Title

Transcriptional control mechanisms of genes of lipid and fatty metabolism in the Atlantic salmon (*Salmo salar* L.) established cell line, SHK-1

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Abstract

The regulatory control mechanisms of lipid and fatty acid metabolism were investigated in Atlantic salmon. We identified sterol regulatory element binding protein (SREBP) genes in salmon and characterised their response, and the response of potential target and other regulatory genes including liver X receptor (LXR), to cholesterol and long-chain polyunsaturated fatty acids (LC-PUFA) in the salmon established cell line, SHK-1. Two cDNAs for SREBPs homologous to mammalian SREBP-1 and SREBP-2 were characterised. We identified three groups of genes whose expression responded differently to the treatments. One group of genes, including cholesterol biosynthetic genes, showed increased expression in response to lipid depletion but supplementary cholesterol or LC-PUFA had no further effect. The expression of a second group of genes belonging to fatty acid biosynthetic pathways, included fatty acid synthase, Δ6 and Δ5 fatty acyl desaturases, also increased after lipid depletion but this was negated by cholesterol or by LC-PUFA supplementation. The expression of a third group of genes including acyl-CoA oxidase, HMG-CoA reductase and Elovl5 elongase was increased by cholesterol treatment but was not affected by lipid depletion or by LC-PUFA. This same pattern of expression was also shown by liver X receptor (LXR), indicating that acyl-CoA oxidase, HMG-CoA reductase and Elovl5 are possible direct targets of LXR. This suggests that salmon Elovl5 may be regulated differently from mammalian Elovl5, which is an indirect target of LXR, responding to LXR-dependent increases in SREBP-1.

Introduction

Fish, especially oily fish such as Atlantic salmon (*Salmo salar*), are unique sources in the human diet of the omega-3 or n-3 long-chain polyunsaturated fatty acids (LC-PUFA) eicosapentaenoate (EPA, 20:5n-3) and docosahexaenoate (DHA, 22:6n-3) [1], that have well-established beneficial effects in a number of inflammatory and pathological conditions, including cardiovascular and neurological diseases [2-4]. Consequently, there is considerable interest in lipid and fatty acid homeostasis in fish, particularly the molecular mechanisms of endogenous LC-PUFA synthesis. In mammals, the nuclear receptors liver X receptors, LXRs, and transcription factors, sterol regulatory element binding proteins, SREBPs, are major regulators of lipid metabolism. In mammals, the LXR subfamily consists of two encoded genes, LXRα and LXRβ [5] and, similarly, the SREBP subfamily is encoded by two genes, SREBF1 and SREBF2 [6]. SREBP-1 and SREBP-2 are major regulators of mammalian fatty acid/lipid and cholesterol biosynthetic genes, respectively, including genes of LC-PUFA synthesis [7]. In vertebrates LC-PUFA synthesis proceeds by consecutive desaturation and elongation steps of the dietary essential precursors linoleic (18:2n-6) and linolenic acids (18:3n-3), catalysed by Δ5 and Δ6 fatty acyl desaturases (Fad) and fatty acyl elongase (Elovl) enzymes [8]. The n-3 LC-PUFA, EPA and DHA, have been shown to regulate SREBP-1 expression through both transcriptional and posttranslational mechanisms and that suppression of SREBP-1 transcription was dependent upon LXRα [9].

In recent years, the regulation of lipid and fatty acid metabolism in fish has received increasing attention [10]. A single LXR cDNA was isolated and characterised from Atlantic salmon showing that it was similar to mammalian LXRα [11]. The LC-PUFA synthesis pathway has been extensively studied and salmon were shown to have multiple Fad proteins with the necessary Δ6 and Δ5 activities [12-14], and Elovls, including Elovl5 and Elovl2 [15]

for biosynthesis of EPA and DHA. Several studies have shown that salmon Fad and Elovl genes are regulated at the transcriptional level after reduced dietary LC-PUFA resulting in increased expression and correspondingly increased LC-PUFA biosynthesis [15-18]. Furthermore, gene promoter studies have implicated SREBPs as important transcriptional regulators of salmon Δ6 Fad [18]. However, there is very little information regarding SREBF genes and their regulatory functions and controls in fish. Molecular studies of SREBPs and other lipid homeostatic regulatory systems in fish have been constrained by a lack of tractable cellular systems, most work having been conducted on tissues from fish at various developmental stages and under different dietary regimes.

As described above, fish, as suppliers of "omega-3", have obvious relevance to human health. However, with the decline in commercial fisheries [19], an increasing proportion of fish, 50 % globally, are now farmed [20] and so research into lipid and fatty acid metabolism in fish is critical with two issues paramount. In Europe aquaculture is largely focussed on carnivorous species and so diets have traditionally been based on fishmeal and fish oil, which themselves are derived from wild fisheries that have reached their sustainable limit as well as being an environmentally and ecologically unsound practice [21]. Therefore, to produce the fish required for the burgeoning human population, sustainable alternatives to marine fish oil are required. Vegetable oils are the prime candidates but they are devoid of LC-PUFA, a problem compounded by the fact that endogenous biosynthesis of LC-PUFA from C_{18} PUFA, as supplied by vegetable oils, is inefficient in vertebrates, including fish [22-23]. As well as supplying essential fatty acids, dietary lipid also supplies energy and the more energy supplied by dietary lipid, the less dietary protein will be used for energy, and so more protein can be "spared" for synthesis of new tissue/flesh [24]. Therefore, recent dietary formulations have shown an upward trend in dietary lipid content, particularly in the case of Atlantic salmon [25]. Although the use of "high-energy" feeds has successfully increased weight

gains, several studies have shown that a potential and, perhaps, detrimental effect of high fat diets is the deposition of excess lipid in tissues [25].

A greater understanding of the molecular basis of lipid and fatty acid homeostasis in fish will enable efficient and effective use of sustainable dietary oils while maintaining the nutritional quality of farmed fish. The aims of the present study were to identify SREBP genes in Atlantic salmon and to characterise their response, and the response of other potential target and regulatory genes, to cholesterol and LC-PUFA in the established salmon cell line, SHK-1. The results show clear patterns of response indicating interaction between SREBPs and LXR in the regulation of lipid and fatty acid metabolism in salmon cells. However, the data also revealed that regulation and control of LC-PUFA biosynthesis in salmon may differ from that in mammals as it appeared that fatty acyl elongase Elovl5 may be a direct target gene of liver X receptor whereas, in mammals, Elovl5 is an indirect target of LXR, responding to LXR-dependent increases in SREBP-1.

Materials and methods

Fish

Atlantic salmon (*Salmo salar*) for the provision of RNA for cDNA cloning and tissues for determining gene expression were obtained from a study described in detail previously [26]. Briefly, tissue samples were obtained from four 2-year old fish maintained in sea pens (Loch Duich, Lochalsh, Scotland) and fed a standard fish oil diet containing 32 % lipid (Skretting ARC, Stavanger, Norway) and formulated to satisfy the nutritional requirements of salmonid fish [27]. All tissue samples were immediately frozen in liquid nitrogen and subsequently stored at -70 °C.

