Chitosan-mediated shRNA knockdown of cytosolic alanine aminotransferase improves hepatic carbohydrate metabolism

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Abstract

Alanine aminotransferase (ALT) catalyses a transamination reaction that links carbohydrate and amino acid metabolism. In this study, we examined the effect of silencing cytosolic ALT (cALT) expression on the hepatic metabolism in *Sparus aurata*. A number of siRNA and shRNA designed to down-regulate cALT expression were validated in HEK-293 cells transfected with plasmids expressing *S. aurata* cALT or mitochondrial ALT (mALT) isoforms: ALT silencing significantly decreased the expression levels of *S. aurata* mRNA cALT1 to 62 % (siRNA) and 48 % (shRNA) of the values observed in control cells. The effect of cALT silencing was analysed in the liver of *S. aurata* 72 h after intraperitoneal injection of chitosan-tripolyphosphate (TPP) nanoparticles complexed with a plasmid encoding a shRNA to down-regulate cALT expression (pCpG-si1sh1). In fish fed diets with different ratio of protein to carbohydrate and treated with chitosan-TPP-pCpG-si1sh1, cALT1 and cALT2 mRNA levels significantly decreased irrespective of the diet. Consistently, ALT activity decreased in liver of treated animals. In the liver of *S. aurata* treated with chitosan-TPP-pCpG-si1sh1 nanoparticles, down-regulation of cALT expression increased the activity of key enzymes in glycolysis (6-phosphofructo-1-kinase and pyruvate kinase) and protein metabolism (glutamate dehydrogenase). Besides showing for the first time that administration of chitosan-TPP-pCpG-si1sh1 nanoparticles silences hepatic cALT expression *in vivo*, our data support that down-regulation of cALT could improve the use of dietary carbohydrates to obtain energy and spare protein catabolism.

Keywords: alanine aminotransferase, siRNA, shRNA, chitosan, gene therapy, *Sparus aurata.*

Introduction

Alanine aminotransferase (ALT; *EC* 2.6.1.2) links amino acid and carbohydrate metabolism through catalysing the reversible transamination reaction between L-alanine and 2-oxoglutarate to form pyruvate and L-glutamate. Two ALT isoforms, ALT1 and ALT2, each encoded by a different gene, have been isolated in mammals (Sohocki et al. 1997; Yang et al. 2002; Jadhao et al. 2004). In teleost fish, the hepatic levels of ALT activity were referred as a good indicator of protein utilisation (Fynn-Aikins et al. 1995; Sanchez-Muros et al. 1998; Metón et al. 1999; Fernández et al. 2007). We previously reported the presence of three ALT isoforms in gilthead sea bream (*Sparus aurata*): two cytosolic isoforms resulting from alternative splicing of cALT gene (cALT1 and cALT2) and a mitochondrial enzyme (mALT) (Metón et al. 2004; Anemaet et al. 2008). cALT1 is expressed mainly in liver, brain, skeletal muscle, intestine and kidney of *S. aurata*. Higher cALT2 mRNA levels are found in heart, gill or spleen. Moderate expression levels of cALT2 are also observed in intestine, kidney and liver. The hepatic expression of cALT2 increases under gluconeogenic conditions such as starvation, while cALT1 is predominant in postprandial periods for utilisation of dietary nutrients. Thus, cALT1 is the most contributing cytosolic isoform to ALT activity in liver of fed fish (Anemaet et al. 2010).

RNA interference (iRNA) confers an effective mechanism for specific silencing of target mRNAs (Fire et al. 1998; Elbashir et al. 2001; Sifuentes-Romero et al. 2011). Plasmids or viral vectors can express small interfering RNAs (siRNAs) as short hairpin RNAs (shRNAs) to induce specific iRNA silencing effects. For the development of iRNA-based therapies and to unravel the molecular knowledge of enzymatic pathways and functional genomic studies, the delivery methods of iRNA-mediating agents is one of the major obstacles. Although viral vectors seem to be highly efficient nucleic acid carriers, safety

concerns such as cytotoxicity, oncogenicity, and immunogenicity limit their potential use. Non-viral vectors such as shRNA expression plasmids have the advantages of no integration into the host chromosome, low cost and high efficiency for gene silencing in fish cell lines and tissues (Su et al. 2008; Zenke and Kim 2008; Terova et al. 2013).

The high positive charge of chitosan allows interaction with negatively charged nucleic acids to form based systems for delivery of plasmid DNA and siRNAs into cells (Roy et al. 1999; Mao et al. 2001; Mansouri et al. 2004; Ramos et al. 2005; Mao et al. 2010; Guo et al. 2010; Ragelle et al. 2013; Ragelle et al. 2014). For aquaculture purposes, chitosan has been used for encapsulation of vaccines. However, the use of shRNA-chitosan particles to perform functional genomic studies in fish is relatively unexplored (Zenke and Kim 2008; Sifuentes-Romero et al. 2011; Borgogna et al. 2011). To our knowledge, no studies have addressed knockdown of ALT gene expression and the effect of ALT silencing on the intermediary metabolism.

In carnivorous fish, dietary amino acids are used not only as building blocks for protein synthesis, but also as important substrates for energy production and gluconeogenesis. Instead, these animals have a limited capacity for dietary carbohydrate utilisation. This metabolic profile mimics non-insulin-dependent diabetes mellitus in mammals after a glucose load and led to consider carnivorous fish as glucose intolerant (Cowey and Walton 1989; Moon 2001; Hemre et al. 2002). We previously reported that amino-oxyacetate (AOA)-mediated inhibition of cALT activity could favour the use of dietary carbohydrates in liver of *S. aurata* (González et al. 2012). In the present study, we hypothesised that administration of chitosan nanoparticles complexed with a plasmid expressing a shRNA to knockdown cALT expression in liver could improve carbohydrate utilisation for energy demands in *S. aurata*, thereby sparing the use of dietary amino acids as an energy source.

