T, Sekiya M, Ohashi K, Hasty AH, Nakagawa Y, Takahashi A, Suzuki H, Yatoh S, Sone H, Toyoshima H, Osuga J, Yamada N: Crucial role of a long-chain fatty acid elongase, Elovl6, in obesity-induced insulin resistance. *Nat Med* 2007;13:1193-1202.
- 52 Das AK, Uhler MD, Hajra AK: Molecular cloning and expression of mammalian peroxisomal trans-2-enoyl-coenzyme A reductase cDNAs. *J Biol Chem* 2000;275:24333-24340.
- 53 Glatz JF, van der Vusse GJ: Cellular fatty acid-binding proteins: their function and physiological significance. *Prog Lipid Res* 1996;35:243-282.
- 54 Giot L, Bader JS, Brouwer C, Chaudhuri A, Kuang B, Li Y, Hao YL, Ooi CE, Godwin B, Vitols E, Vijayadamodar G, Pochart P, Machineni H, Welsh M, Kong Y, Zerhusen B, Malcolm R, Varrone Z, Collis A, Minto M, Burgess S, McDaniel L, Stimpson E, Spriggs F, Williams J, Neurath K, Ioime N, Agee M, Voss E, Furtak K, Renzulli R, Aanensen N, Carrola S, Bickelhaupt E, Lazovatsky Y, DaSilva A, Zhong J, Stanyon CA, Finley RL, Jr., White KP, Braverman M, Jarvie T, Gold S, Leach M, Knight J, Shimkets RA, McKenna MP, Chant J, Rothberg JM: A protein interaction map of *Drosophila melanogaster*. *Science* 2003;302:1727-1736.
- 55 Dolt KS, Karar J, Mishra MK, Salim J, Kumar R, Grover SK, Qadar Pasha MA: Transcriptional downregulation of sterol metabolism genes in murine liver exposed to acute hypobaric hypoxia. *Biochem Biophys Res Commun* 2007;354:148-153.
- 56 Kato M, Wickner W: Ergosterol is required for the Sec18/ATP-dependent priming step of homotypic vacuole fusion. *Embo J* 2001;20:4035-4040.
- 57 Shimano H, Horton JD, Hammer RE, Shimomura I, Brown MS, Goldstein JL: Overproduction of cholesterol and fatty acids causes massive liver enlargement in transgenic mice expressing truncated SREBP-1a. *J Clin Invest* 1996;98:1575-1584.
- 58 Eberle D, Hegarty B, Bossard P, Ferre P, Foufelle F: SREBP transcription factors: master regulators of lipid homeostasis. *Biochimie* 2004;86:839-848.
- 59 Bennett MK, Lopez JM, Sanchez HB, Osborne TF: Sterol regulation of fatty acid synthase promoter. Coordinate feedback regulation of two major lipid pathways. *J Biol Chem* 1995;270:25578-25583.