Cell culture and treatments

The Atlantic salmon established cell line, SHK-1, originated from salmon head kidney tissue and are likely leucocyte-derived, showing both macrophage- or dentritic-like phenotype [28,29]. The cells were routinely cultured in 75 cm^2 flasks (Nunc, Denmark) at 25 ˚C in Leibovitz's L-15 with GlutaMAX™-l supplemented with L-glutamine (4 mM final conc.), penicillin G (50 U/ml), streptomycin (50 µg/ml), 0.08 % mercaptoethanol, and 5 % foetal bovine serum (FBS) (normal medium). All components were from Gibco®/Invitrogen, UK with the exception of FBS that was from Biosera, UK. Lipid-free medium was as above except the serum supplement was 5 % delipidated (charcoal-treated) FBS (Gibco®/Invitrogen, UK). For sub-culturing, the cell monolayer was washed twice with phosphate buffer saline (PBS) without $CaCl_2$ or $MgCl_2$ (Gibco®/Invitrogen, UK), cells detached by incubation with 0.05 % trypsin/ 0.02 % EDTA, re-suspended in normal medium and redistributed in daughter flasks at a ratio of 1:3.

For experiments, viable cells were counted after harvesting using 0.4 % Trypan Blue (Sigma, UK), a Neubauer haemocytometer and inverted microscope (IMT-2, Olympus), and seeded in 6-well plates (Nunc, Denmark) at a density of 0.4×10^6 cells/well in a volume of 3 ml. Cells were approximately 70% confluent after 48 h growth (24 h in normal media followed by 24 h in delipidated medium), after which medium was aspirated, cells washed twice with PBS and the experimental conditions applied. Cells were exposed to either cholesterol, EPA or DHA in ethanol at concentrations of 20 µM for each, with parallel control cultures receiving ethanol carrier alone. Cells were harvested at 24 and 72 h after treatment. The medium was removed, cells washed twice with 1 ml of PBS per well, the monolayer scraped from the well in 0.5 ml of PBS and transferred to a microfuge tube before RNA extraction as below.

In addition, lipid contents of cells at 0 h and 72 h (control, cholesterol, EPA and DHA) were determined using 6 wells per treatment/time point (triplicate samples of two pooled wells). The cell layers were washed with PBS, scraped in 2 ml of PBS per well and the content of two wells (4 ml) transferred to a glass test tube and centrifuged to pellet the cells. The PBS was removed, 5 ml chloroform/methanol (as above) added and the cells extracted by mixing with a glass Pasteur pipette. One ml of 0.88 % KCl were then added and the extracts thoroughly mixed by vortexing. The tubes were centrifuged and the lower organic layers removed to clean tubes and solvent evaporated under a stream of nitrogen. Lipid extracts were desiccated for 2 h and total lipid contents determined gravimetrically.

RNA extraction and cDNA synthesis

Cell samples were centrifuged at 3000 g for 10 min, the PBS removed, 0.5 ml of TRI Reagent was added and total RNA extracted following the manufacturer's instructions (Ambion, UK). Three replicates wells were taken per treatment and time point. Total RNA was extracted from salmon tissues by homogenisation in TRI Reagent® RNA extraction buffer following the manufacture instructions. Quantity and quality of isolated RNA was determined by spectrophotometry with an ND-1000 Nanodrop (Labtech Int., East Sussex, UK) and electrophoresis using 1 µg of total RNA in a 1 % agarose gel. Prior to cDNA synthesis, 1 µg of total RNA was incubated at 70 °C for 5 min followed by 2 min on ice. For cDNA synthesis, 1µL of a 3:1 mix of random hexamers (400 ng/µL)/oligo dT (500 ng/µL), 2 µL dNTP (5 mM), 1µL of reverse transcriptase, and 1µL of RT enhancer (DNAse) were mixed with kit buffer in a final volume of 20 µL (Verso™ cDNA kit, ABgene, UK), and incubated at 42 °C for 60 min, followed by 95 °C for 2 min to inactivate the enzymes. The resulting cDNA was diluted 10-fold with milliQ water.

Isolation of SREBP-1 and SREBP-2 cDNAs

The Atlantic salmon gene index (http://compbio.dfci.harvard.edu/tgi/) was interrogated using human SREBP-1 (GenBank ID: NM_001005291) and SREBP-2 (GenBank ID: NM_004599) and homologous transcribed contig sequences TC148424 and TC166313 were identified for SREBP-1 and SREBP-2, respectively. The SREBP-1 and SREBP-2 transcribed contigs each consisted of the 3' end of the cDNAs including the stop codon and 2.0 and 1.4 kb of 3' untranslated sequence, respectively. Rapid Amplification of cDNA Ends (RACE)-PCR was performed and RACE cDNAs generated from 1 µg of salmon liver total RNA as described in the manual using the SMART™ RACE kit (Clontech, USA). The 5' RACE amplicons were generated by two rounds of PCR using SREBP-1 (Srebp1 5'R1 and Srebp1 5'R2) or SREBP-2 (Srebp2 5'R1 and Srebp2 5'R2) gene-specific degenerate primers. Primers were designed by selecting conserved areas, from alignments of DNA sequences of zebrafish (*Danio rerio*) (Ensembl transcript ID: SREBP1 ENSDART00000092665, SREBP2 ENSDART00000092690), pufferfish (*Tetraodon negroviridis*) (Ensembl transcript ID: SREBP1 ENSTNIT00000015119, SREBP2 ENSTNIT00000013959) and stickleback (*Gasterosteus aculeatus*) (Ensembl transcript ID: SREBP-1 ENSGACT00000015997, SREBP-2 ENSGACT00000009636). The final full-length PCR products were obtained by two rounds of PCR using nested primers designed to amplify end to end full length cDNAs (Srebp1Full-F1, Srebp1Full-R1 and Srebp1Full-F2, Srebp1Full-R2 for SREBP-1; Srebp2Full-F1, Srebp2Full-R1 and Srebp2Full-F2, Srebp2Full-R2 for SREBP-2). All PCRs were run at an annealing temperature of 60 °C and the extension time was 1 min/Kb of predicted PCR product, and 3 min were applied for unpredictable RACE PCR products. All primers were designed using PrimerSelect Ver. 6.1 program (DNASTAR, www.dnastar.com) (Table 1).

Sequence analysis

Sequencing was performed using a Beckman 8800 autosequencer, and Lasergene SEQman software (DNASTAR) used to edit and assemble DNA sequences. As SREBP sequence annotation was incomplete in fish species with sequenced genomes, and in order to predict the full-length polypeptide sequences, salmon SREBP cDNA sequences were used to interrogate pufferfish, stickleback and zebrafish genomes (www.Ensembl.org) using TBlastX. Regions of chromosomal DNA sequences containing homologous sequences were processed with GeneWise2 (www.ebi.ac.uk) using the salmon sequence as a key to generate predicted polypeptide sequences. Multiple alignments of deduced protein sequences were generated by ClustalW [30], and the neighbour joining method [31] and MEGA version 4 [32] used to deduce and bootstrap phylogenetic trees.