Material and Methods

Experimental design and procedures

Gilthead seabream (*Sparus aurata*) juveniles (5 g) were obtained from Piscimar (Andromeda Group, Burriana, Castellón, Spain) and maintained in our facilities distributed in aquaria of 260 L at a temperature of 21 °C. Facilities and maintenance procedures were previously described (Fernández et al. 2007). To validate chitosan-TPP nanoparticles as a vehicle to deliver the expression plasmids pCpG-siRNA (long lasting expression plasmid of siRNAs; InvivoGen, San Diego, CA, USA), pCpG-siRNA-Scramble (control plasmid that expresses a scramble sequence with no homology with known sequences; InvivoGen, San Diego, CA, USA), and pCpG-si1sh1 (expression plasmid encoding a shRNA to down-regulate cALT expression) to *S. aurata* liver, fish were fed a commercial diet (Microbaq, from Dibaq Diproteg, S.A., Segovia, Spain) for 30 days at 2 % body weight (BW). Thereafter, fish were intraperitoneally injected with chitosan-tripolyphosphate (TPP) nanoparticles complexed with pCpG-siRNA (10 μg of plasmid per gram of BW). In a second experiment, to determine the metabolic effect of pCpG-si1sh1, fish were fed for 30 days with two diets differing in the protein to carbohydrate ratio, P60 and P45 (Table 1) before treatment. For both experiments and to obtain tissue samples, animals were anesthetised with MS-222 (1:12,500) before handling to prevent stress (9:30 a.m.) and killed by cervical section. Blood was collected, and liver samples were immediately frozen in liquid N_2 and kept at -80 °C until use. The experimental procedures complied with the guidelines of the Animal Use Committee of the Universitat de Barcelona.

Construction of the expression plasmids

The coding sequence of cALT1 was obtained by PCR using oligonucleotides JDAL16 and IMAL20 (Table 2) and the previously described pcALT-GFP as template [9]. To amplify the coding domain sequence of mALT, a PCR using primer pair JDAL26/IMAL24 (Table 2) and the previously described pmALT-GFP as template was carried out [9]. The coding sequence of cALT2 was obtained by RT-PCR using total RNA isolated from *S. aurata* liver and oligonucleotides JDAL36 and IMAL20 (Table 2), designed from the previously isolated cALT2 messenger (Anemaet et al. 2008). For the three constructs, PCR products were ligated into pcDNA3 (Life Technologies, Carlsbad, CA, USA) previously digested with *Hind*III and *Bam*HI, to generate pcDNA3-cALT1, pcDNA3-mALT and pcDNA3-cALT2, respectively.

To obtain pCpG-si1sh1, primers JDsi1sh1s and JDsi1sh1as (Table 2) were mixed at a final concentration of 25 μM each, heated at 90 ºC for 1 min and then cooled down at room temperature. One hundred ng of the double-stranded product were ligated into pCpG-siRNA previously digested with *Hin*dIII and *Acc*65I.

Cell culture and transfection

The human embryonic kidney derived cell line HEK-293 (ATCC CRL-1573) was cultured in modified Essential medium (MEM), supplemented with 10% foetal bovine serum, 90 IU/l penicillin, 90 μg/ml streptomycin and 2 mM glutamine. The cells were grown at 37 ºC in 5 % CO_2. One day before transfection, 5 x 10^5 cells were seeded per well in six-well plates. For gene knockdown experiments using siRNAs, transfections were performed using 3 to 300 ng

cALT1, cALT2 or mALT expression vectors, 20 to 100 nM siRNA and 4 µl of lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) following manufacturer instructions. For gene silencing assays with shRNA expression vectors, the calcium phosphate co-precipitation method was used to transiently transfect the cells with 30 ng pcDNA3-cALT1 and 30 to 600 ng pCpG-si1sh1 or pCpG-siRNA-Scramble. To correct for variations in transfection efficiency, 300 ng of CMV-β plasmid (*lac*Z) was included in each transfection. To ensure equal DNA amounts, empty plasmids were added in each transfection. The cells were harvested 24 to 72 h later washed in PBS, lysed and used to isolate total RNA. β-Galactosidase activity in the clear lysate was determined as described elsewhere (Metón et al. 2006). siRNA1 targeting cALT1 (Table 2) was custom designed and synthesised by Bionova Cientifica, S.L. (Madrid, Spain).

Semi-quantitative RT-PCR

Total RNA was isolated from *S. aurata* liver by the Total Quick RNA Cells & Tissues kit (Talent, Trieste, Italy). Three µg of total RNA was reverse-transcribed to cDNA by incubation with 5x First Strand Buffer (Life Technologies, Carlsbad, CA, USA), 10 mM dithiothreitol, 0.5 mM dNTPs, 6.25 ng/µl random hexamers, 1 U/µl RNasin (Promega, Southampton, UK) and 10 U/µl Moloney murine leukemia virus RT (Life Technologies, Carlsbad, CA, USA) for 1 h at 37 ºC. The cDNA product and specific oligonucleotides to amplify alpha peptide of *Escherichia coli* β-galactosidase (JDpepalphaS and JDpepalphaAS; Table 2) and *S. aurata* β-actin (BA0199 and BA0299; Table 2) were used subsequently for PCR. After initial denaturation at 95 ºC for 3 min, a number of cycles between 20 and 35 at

95º for 30 sec, 68 ºC for 30 sec, and 72 ºC for 1 min, with a final extension step at 72 ºC for 7 min, were performed to verify that the PCR products amplify linearly and to determine the optimal number of cycles allowing detection without saturation of the signal. The PCR product was analysed in a 2% agarose gel.

Quantitative RT-PCR

Five micrograms of total RNA isolated from HEK-293 cells or *S. aurata* liver were reverse-transcribed to cDNA using Moloney murine leukemia virus RT (Life Technologies, Carlsbad, CA, USA) for 1 h at 37 ºC in the presence of random hexamer primers. The cDNA product was used for subsequent quantitative real time PCR (qRT-PCR). The mRNA levels of *S. aurata* cALT1, cALT2 and mALT were determined in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using 0.4 μM of each primer, 10 μl of SYBR Green (Applied Biosystems), and 1.6 μl of the diluted cDNA product in a final volume of 20 μl. Primer pairs used were JDc1s/JDc1as, JDc2s/JDc2as and JDm1s/JDm1as for *S. aurata* cALT1, cALT2 and mALT, respectively (Table 2). The amount of mRNA for the genes of interest in transiently transfected HEK-293 cells was normalised with human hypoxanthine-guanine phosphoribosyltransferase (HPRT) using primer pair JDRTPHPRTs/JDRTPHPRTas (Table 2). For *in vivo* experiments, normalisation of mRNA levels was carried out assessing *S. aurata* β-actin expression with primer pair JDBAs/JDBAas (Table 2). To correct for variations in transfection efficiency in the experiments performed on HEK-293 cells, mRNA were normalised with β-galactosidase expression using oligonucleotides JDpcmv1s and JDpcmv1as (Table 2). Variations in gene expression were calculated by the standard ΔΔCt method.

Western blot analysis

Forty micrograms of hepatic protein extract was loaded per lane in a 10% PAGE-SDS gel. After electrophoresis the gel was equilibrated in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol, pH 8.3) and electroeluted onto NytranN nylon membranes (Whatman, Kent, UK) for 3 h at 60 V at 4 °C. A rabbit polyclonal antibody raised against *S. aurata* ALT was used as primary antibody (1:1000). Immunodetection of ALT protein was performed with the Immun-Star™ Substrate Kit (Bio-Rad, Hercules, CA, USA).

Enzyme activity assays

Enzyme activities were assayed in crude extracts obtained from powdered frozen liver (1:5, w/v) homogenised in buffer H (50 mM Tris-HCl pH 7.5, 4 mM EDTA, 50 mM NaF, 0.5 mM PMSF, 1 mM DTT and 250 mM sucrose) with a PTA-7 Polytron (Kinematica GmbH, Littau-Luzern, Switzerland) (position 3, 30 s). After centrifugation at 20,000 g for 30 min at 4 ºC, the supernatant was collected and used to determine enzyme activities. ALT and aspartate aminotransferase (AST; *EC* 2.6.1.1) were assayed using commercial kits (Linear Chemicals, Montgat, Barcelona, Spain). Assays for 6-phosphofructo-1-kinase (PFK-1; *EC* 2.7.1.11), pyruvate kinase (PK; *EC* 2.7.1.40), fructose-1,6-bisphosphatase (FBPase-1; *EC* 3.1.3.11), glucose-6-phosphate dehydrogenase (G6P-DH; *EC* 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PG-DH; *EC* 1.1.1.44) and glutamate dehydrogenase (GDH; *EC* 1.4.1.2) were previously described (Metón et al. 1999; Bibiano Melo et al. 2006). Protein content in

extracts was determined by the Bradford method at 30 °C using bovine serum albumin as a standard (Bradford 1976). Spectrophotometric determinations were performed at 30 °C in a Cobas Mira S analyser (Hoffman-La Roche, Basel, Switzerland).

Preparation of chitosan-TPP-plasmid nanoparticles

Chitosan-TPP-plasmid nanoparticles were prepared using the modified version of the ionic gelation method (Calvo et al. 1997). Briefly, low molecular weight chitosan (Sigma-Aldrich, MA, USA) was added to acetate buffer solution at pH 5.0 to reach a final concentration of 2 mg/ml. The mixture was shaken for 3 hours under vigorous magnetic stirring in order to get chitosan dissolved in acetate buffer. Then it was filtered to remove any traces of undissolved chitosan. Three-hundred μg of plasmid DNA (pCpG-siRNA, pCpG-siRNA-Scramble or pCpG-si1sh1), previously linearised by restriction with *Sac*I (Fermentas, Madrid, Spain), were added to 1.2 ml of a 0.84 mg/ml TPP (Panreac, Castellar del Vallès, Barcelona, Spain) solution, and then the TPP-plasmid solution was added dropwise to 3 ml of the previously prepared chitosan solution (1:0.4 chitosan:TPP ratio) under magnetic stirring at 800 rpm (Fàbregas et al. 2013). Nanoparticles were recovered by centrifugation (12000 rpm at 15 ºC for 10 min). The chitosan-TPP-plasmid complexes were gently rinsed twice with ultrapure water and resuspended through vortex shaking with a 2 ml mannitol solution at 2% w/v, which acted as a cryoprotectant during lyophilisation. Nanoparticles were subjected to a freeze-drying process at -47 ºC. In order to remove internal residual water molecules that may not be frozen, a secondary drying was held at 25 ºC. Two hours before administration to fish, the samples were resuspended in 0.5 ml of 0.9 % NaCl solution.