Quantitative PCR (qPCR)

Oligonucleotide primers for target genes (Table 1), SREBP-1, SREBP-2, LXR, Δ5Fad and Δ6Fad_a, Elovl2 and Elovl5a elongases, fatty acid synthase (FAS), acyl-CoA oxidase (ACOX), 7-dehydrocholesterol reductase (DHCR7), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoAR), mevalonate kinase (MVK), isopentenyl-diphosphate delta-isomerase 1 (IDI1) and reference genes (β-actin, EF1α, Gapdh and α-tubulin) were used at 0.3 µM with one-fortieth of the cDNA synthesis reaction (5 µl of a 1:10 dilution) and 10 µl of SYBR-green qPCR mix (ABgene, UK) in a total volume of 20 µl. Reactions were run in a Techne Quantica thermocycler at annealing temperatures of 58 ºC for LXR and IDI1, 60 ºC for Δ5Fad, Δ6Fad, Elovl2, Elovl5a, ACOX, FAS, HMG-CoAR and DHCR7, 61 ºC for MVK, β-actin, EF1α, Gapdh, α-tubulin and 18s, and 63 ºC for SREBP-1 and SREBP-2 to give PCR products of 210, 140, 191, 180, 145, 141, 230, 159, 224, 183, 196, 120, 175, 204, 124, 189,

151 and 147 bp, respectively. Each qPCR product was sequenced to confirm identity and each was 100 % identical to its predicted sequence. Quantification was achieved by a parallel set of reactions containing standards consisting of serial dilution of spectrophotometrically determined, linearised plasmid containing the above mentioned cDNA sequences. Normalisation of copy number across biological samples was achieved by using a normalisation factor (NF), based on the geometric mean expression of two reference genes (β-actin and EF1α) determined using geNorm software [33]. For comparison of expression levels of target genes across diverse tissues, the target gene copy number was normalized to 18s ribosomal RNA copy number.

Statistical analysis

All data are presented as means ± SD (n = 3 for cell studies or 4 for tissue expression). Statistical analysis was performed using the Minitab v.15.1 statistical software package (Minitab Inc., USA). Data were assessed for normality with the Kolmogorov-Smirnov test and for homogeneity of variances by Bartlett's test and examination of residual plots and, where necessary, data were transformed to improve normality. Data were analysed by full factorial two-way analysis of variance (ANOVA) using time and treatment as the fixed factors, with post hoc multiple comparisons applied using Tukey's test [34]. A significance of $p < 0.05$ was applied to all statistical tests performed.

Results

Atlantic salmon SREBP-1 and SREBP-2 isolation

Several rounds of RACE cDNA synthesis using primers designed to salmon SREBP-like sequences yielded multiple amplicons that, when assembled, predicted full-length cDNA sequences coding for two SREBP-like proteins. The existence of these as complete expressed

transcripts was confirmed by “end to end” RT-PCR which generated two distinct cDNAs, each of which contained an entire open reading frame for a protein with high identity to mammalian SREBPs.

Alignment and phylogenetic analysis of the salmon proteins with other previously characterised SREBPs from invertebrate and vertebrate species including SREBPs predicted by alignment to fish genome sequences, show that all vertebrates possess two SREBP proteins that robustly cluster in two different clades containing mammalian SREBP-1 and SREBP-2 sequences respectively, and that homologous genes for each of the SREBP forms exist in other fish species including salmon (Fig.1 and supplementary Figure). In addition, the deduced protein sequences showed that the salmon SREBP-1 sequence was more similar to the mammalian SREBP-1a isoform (Fig. 2). Thus, taking all of the sequence evidence, salmon SREBP-1 cDNA contained an open reading frame of 3516 bp (1172 amino acids) and a 3’ and 5’ untranslated regions of 174 and 838 bp respectively (GenBank accession number HM561860). The deduced salmon SREBP-1 protein sequence displayed 54 % and 70 % identity with human SREBP-1a and zebrafish SREBP-1 sequences. The full length cDNA of salmon SREBP-2 was 4884 bp which contained an open reading frame of 3243 bp (1081 amino acids) and a 5’ and 3’ untranslated regions of 233 and 1408 bp respectively (GenBank accession number HM561861). The deduced salmon SREBP-2 protein sequence displayed 49 % and 70 % identity with human and zebrafish SREBP-2 sequences.

Tissue distribution of salmon SREBP-1 and SREBP-2 mRNAs

Expression profiles of salmon SREBP-1 and SREBP-2 were very similar in each of the tissue tested with highest levels of expression in intestine (pyloric caeca), then brain > gill > liver > heart, spleen, kidney, red and white muscle (Fig. 3).

Response of SHK-1 cells exposed to cholesterol, EPA and DHA

Transcriptional responses of SHK-1 cells are presented in Figs. 4-6. We manipulated the lipid content of SHK-1 cells by growing cells over an extended period in lipid-free medium, and by treatment with excess cholesterol, EPA or DHA. After 72 h in lipid-free medium, cells were still sub-confluent but showed decreased lipid content (Table 2). There was a clear effect of time on SREBP-1, Δ5Fad, Δ6Fad, MVK and FAS. Message levels of these genes increased over the 72 h period. Supplementation of EPA, DHA or cholesterol prevented the increase in mRNA expression of Δ6Fad and FAS, showed a similar though not significant effect on Δ5Fad and MVK, and highly decreased expression of SREBP-1. There was also a smaller increase in expression of SREBP-2 after 72 h, which was not affected by DHA or EPA. Treatment with DHA also caused an increase in HMG-CoAR mRNA after 72 h.

Supplemental cholesterol caused a dramatic increase in LXR mRNA expression, up to 18-fold at 72 h after treatment. An increase in the expression of both salmon SREBP-1 and SREBP-2 mRNAs was also observed, with the largest effect seen on SREBP-1. The increased levels of these transcription factor mRNAs were accompanied by increased expression of Elovl5a, ACOX and HMG-CoAR at both the 24 h and 72 h time-points. The expression of IDI1 mRNA also increased, but only at 24 h after treatment, whilst no effect on DHR7 expression was observed with any treatment. The pattern of expression of Elovl2 mRNA was different from all other genes. Elovl2 was reduced by DHA after 24 h only, and highly decreased 72 h following cholesterol supplementation.

Discussion

Lipid and protein pathways dominate intermediary metabolism in carnivorous fish, such as Atlantic salmon, as carbohydrate is only a minor nutrient in their diet [25]. However, although the regulation of lipid biosynthesis and metabolism in Atlantic salmon is of great importance for nutrition and quality of farmed fish and also for understanding key life history transitions in wild fish, studies at the molecular level have been hampered by a lack of suitable experimentally tractable systems and manipulatable candidate genes. Previous studies in mammalian species [6] and in salmon [16,18] have indicated the importance of SREBP transcription factors, particularly in relation to the control of lipid and cholesterol biosynthesis. One aim of the present study was to identify and compare salmon SREBPs with characterised SREBPs from other species. We have synthesised and sequenced two complete SREBP cDNAs from Atlantic salmon, which enabled, by homology searching, SREBPs to be structurally characterised in other fish species with sequenced genomes. To verify whether fish species contain multiple genes for SREBPs, the salmon cDNA sequences were used to search for similar sequences in the zebrafish, pufferfish, medaka and stickleback genomes. In each species two SREBP genes were identified and in all fish each of these was clearly related to SREBP-1 or SREBP-2 from mammals. Thus, teleost fish contain homologues of mammalian SREBP-1 and SREBP-2 and these homologues must have arisen from a gene duplication preceding the evolutionary divergence of teleosts and mammals, but after the vertebrate-invertebrate division, since invertebrates contain only one SREBP gene [35]. Neo-functionalisation of duplicated SREBP gene isoforms, following the early vertebrate whole genome duplication, may have been driven by the necessity to independently regulate fatty acid and cholesterol biosynthesis and metabolism [36].