Characterisation of the nanoparticles

Morphological characterisation of the nanoparticles was performed by atomic force microscopy using peak force tapping mode (Multimode 8 AFM attached to a Nanoscope III Electronics, Bruker, USA). The Z potential values, as a measure of surface charge, were determined by means of laser Doppler microelectrophoresis using a Zetasizer NanoZ (Malvern Instruments, Malvern, UK) equipped with DTS1060 capillary cells (Malvern Instruments).

Statistics

Data were analysed by one-way and two-way ANOVA using a computer program (IBM SPSS Statistics, Armonk, NY, USA). One-way statistical analysis with two levels was determined using Student's *t*-test. One-way ANOVA statistical differences among three or more levels and two-way ANOVA were determined with the Scheffé post hoc test.

Results

siRNA-mediated knockdown of *S. aurata* cALT isoforms in HEK-293 cells

HEK-293 cells were used to validate candidate siRNA sequences to knockdown *S. aurata* cALT expression *in vitro*. After transfection of HEK-293 cells with constructs expressing *S.*

aurata cALT1 (pcDNA3-cALT1), cALT2 (pcDNA3-cALT2) or mALT (pcDNA3-mALT), the expression level of ALT isoforms was assessed by measuring ALT activity in cytosolic and mitochondrial cellular fractions. Thereafter, four different siRNAs designed to silence *S. aurata* cALT isoforms were assayed to select the siRNA responsible for the highest knockdown effect on *S. aurata* cALT1 expression. Twenty-four h after transfection with 20 nM of each siRNA, 30 ng of pcDNA3-cALT1 and 300 ng of pCMV-β as an internal control for transfection efficiency, the cells were lysed and RNA isolated to perform a qRT-PCR assay to determine cALT1 expression levels. The highest ALT gene silencing effect was observed using the siRNA herein named siRNA1. Two siRNAs (siRNA2 and siRNA4) did not show any effect on cALT1 mRNA levels (data not shown). Therefore, siRNA1 was selected to perform subsequent functional genomic studies. Optimal concentration of the *S. aurata* cALT1 plasmid and time post-transfection (24 to 72 h) to improve an ALT knockdown effect in HEK-293 cells co-transfected with 20 nM siRNA1 was assayed through analysis of cALT1 expression by qRT-PCR. To this end, cALT1 silencing efficiency of siRNA1 was firstly analysed 24 h post-transfection in HEK-293 cells co-transfected with different amounts of the *S. aurata* cALT1 expression plasmid (pcDNA3-cALT1, ranging from 3 ng to 300 ng). As shown in Fig. 1a, a significant cALT1 silencing effect trigger by siRNA1 was observed when cells were transfected with at least 30 ng of pcDNA3-cALT1, which reduced *S. aurata* cALT1 mRNA levels to about 62 % of the values observed in cells not treated with siRNA. The decreased cALT1 expression observed using 30 ng of pcDNA3-cALT1 was even higher, although not significantly different, than using 300 ng of pcDNA3-cALT1.

In HEK-293 cells transfected with 20 ng of siRNA1, 30 ng de pcDNA3-cALT1 and 300 ng of pCMV-β, qRT-PCR assays were performed to determine cALT1 expression from data obtained after 24 h, 48 h and 72 h post-transfection. The highest decrease in cALT1

mRNA levels (to 66 % of the values in non-treated cells) was observed 24 h after cell transfection. Extended post-transfection times did not allow a greater knockdown effect on cALT1 expression (Fig. 1b).

In addition, we also studied the effect of siRNA1 on the expression of other *S. aurata* ALT isoforms (cALT2 and mALT) expressed in HEK-293 cells. To this end, HEK-293 cells were transfected in the presence or absence of 50 nM siRNA1, 300 ng of pCMV-β and 30 ng of either pcDNA3-cALT2 or pcDNA3-mALT. As expected, transfection with siRNA1 caused a significant knockdown effect in cALT2 mRNA levels to 79 % of the values found in the cells not treated with siRNA (Fig. 1c). However, siRNA1 did not affect mALT expression in transfected cells.

shRNA-mediated knockdown of *S. aurata* cALT1 in HEK-293 cells

Since siRNA1 was able to knockdown the expression of *S. aurata* cALT isoforms at mRNA level in HEK-293 cells, and considering that siRNAs have a short half-life inside the cells, we designed a shRNA based on the siRNA1 sequence and cloned it into pCpG-siRNA, a plasmid designed for long lasting expression of siRNAs *in vivo* as the plasmid does not induce inflammatory responses and gene silencing by methylation in vertebrate hosts. The cALT1 silencing efficiency *in vitro* of the resulting plasmid (pCpG-si1sh1) was assayed by transfection of HEK-293 cells with 30 ng of pcDNA3-cALT1, 300 ng of pCMV-β and different amounts of pCpG-si1sh1. Forty-eight h following transfection, the cells were lysed and total RNA isolated to determine cALT1 mRNA levels by qRT-PCR. Transfection with 300 ng and 600 ng pCpG-si1sh1 caused a significant decrease in cALT1 mRNA levels to about 48 % and 60 %, respectively, of the values observed in control cells (Fig. 2).

Administration of chitosan-TPP-pCpG-siRNA allows expression of E. coli β-galactosidase alpha peptide in the liver of *S. aurata*

To study the effect of cALT silencing on intermediary metabolism *in vivo*, we focused first on finding an appropriate method to deliver pCpG-si1sh1 into *S. aurata* liver cells. To assess the use of chitosan-TPP as a vehicle to deliver recombinant DNA to *S. aurata*, chitosan-TPP nanoparticles were complexed with the plasmid pCpG-siRNA, which encompasses an EM7-LacZ alpha peptide cassette that allows expression of amino acids 10-85 of *E. coli* β-galactosidase. Atomic force microscopy of chitosan-TPP nanoparticles showed a rounded morphology with mean diameter size of 193.3 ± 52.8 nm (n=3). The mean Z potential of chitosan-TPP samples was 32,0 ± 1,0 mV (n=3). Similarly as for chitosan-TPP, images of chitosan-TPP particles complexed with plasmid DNA (pCpG-siRNA) obtained with atomic force microscopy indicated a mean size of 246.0 ± 73.7 nm (n=3). The mean Z potential of chitosan-TPP-plasmid DNA particles was 14.4 ± 1.3 mV (n=3) (Fig. 3a). Two groups of fish received an intraperitoneal injection of either chitosan-TPP-pCpG-siRNA (10 μg of plasmid per gram BW) or chitosan-TPP nanoparticles (control fish). Twenty-four h after injection, the liver was excised and total RNA was isolated. Expression of bacterial β-galactosidase alpha peptide and *S. aurata* β-actin (endogenous constitutive expression gene) was analysed by semi-quantitative RT-PCR in the liver of fish administered with chitosan-TPP-pCpG-siRNA and control animals. Since the bacterial β-galactosidase alpha peptide is not present in vertebrate tissues, as expected the mRNA levels of this gene were not detected in control animals. However, bacterial β-galactosidase alpha peptide expression was observed in the

liver of all individuals administered with the chitosan-TPP-pCpG-siRNA complex (Fig. 3b). Although β-galactosidase alpha peptide mRNA was detected in the liver of all treated fish, the expression levels differed depending on the individual. This may be due to variations in the quantity of nanoparticles reaching the liver or in the number of transfected hepatic cells. To exclude whether administration of chitosan-TPP and chitosan-TPP complexed with pCpG-siRNA nanoparticles might affect blood glucose levels and the hepatic ALT activity, these parameters were analysed in *S. aurata* intraperitoneally injected with saline, chitosan-TPP or chitosan-TPP-pCpG-siRNA. No differences in glycemia levels and ALT activity were found among the three groups of fish 72 h after treatment (data not shown).

Effect of cALT silencing on liver intermediary metabolism

The fact that administration of chitosan-TPP-pCpG-siRNA nanoparticles allowed expression of bacterial alpha peptide in the liver, prompted us to prepare chitosan-TPP complexed with pCpG-si1sh1 and analyse metabolic effects due to cALT silencing in *S. aurata*. To this end, chitosan-TPP, chitosan-TPP-pCpG-siRNA-Scramble (10 μg of plasmid per gram BW) or chitosan-TPP-pCpG-si1sh1 (10 μg of plasmid per gram BW) were intraperitoneally injected to three groups of *S. aurata* fed on Microbaq diet. Seventy-two h following treatment, cALT1 and cALT2 mRNA levels, immunodetectable ALT protein and enzyme activity corresponding to ALT and AST were determined in liver samples. As shown in Figs. 4a and 4b, cALT1 mRNA levels and ALT enzyme activity in *S. aurata* administered with chitosan-TPP-pCpG-si1sh1 significantly decreased to a 63 % and 70 %, respectively, of the values found in the group of animals treated with chitosan-TPP. Although not significant,

a similar tendency to decrease cALT2 expression was also observed in pCpG-si1sh1-treated fish (88 % of the values in controls). Consistently, administration of pCpG-si1sh1 caused a significant reduction of immunodetectable ALT protein levels in liver crude extracts compared to the values obtained in controls (fish intraperitoneally injected with chitosan-TPP or chitosan-TPP-pCpG-siRNA-Scramble). No significant differences were found in AST activity values among the three groups of animals.

To determine whether knockdown of cALT expression mediated by pCpG-si1sh1 depends on the nutrient composition of the diet, we intraperitoneally injected chitosan-TPP, chitosan-TPP-pCpG-siRNA-Scramble or chitosan-TPP-pCpG-si1sh1 to two groups of *S. aurata* fed 25 days on diets differing in the protein/carbohydrate ratio, diet P60 (high protein and low carbohydrate content) and P45 (low protein and high carbohydrate content). ALT activity and mRNA levels of cALT1, cALT2 and mALT isoforms were analysed in liver samples of *S. aurata* 72 h after treatment (Fig. 5). Consistent with previous results, the highest ALT activity was found in the animals fed the high protein/low carbohydrate diet (P60) (Metón et al. 1999; Fernández et al. 2007; González et al. 2012). Correspondingly, increased cALT1 and cALT2 mRNA levels were observed in the animals fed diet P60. However, mALT expression remained unaffected by diet composition. Administration of chitosan-TPP-pCpG-si1sh1 decreased cALT1 and cALT2 expression to 53 % and 72 %, respectively, of the values observed in *S. aurata* fed diet P60, and to 55 % and 57 %, respectively, of the mRNA levels in those fed diet P45. Knockdown of cALT expression caused a concomitant decrease in ALT activity to 59 % and 74 % of the values determined in control animals fed diets P60 and P45, respectively. cALT silencing mediated by chitosan-TPP-pCpG-si1sh1 did not affect mALT expression levels irrespective of the diet supplied.