In mammals two major forms of SREBP-1, SREBP-1a and SREBP-1c, are expressed as a result of alternative splicing and are differentially expressed across tissues [37]. SREBP-1c is highly expressed in liver whilst in other tissues, particularly lipogenic tissues and in cultured

human cells, SREBP-1a predominates. SREBP-2 is more generally expressed. We found no evidence of alternatively spliced mRNAs in salmon, although this cannot at present be excluded since SREBP cDNAs were only synthesised from liver RNA. Furthermore, tissue expression profiling showed that both salmon SREBP genes were expressed at the same level in each of the tissues examined. However, the qPCR primers for SREBP-1 were designed in an area that corresponds to an identical region in both mammalian SREBP-1a and SREBP-1c and thus the expression patterns of SREBP-1 in the present study represent the expression of either transcript.

Alignments and phylogenetic analysis of the deduced amino acid sequence of SREBP proteins derived from genome sequences of other fish species, bird, amphibian and human and also from insect and worms show areas of very strong sequence conservation (supplementary Fig. and Fig. 1). The features which characterize mammalian SREBPs such as the transcription activation domain, proline/serine-rich domain, DNA binding domain including the tyrosine that characterises SREBP DNA binging motifs (bHLH-LZ) domains, the serine/proline/glycine-rich domain, the two transmembrane domains and the regulatory carboxyl-terminus domain are conserved in fish SREBPs [38]. A site cleaved by caspase 3 and 7 is also conserved in both salmon SREBPs [39] (supplementary Fig.1).

Mammalian SREBP-1 and SREBP-2 regulate distinct, but overlapping sets of target genes. SREBP-1 predominantly acts to increase the expression of genes involved in fatty acid synthesis, including FAS, Fad and Elovl genes [7]. SREBP-2 predominantly regulates genes of cholesterol biosynthesis, although there is limited reciprocal regulation of the two pathways by each SREBP protein. Furthermore, SREBP-1 activity is increased by depletion of LC-PUFA, while SREBP-2 is increased by depletion of cholesterol [6].

Previous studies have indicated the role of SREBPs in regulating genes such as thosse of cholesterol and LC-PUFA biosynthsis involved in the adaptation to vegetable oil-based

diets in salmon liver [10, 13-17]. In order to study this process in more detail we searched for a cellular model for salmon lipid homeostasis. Due to the scarcity of salmon cell lines and the absence of a hepatocyte-like model, we targeted the macrophage-like SHK-1 cell line [28,29]. This was based on the rationale that the critical transcriptional regulators of macrophage lipid homeostasis are the same as those in liver, and they regulate similar target genes [40]. In addition, macrophages have been shown to reflect diet-induced physiological responses and exhibit associated gene expression changes similar to liver [41]. Therefore, in SHK-1 cells we have measured mRNA expression of genes of fatty acid and LC-PUFA biosynthesis, cholesterol biosynthesis and peroxisomal β-oxidation as well as levels of SREBP-1, SREBP-2 and LXR mRNAs. In response to depletion of lipid (i.e. cholesterol and fatty acids), there were increases in expression of cholesterol biosynthetic enzymes IDI1 and MVK. Treatment with cholesterol or with EPA/DHA had no further effect. The expression of a second group of genes also increased after lipid depletion but this was negated by cholesterol or by EPA/DHA. This group contained genes belonging to fatty acid biosynthetic pathways and included FAS, Δ6Fad and Δ5Fad. The expression of a third group of genes was increased by cholesterol treatment but was not affected by lipid depletion or by EPA/DHA and included Elovl5, ACOX and, surprisingly, HMG-CoAR. Expression of LXR was also increased by cholesterol and not affected by lipid depletion or by EPA/DHA. Thus, based on the similarities of expression profiles, it is possible that the genes of Elovl5, ACOX and HMG-CoAR are direct targets of LXR. For ACOX, this conclusion is supported by the observation that LXR agonists cause increases in ACOX and in peroxisomal lipid oxidation in mice [42]. However, salmon Elovl5 may be regulated differently from mammalian Elovl5, which is an indirect target of LXR, responding to LXR-dependent increases in SREBP-1 [43]. Similarly the response of HMG-CoAR is surprising since in mammalian systems LXR may in fact negatively regulate this gene, possibly through a post-transcriptional mechanism

[44]. HMG-CoAR catalyses an early step in cholesterol and terpenoid biosynthesis and in mammals is primarily regulated by post-translational and proteolytic mechanisms, although it is also a SREBP target [7]. A previous study on salmon liver also showed HMG-CoAR to behave contrary to expectation, in that there was no increase in mRNA expression of this gene in response to low dietary cholesterol, despite increases in other genes of cholesterol biosynthesis [16]. Clearly these results indicate that HMG-CoA is likely to be regulated differently in salmon compared to mammals.

All other measured genes that responded to lipid depletion are likely to be targets of SREBP transcription factors. Since these fell into two groups, that is those whose expression was unaffected by cholesterol or EPA/DHA and those whose expression was decreased by cholesterol or EPA/DHA, it may be that these groups represent preferential targets of SREBP-1 and SREBP-2. Notably, although expression levels of SREBP-1 and SREBP-2 were both increased by supplementary cholesterol, only SREBP-1 was reduced after EPA/DHA treatment, indicating that FAS, Δ6Fad and Δ5Fad, also reduced after EPA/DHA treatment, are more likely to be SREBP-1 targets. Similar to SHK-1 cells, the expression of both rodent and human Δ5 and Δ6 desaturase genes is higher under a low fat diet [45,46]. Furthermore the human Δ6 desaturase gene contains a SREBP-1 response element which mediates LC-PUFA suppression of expression [47] and this element is conserved in the salmon gene [18]. However, it will be necessary to more fully characterise the salmon LXR with regard to its activation by synthetic ligands in order to fully distinguish LXR and SREBP regulation. For example, in mammals the FAS gene is a direct target of both LXR and SREBP-1 and the promoter recognition sequences for these transcription are conserved in birds [48] Thus, in salmon, either the FAS gene is not a target of LXR, or LXR-effects are masked by SREBP-1, which may be also be the case for the other genes sharing SREBP expression profiles in SHK-1 cells.

Although SREBP mRNA was increased in SHK-1 cells after supplementary cholesterol, this was not reflected in increases in putative target gene expression after cholesterol treatment. Proteolytic activation of SREBPs occurs after sensing lipid levels and so it is likely that, although mRNA and perhaps SREBP protein was increased following cholesterol treatment, this was not reflected in increased SREBP proteolytic activation pathways. These increases in SREBP mRNA may be secondary to increases in LXR. The mammalian SREBP-1 gene is regulated by LXRα that is activated by binding oxysterols (cholesterol metabolites) arising from increased intracellular cholesterol [49,50]. The LXRs play a critical role in reverse cholesterol transport by responding to elevated oxysterol concentrations through regulation of cholesterol 7α-hydroxylase which catalyses the rate-limiting step in bile acid synthesis, and by up-regulating ATP-binding cassette transporters, ABCG5 and ABCG8, which pump free cholesterol from hepatocytes into bile or from macrophages to the circulation [51]. Thus, one explanation for the increase in SREBP mRNA in SHK-1 cells after supplementary cholesterol is that, as in mammals, consequent increases in cholesterol metabolites upregulate LXR, which in turn activates SREBP genes.