In order to unravel functional genomic effects associated with silencing of cALT expression, the activity of key enzymes in carbohydrate and protein metabolism were also

determined in the liver of fish fed either diet P60 or P45 and administered with chitosan-TPP, chitosan-TPP-pCpG-siRNA-Scramble or chitosan-TPP-pCpG-si1sh1. In agreement with previous results, the hepatic activity of glycolytic enzymes PFK-1 and PK and key enzymes of the pentose phosphate pathway (G6P-DH and 6PG-DH) was higher in the liver of *S. aurata* fed the low protein/high carbohydrate diet (P45) (Metón et al. 1999; Fernández et al. 2007). Knockdown of cALT expression mediated by administration of chitosan-TPP-pCpG-si1sh1, significantly increased PFK-1 (1.4- and 1.9-fold increase compared to controls injected with chitosan-TPP and fed diets P60 and P45, respectively) and PK activity (2.7- and 3.2-fold increase compared to fish injected with chitosan-TPP and fed diets P60 and P45, respectively) (Figs. 6a and 6b). For both enzymes, cALT silencing caused a higher effect on *S. aurata* fed diet P45. However, no significant changes were detected in the gluconeogenic FBPase-1 activity in the liver of animals fed either diet P60 or P45 and treated with chitosan-TPP, chitosan-TPP-pCpG-siRNA-Scramble or chitosan-TPP-pCpG-si1sh1 (Fig. 6c).

A tendency to present increased activity values (about 1.2-fold) was also found for key enzymes of pentose phosphate pathway (G6P-DH and 6PG-DH) in the liver of *S. aurata* administered with chitosan-TPP-pCpG-si1sh1 and fed diet P60 or P45 (Figs. 7a and 7b). Concerning GDH, an enzyme with a major role in nitrogen balance, the highest hepatic activity values were observed when feeding the high protein/low carbohydrate diet (P60). Treatment with chitosan-TPP-pCpG-si1sh1 significantly increased GDH activity (1.7- and 1.3-fold in the liver of *S. aurata* fed diet P60 or P45, respectively) (Fig. 7c).

Discussion

In previous studies we reported that ALT activity levels in *S. aurata* liver depends on the nutritional composition of the diet (Metón et al. 1999; Fernández et al. 2007; Anemaet et al. 2008). Moreover, we found that the AOA-dependent inhibition of cytosolic ALT activity may improve the use of dietary carbohydrates in *S. aurata* through a mechanism involving increased PK activity in liver (González et al. 2012). The aim of the present study was to analyse functional genomic effects associated with gene knockdown of *S. aurata* cALT isoforms in liver. To this end, *S. aurata* fingerlings were intraperitoneally administered with chitosan-TPP complexed with a plasmid expressing shRNAs that encoded siRNA for targeting cALT isoforms. In addition to study the effect of cALT knockdown on ALT expression, the activity of key enzymes involved in metabolic pathways related to carbohydrate and protein metabolism was also determined in the liver of fish subjected to cALT silencing.

In order to down-regulate the expression of cALT isoforms, we firstly validated *in vitro* different specifically designed siRNAs. At present there is no availability of a stable hepatic cell line derived from *S. aurata* that is easily transfectable, and therefore selected siRNAs were validated in HEK-293 cells co-transfected with constructs expressing *S. aurata* ALT isoforms. This allowed us to choose siRNA1 to perform further experiments, as it produced the more pronounced knockdown effect on cALT expression and did not affect mALT mRNA levels. siRNA1 can interact with positions 519-537 and 604-622 of cALT1 and cALT2 mRNA sequences (GenBank no. AY206502 and DQ334748), respectively, and down-regulate both cALT1 and cALT2 expression. In fact, siRNA1 targets both isoforms, which are produced by alternative splicing of cALT gene (Anemaet et al. 2008). However, *in vitro* and *in vivo* data indicate that siRNA1 appeared to silence cALT1 expression somewhat more efficiently than cALT2, possibly as a result of secondary structure differences between

cALT1 and cALT2 messengers. Indeed, cALT1 lacks an extra 85 bp-exon present in cALT2 (Anemaet et al. 2008).

Based on *in vitro* results with siRNA1, and with the intention of extending siRNA1 half-life inside the cells, a siRNA1-based shRNA was designed and cloned into pCpG-siRNA to obtain pCpG-si1sh1. In fish species other than zebrafish, siRNAs for gene silencing was firstly used in rainbow trout embryos. The authors showed the ability of specific siRNAs to target foreign and endogenous cell gene expression (Boonanuntanasarn et al. 2003). Since then, a few studies have reported the use of shRNAs to produce siRNAs and knockdown the expression of specific genes in fish-derived cell lines and fish species (Su et al. 2008; Zenke and Kim 2008; Terova et al. 2013). Our *in vitro* results indicate that administration of a shRNA-based vector (pCpG-si1sh1) can be efficiently used to knockdown cALT mRNA levels, and thus reduce the economic cost of using synthesised siRNAs.