The elongase Elovl2 was not consistently changed by any treatment, and thus could not be included in any of the groups of genes to which we were able to infer a possible regulatory mechanism. However, expression of Elovl2 mRNA was drastically reduced following cholesterol treatment at the highest lipid depletion point. Since EPA/DHA biosynthesis requires Elovl5, Elovl2 and Fad genes [12,15], and each of these genes is regulated differently in response to cellular lipid status, the factors that determine levels of LC-PUFA synthesis in salmon are likely to be complex.

In conclusion, these results indicate that the established cell line is a useful system in which to further study the molecular basis of lipid homeostasis in Atlantic salmon. The present study has revealed that SREBP and LXR interact in the regulation of range of genes

key to lipid homeostasis. In some cases the interactions appear generally similar to those being elucidated in mammalian systems. However, the present study has revealed that Elovl5, an important enzyme for the conversion of the C_{18} PUFA, 18:3n-3, to LC-PUFA, EPA and DHA, appears to be regulated differently in salmon compared to mammals, possibly being a direct target for LXR, rather than being regulated primarily by SREBP-1, albeit in an LXR-dependent pathway. This finding requires confirmation by direct promoter analyses involving the identification of functional binding sites of the salmon *elovl5* gene, similar to those studies recently performed on fish *fad* genes [18]. The regulation of endogenous LC-PUFA biosynthesis pathway in salmon is particularly important considering the critical role of n-3 LC-PUFA for human health, the role of oily fish such as salmon for provision of n-3 LC-PUFA in the human diet, and the changes to salmon feed driven by environmental, ecological and sustainability issues.

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Figure captions

Fig.1. Phylogenetic tree comparing the deduced AA sequences of salmon SREBPs with SREBP proteins from fish and other organisms. The tree was constructed using the neighbor joining method with MEGA4 [31,32]. The horizontal branch length is proportional to AA substitution rate per site. The numbers represent the frequencies with which the tree topology presented was replicated after 1000 iterations.

Fig.2. SREBPs protein structure. A: organization of SREBPs trans membrane precursor protein and nuclear, mature transcription factor. B: Amino terminus of SREBP-1 proteins highlighting the higher similarity of fish SREBP-1 to human SREBP-1a. [Adapted from 38].

Fig.3. Tissue expression profile of SREBP-1 and SREBP-2. Gene expression is represented as copy number relative to 18s RNA and values are means ± S.D. (n = 4). Bars bearing different letters are significantly different ($p < 0.05$; ANOVA, Tukey's test). PC, pyloric caeca; RM, red muscle; WM, white muscle.

Fig.4. Expression of liver X receptor (LXR), sterol regulatory element binding protein-1 and -2 (SREBP-1, SREBP-2) in SHK-1 cells exposed to 20 μM cholesterol, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) or carrier alone (control). Expression is represented in copy numbers. Normalization was performed by geNorm using a normalization factor (NF) based on the geometric mean of β-actin and elongation factor-1α reference genes. Values are means ± S.D. (n = 3). Bars bearing different letters are significantly different ($P < 0.05$; ANOVA, Tukey's test).

Fig. 5. Expression of acyl-coenzyme A oxidase (ACOX), fatty acyl elongases 2 and 5a (Elovl2, Elovl5a), Δ5 and Δ6 fatty acyl desaturases (Δ5Fad, Δ6_a Fad) and fatty acid synthase (FAS) in SHK-1 cells exposed to 20 μM cholesterol, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) or carrier alone (control). Data, normalisation and statistics are all as described in legend to Fig. 4.

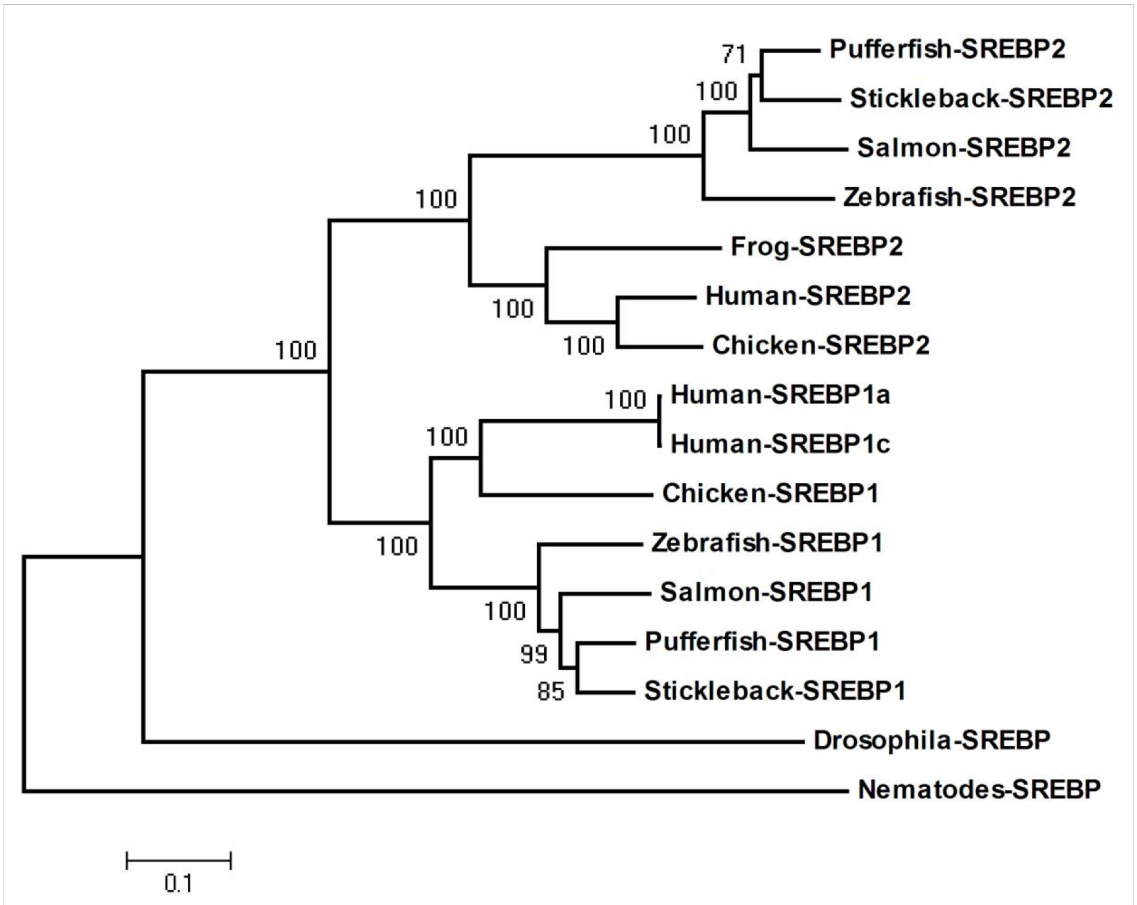
Fig. 6. Expression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA R), mevalonate kinase (MVK), isopentenyl-diphosphate delta isomerase 1 (IDI1) and 7-dehydrocholesterol reductase (DHCR7) in SHK-1 cells exposed to 20 μM cholesterol, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) or carrier alone (control). Data, normalisation and statistics are all as described in legend to Fig. 4.

Table 1. Primers used for cDNA isolation and qRT-PCR.			
Gene name	Forward primer 5'→3'	Reverse primer 5'→3'	Repository ID
Srebp1 5'R1	-	gatccaggttatccarygamggatcgtc	
Srebp1 5'R2	-	agsgcygtgtcaatatcgcttggrtcat	
Srebp1-full-1	ataacgagacagcaaaaatgaact	acgcaggggaatagaaaagaaacaga	
Srebp1-full-2	tgacacagcgctgctcaatgaca	gctgcaggccaaatgccaaaaacctc	
Srebp2 5'R1	-	cccccatcaysacaggcacrgtggtc	
Srebp2 5'R2	-	atggtctggcmcgtytgsagtagtctct	
Srebp2-full-1	tctggggcgttggtgaggtgttac	caggctggcagtgtgaagattgaagg	
Srebp2-full-2	gacatcttggttctttccttcagc	ggtgactgttctgggatctgtatgag	
qSrebp1	gccatgcgcaggttgtttcttca	tctggccaggacgcatctcacact	TC148424[b]
qSrebp2	tcgcggcctcctgatgatt	agggctaggtgactgttctgg	TC166313[b]
LXR	gccgccgctatctgaaatctg	caatccggcaaccaatctgtagg	FJ470290[a]
Fadsd5	gtgaatggggatccatagca	aaacgaacggacaaccagac	AF478472[a]
Fadsd6	ccccagacgtttgtgtcag	cctggattgttgctttggat	AY458652[a]
Elovl5a	acaagacaggaatctctttcagattaa	tctggggttactgtgctatagtgtac	AY170327[a]
Elovl2	cgggtacaaaatgtgctggt	tctgtttgccgatagccatt	TC91192[b]
DHCR7	cccggcaggcctcataaacaagta	aaggcaaaggtggacacagcatag	TC166313[b]
HMGCR	ccttcagccatgaactggat	tcctgtccacaggcaatgta	DW561983[a]
IDI1	tcagcgattgtgtagaccgtattc	gcaccatcgccacaccaaaaa	CK875291[a]
MVK	aggtggggtgaggtggagatgga	tgcgagggaccttggtgtttgtg	TC101231[b]
ACOX	aaagccttcaccacatggac	taggacacgatgccactcag	TC145297[b]
FAS	accgccaagctcagtgtgc	caggccccaaaggagtagc	DW551395[a]
β-actin	atcctgacagagcgcggttacagt	tgcccatctcctgctcaaagtcca	AF012125[a]
Gapdh	tctggaaagctgtggagggatgga	aaccttcttgatggcgtcgtagc	NM_001123561[a]
EF1α	tctggagacgctgctattgttg	gactttgtgaccttgccgcttgag	AF321836[a]
α-tubolin	gtttcgacggggccctcaatgtg	ctcatggtaggccttctcagc	NM_001141467[a]
18s	ggcgccccctcgatgctctta	cccccggccgtccctcttaat	AJ427629[a]
[a] GeneBank (http://www.ncbi.nim.nih.gov/)			
[b] Atlantic salmon Gene Index (http://compbio.dfci.harvard.edu/tgi/)			

Table 2. Effect of time in culture and lipid supplementation on total lipid content of SHK1 cells. Values (in mg/well) are means ± S.D (n = 3). Mean values bearing different superscript letters are significantly different ($p < 0.05$; ANOVA, Tukey's test). DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

Time (h)	Supplement	Lipid content
0	None	0.31 ± 0.02^{b}
72	None	0.18 ± 0.01^{c}
72	Cholesterol	0.36 ± 0.05^{b}
72	EPA	0.63 ± 0.14^{a}
72	DHA	0.60 ± 0.15^{a}

Fig.1.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Stickleback-SREBP2
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Zebrafish-SREBP2
Frog-SREBP2
Human-SREBP2
Chicken-SREBP2
Human-SREBP1a
Human-SREBP1c
Chicken-SREBP1
Zebrafish-SREBP1
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Nematodes-SREBP
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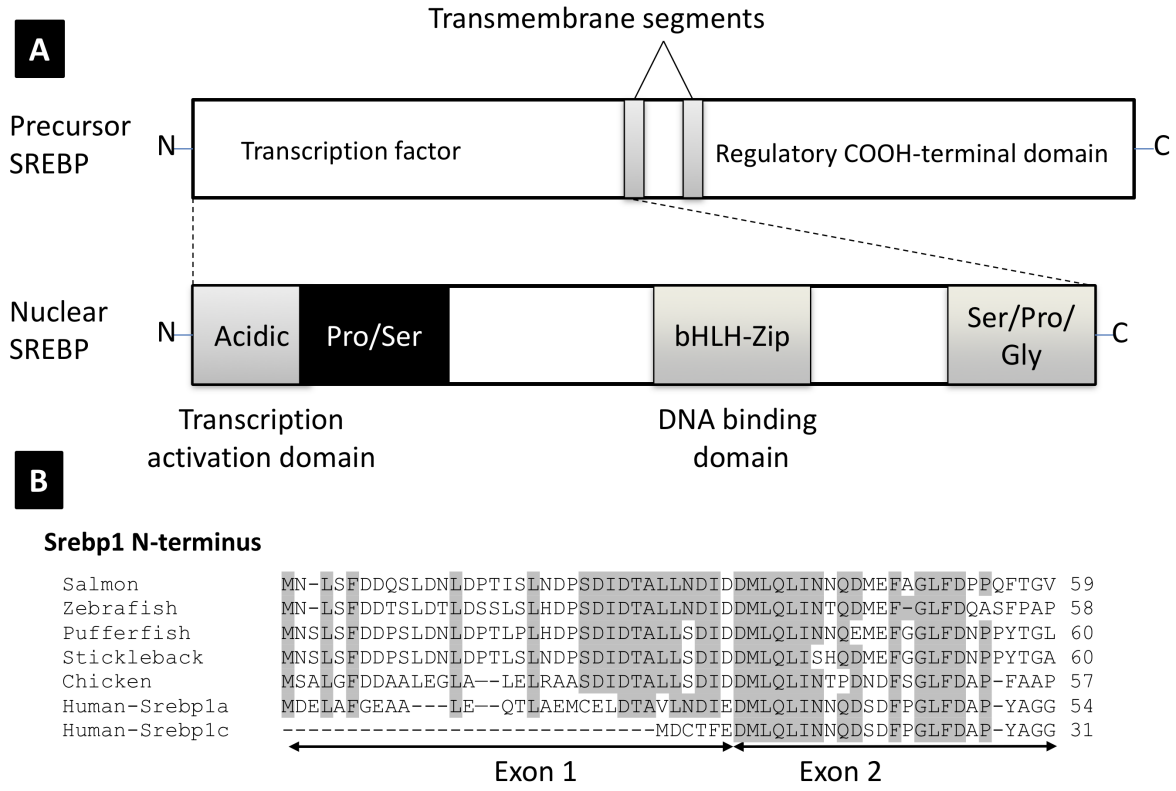
Fig.2.