Based on its beneficial properties compared to viral vectors, chitosan-DNA complexes have increased their use as carrier systems for nucleic acids delivery into cells in culture or animal tissues (Kiang et al. 2004; Huang et al. 2005; Lavertu et al. 2006; Howard et al. 2006; Katas and Alpar 2006; Wang et al. 2009; Mao et al. 2010; Techaarpornkul et al. 2010; Ballarín-González et al. 2013). The low toxicity and the capacity of chitosan to form nanoparticles with nucleic acids represent good characteristics to explore the potential of chitosan-DNA particles for hepatic ALT expression silencing and its downstream metabolic effects. In this regard, it was reported that chitosan nanoparticles complexed with DNA located mainly in liver and kidney following intravenous injection in mice, causing minimal toxicity effects (Mao et al. 2001). In the present study, intraperitoneal administration of chitosan-TPP complexed with the empty vector pCpG-siRNA allowed us to detect expression of bacterial β-galactosidase alpha peptide in the *S. aurata* liver, which suggests that the use of chitosan-TPP complexed with plasmid DNA could be a good strategy to analyse functional

genomic effects caused after silencing cALT expression in the liver. Furthermore, no damage or death of animals was found after treatment with chitosan-TPP-DNA particles. Therefore, we have used chitosan-TPP-pCpG-si1sh1 nanoparticles in order to express a specific shRNA with the aim to induce a siRNA-mediated knockdown of cALT expression in the *S. aurata* liver and to study functional genomic effects derived from cALT silencing *in vivo*.

Our findings indicate that intraperitoneal delivery of chitosan-TPP-pCpG-si1sh1 nanoparticles to *S. aurata* down-regulated hepatic mRNA levels of both cALT1 and cALT2 and caused a concomitant decrease in ALT activity irrespective of the diet supplied. Consistent with previous observations, the highest cALT1 and cALT2 expression and ALT activity values were found in the group of animals fed the high protein/low carbohydrate diet (P60). This results confirm that the expression of cALT isoforms represents a biomarker that is sensitive to fish nutritional status and changes in the nutrient composition of the diet (Metón et al. 1999; Fernández et al. 2007).

To analyse the effect of cALT knockdown on the *S. aurata* intermediary metabolism, activity of key enzymes in glycolysis, gluconeogenesis, pentose phosphate pathway and protein metabolism was determined in the liver of *S. aurata* treated with chitosan-TPP-pCpG-si1sh1. Silencing of cALT isoforms led to increased activity of glycolytic enzymes (PFK-1 and PK), whereas did not affect the gluconeogenic FBPase-1. Concerning the glycolytic enzymes, the effect of cALT down-regulation was significantly more pronounced when feeding the low protein/high carbohydrate diet (P45). In this regard, the hepatic activity of PFK-1 and PK were previously found to correlate positively with the carbohydrate content of the diet and negatively with dietary protein in *S. aurata* (Metón et al. 1999; Fernández et al. 2007). Taken together, our findings suggest that cALT knockdown stimulates the glycolytic flux in the *S. aurata* liver. In a previous study where cytosolic ALT activity was inhibited by addition of AOA, we also reported increased PK activity in this species (González et al.

2012). Interestingly, a tendency to increase activity of key enzymes in the oxidative phase of the pentose phosphate pathway (G6P-DH and 6PG-DH) was also observed in the liver of *S. aurata* treated with chitosan-TPP-pCpG-si1sh1 nanoparticles, supporting that carbohydrate mobilisation occurs in the liver as a result of decreased cALT expression.

Moreover, silencing of cALT expression increased hepatic GDH activity. *S. aurata* fed the high protein/low carbohydrate diet (P60) exhibited the highest GDH activity levels as a result of cALT down-regulation. GDH links amino acids, carbohydrate metabolism and cellular bioenergetic processes by producing α-ketoglutarate, which can replenish tricarboxylic acid cycle intermediates or generate glutamate for metabolic purposes. Contradictory results have been reported regarding the effect of dietary nutrients on GDH activity (Cowey and Walton 1989; Gómez-Requeni et al. 2003; Figueiredo-Silva et al. 2010; Liu et al. 2012). This may be explained by the fact that GDH undergoes a complex allosteric regulation by amino acids and metabolites, such as activation by ADP and leucine, and inhibition by GTP, ATP and palmitoyl-CoA (Stanley 2009). The rationale behind increased GDH activity resulting from silenced cALT expression in the *S. aurata* liver remains unclear.

In conclusion, our results demonstrate for the first time that chitosan-TPP particles complexed with a shRNA expression vector, can efficiently knockdown mRNA levels of cALT isoforms *in vivo*. Indeed, down-regulation of ALT expression in liver of *S. aurata* might be a good strategy to improve the use of dietary carbohydrates as metabolic fuel, via glucose oxidation through glycolysis or the pentose phosphate pathway, and thus promote the sparing of dietary protein catabolism and increase its utilisation for growth. Development of long-term nucleic acid delivery methods to silence gene expression *in vivo* will be necessary to further assess the effects of cALT silencing on the use of dietary carbohydrates and growth performance in fish in culture.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Table 1. Composition of the diets supplied to *S. aurata* in this study.

	P60	P45	Microbaq
Formulation (%)			
Mineral mixture[a]	0.9	0.9	1.7
Brown fish meal[b]	84.7	63.9	60.5
Fish oil[c]	5.0	8.4	13.5
Starch[d]	7.2	24.6	-
Wheat meal	-	-	11.4
Wheat gluten	-	-	8.3
Soluble fish extract	-	-	2.5
Yeast extract	-	-	1.0
Soy lecithin	-	-	1.0
Vitamin mixture[e]	0.2	0.2	0.1
Carrageenan[f]	2.0	2.0	-
Chemical analysis (%)			
Protein	60	45.3	51.0
Carbohydrates[g]	7.2	24.6	16.3
Fat	14.7	15.7	20.1
Moisture	2.3	1.7	2.1
Celluloses	1	1	0.5
Phosphate	1.3	1.3	1.2
Ash	14.6	11.2	10.5
Gross energy (kJ/g)[h]	21.3	21.2	23.0