A
Transmembrane segments
Precursor SREBP
N
Transcription factor
Regulatory COOH-terminal domain
C
Nuclear SREBP
N
Acidic
Pro/Ser
bHLH-Zip
Ser/Pro/ Gly
C
Transcription activation domain
DNA binding domain
B
Srebp1 N-terminus
Salmon MN-LSFDDQSLDNLDPTISLNDPSDIDTALLNDIDDMLQLINNQDMEFAGLFDPPQFTGV 59
Zebrafish MN-LSFDDTSLDTLDSSLSLHDPSDIDTALLNDIDDMLQLINTQDMEF-GLFDQASFPAP 58
Pufferfish MNSLSFDDPSLDNLDPTLPLHDPSDIDTALLSDIDDMLQLINNQEMEFGGLFDNPPYTGL 60
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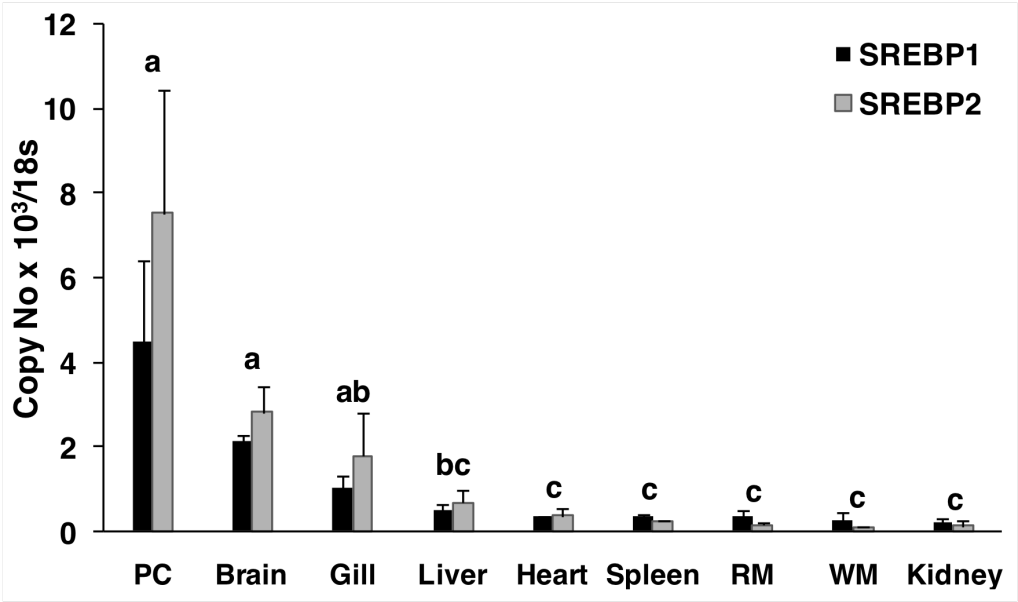
Chicken MSALGFDDAALEGLA--LELRAASDIDTALLSDIDDMLQLINTPDNDFSGLFDAP-FAAP 57
Human-Srebp1a MDELAFGEAA---LE--QTLAEMCELDTAVLNDIEDMLQLINNQDSDFPGLFDAP-YAGG 54
Human-Srebp1c ----------MDCTFEDMLQLINNQDSDFPGLFDAP-YAGG 31
Exon 1
Exon 2

Fig.3.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Heart
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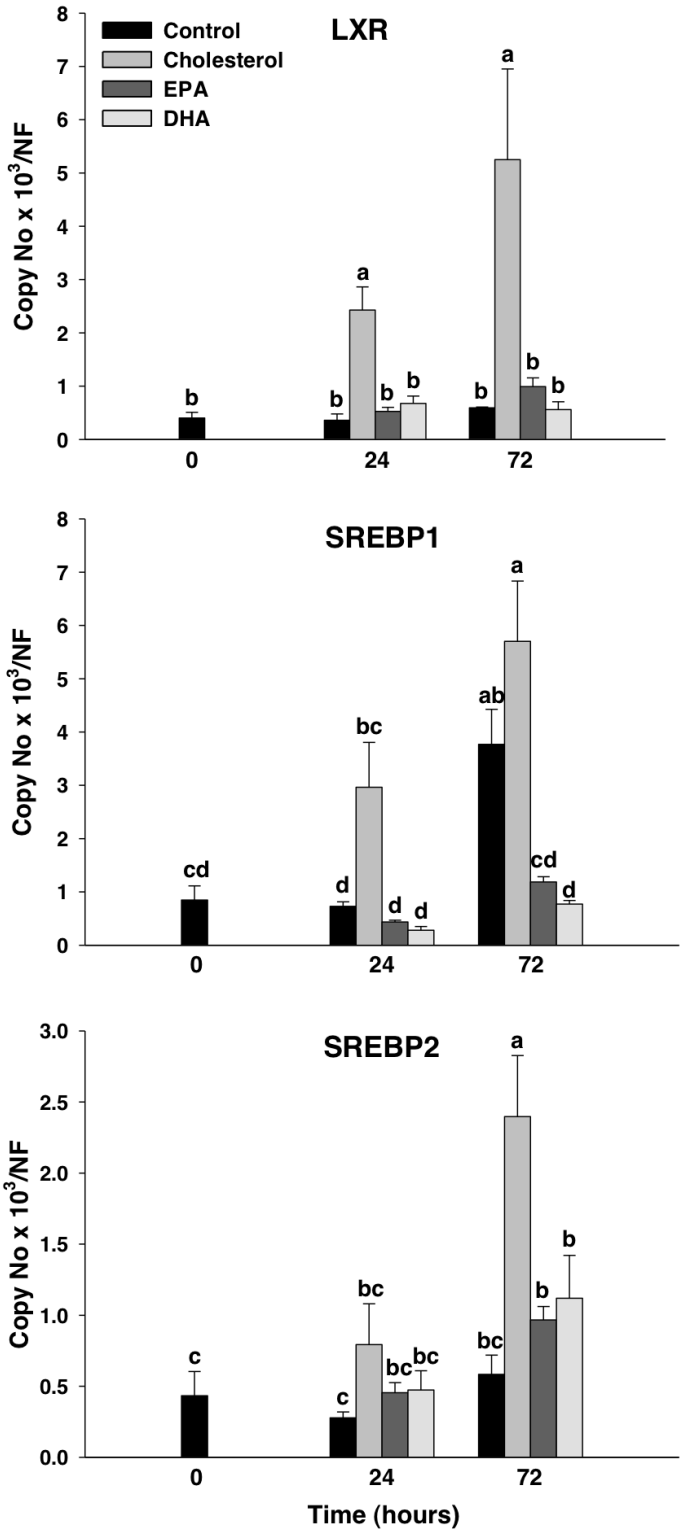
WM
Kidney

Fig.4.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24
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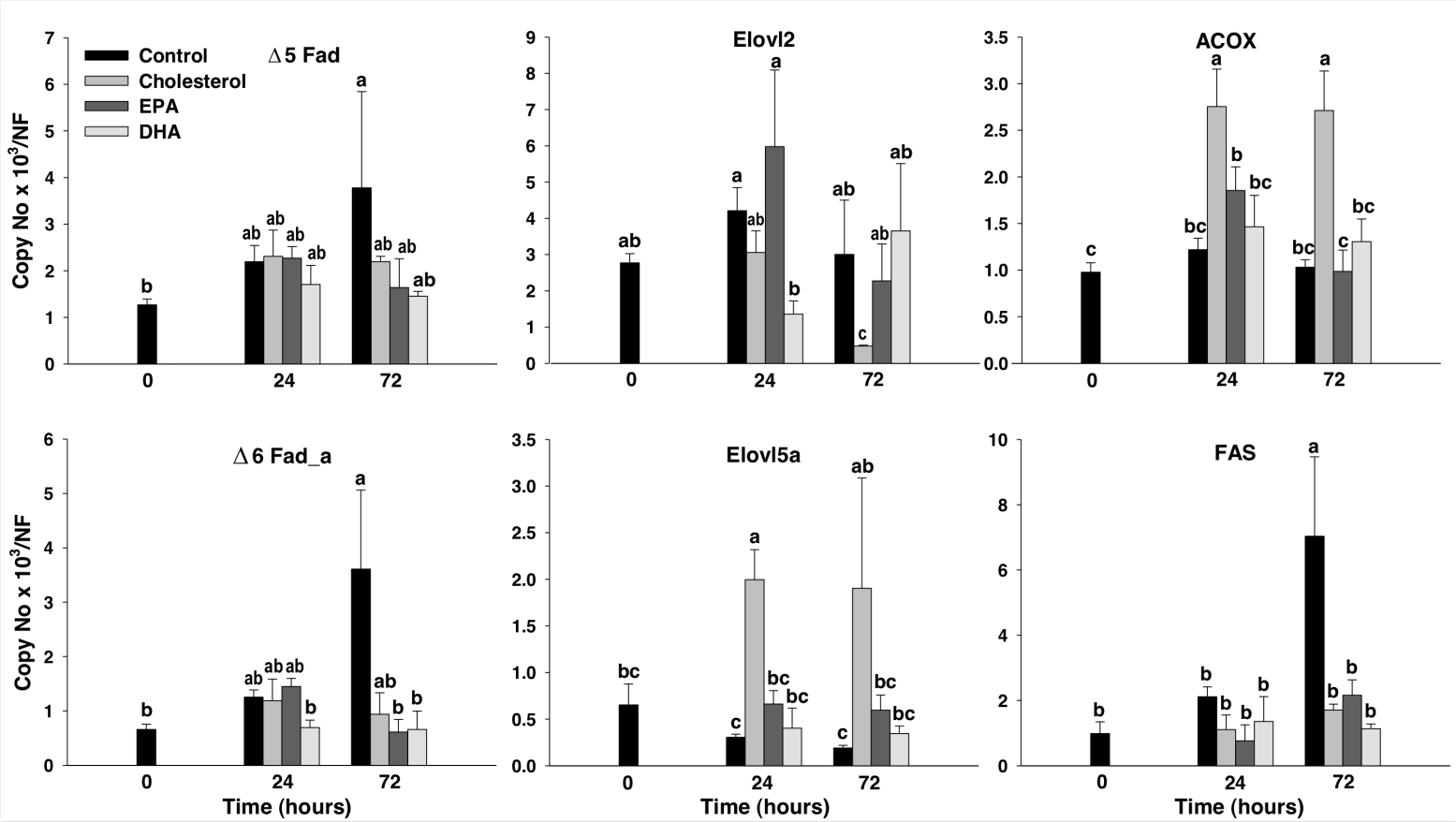
Fig.5.


Control
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Δ5 Fad
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Δ6 Fad_a
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Time (hours)
0
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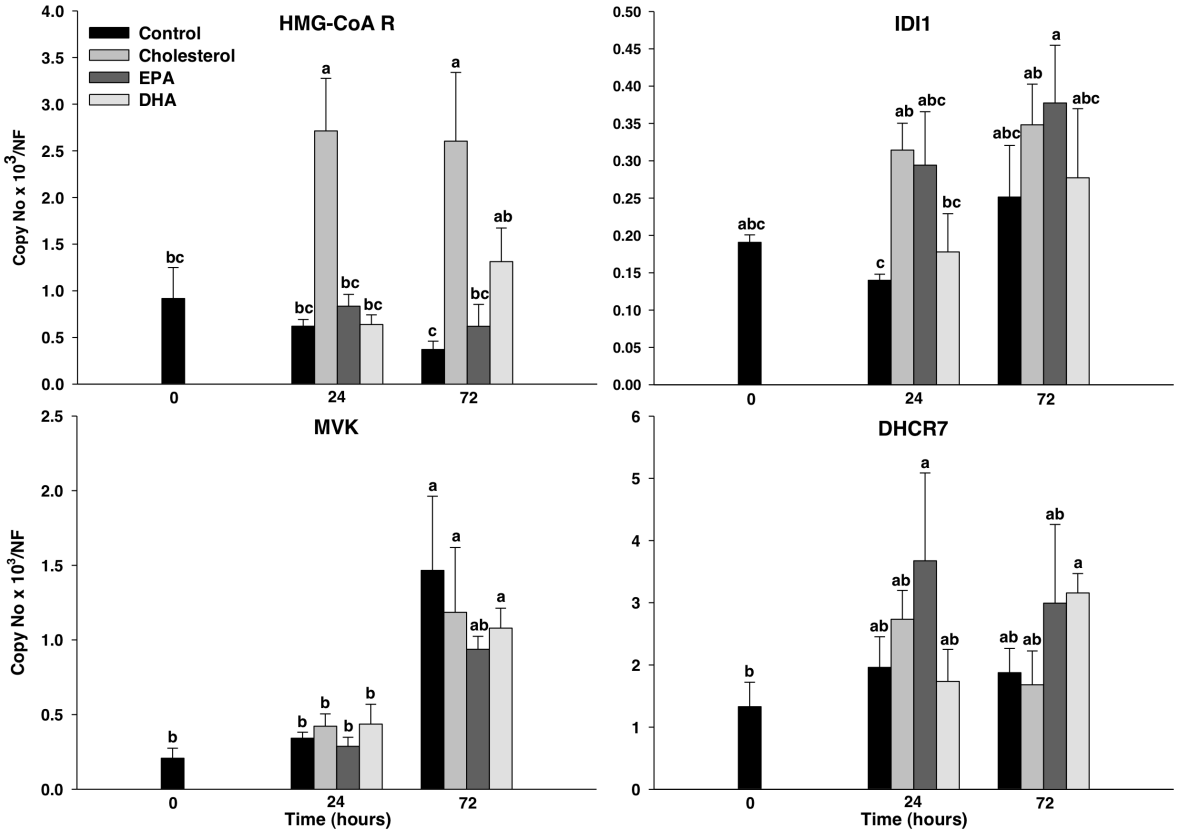
Fig.6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IDI1
MVK
DHCR7
Control
Cholesterol
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