[a]Mineral mixture provided (mg/Kg): $CaHPO_4 \cdot 2H_2O$, 7340; MgO, 800; KCl, 750; $FeSO_4 \cdot 7H_2O$, 60; ZnO, 30; MnO2, 15; $CuSO_4 \cdot 5H_2O$, 1.7; $CoCl_2 \cdot 6H_2O$, 1.5; KI, 1.5; Na_2SeO_3, 0.3. [b]Corpesca S.A. Super-Prime fish meal (Santiago de Chile, Chile). [c]Cod liver oil from A.F.A.M.S.A. (Vigo, Spain). [d]Pregelatinised corn starch from Brenntag Química S.A. (St. Andreu de la Barca, Barcelona, Spain). [e]Vitamin mixture provided (mg/Kg): choline chloride, 1200; myo-inositol, 400; ascorbic acid, 200; nicotinic acid, 70; all-rac-tocopherol acetate, 60; calcium pantothenate, 30; riboflavin, 15; piridoxin, 10; folic acid, 10; menadione, 10; thiamin-HCl, 8; all-trans retinol, 2; biotin, 0.7 cholecalciferol, 0.05; cyanocobalamin,

0.05. [f]Iota carrageenan (Sigma-Aldrich). [g]Carbohydrates were calculated by difference. [h]Calculated from gross composition (protein 24 kJ/g, lipids 39 kJ/g, carbohydrates 17kJ/g).

Table 2. Primers used in the present study.

Primer	Sequence (5' to 3')
JDAL16	GA**AAGCTT**AACATGTCCCACCAGGCGGC
IMAL20	GA**GGATCC**CGTGAGAACTCTTGTGTGAAGCG
JDAL26	GA**AAGCTT**AACATGTCGGCTACAAGGATG
IMAL24	GA**GGATCC**CGTGAGAACTCTTGTGTGAAGCG
JDAL36	GA**AAGCTT**TTCATGTTTCAGATATCAGTCCAGAG
JDsi1sh1s	GTACCTCGAAGAGAGATGGTGGAATCTCAAGAGG
	ATTCCACCATCTCTCTTCTTTTTGGAAA
JDsi1sh1as	AGCTTTTCCAAAAAGAAGAGAGATGGTGGAATCC
	TCTTGAGATTCCACCATCTCTCTTCGAG
siRNA1	GAAGAGAGAUGGUGGAAUCTT
JDpepalphaS	ATGGACCCTGTTGTGCTGCAAAGG
JDpepalphaAS	TAGCTCAGGTTTACTTCTGGCACC
BA0199	GACAACGGATCCGGTATGTGC
BA0299	GACCTGTCCGTCGGGCAGCTC
JDc1s	TTACCCTGAACTCCTGAAAGACAAC
JDc1as	CCGCAGGCCTCGAGAATAC
JDc2s	TCACACTCTCTATCTGCTCTCGTGC
JDc2as	GAGAGGTTGAGGAGTGAGCAGAAAG
JDm1s	CCAGGGTCCGCTCACTGAT
JDm1as	GTCGCGCTCACACTCGATAA
JDRTPHPRTs	CATTGTAGCCCTCTGTGTGCTC
JDRTPHPRTas	CCAGCAGGTCAGCAAAGAATTT
JDBAs	CGTCCACCGCAAATGCTTC
JDBAas	GTTGTTGGGCGTTGGTTGG
JDpcmv1s	ACAACCCGTCGGATTCTCC
JDpcmv1as	CCCATTACGGTCAATCCGC

The following primers contain restriction sites indicated in bold and underlined: JDAL16, JDAL26 and JDAL36, *Hin*dIII; IMAL20 and IMAL24, *Bam*HI.

Figure captions

Fig. 1. siRNA-mediated down-regulation of *S. aurata* cALT isoforms in HEK-293 cells expressing piscine cALT1, cALT2 and mALT. **a** HEK-293 cells grown in 6-well plates were transfected with or without siRNA1 (20 nM), different amounts of an expression plasmid encoding *S. aurata* cALT1 (3 ng, 30 ng or 300 ng) and CMV-β (300 ng). Twenty-four h following transfection, cells were lysed and RNA isolated. **b** HEK-293 cells grown in 6-well plates were transfected with or without siRNA1 (20 nM), an expression plasmid encoding *S. aurata* cALT1 (30 ng) and CMV-β (300 ng). Cells were lysed and RNA isolated at 24 h, 48 h and 72 h following transfection. **c** HEK-293 cells grown in 6-well plates were transfected with or without siRNA1 (50 nM), 30 ng of expression plasmids encoding *S. aurata* cALT2 and mALT and CMV-β (300 ng). Twenty-four h following transfection, cells were lysed and RNA isolated. mRNA levels of *S. aurata* ALT isoforms relative to β-galactosidase (internal control of transfection) and human HPRT were determined by qRT-PCR. Normalised mRNA levels in the cells transfected without siRNA1 were set at 1. The data represent the mean ± S.D. values of three independent experiments performed in duplicate. Statistical significance related to cells transfected without siRNA1 is indicated as follows: $^{**}P < 0.01$; $^{***}P < 0.001$.

Fig. 2. shRNA-mediated down-regulation of *S. aurata* cALT1 expressed in HEK-293 cells. HEK-293 cells grown in 6-well plates were transfected with different amounts of pCpG-si1sh1 (0, 30 ng, 300 ng or 600 ng), an expression plasmid encoding *S. aurata* cALT1 (30 ng) and CMV-β (300 ng). Forty-eight h following transfection, cells were lysed and RNA isolated. mRNA levels of *S. aurata* cALT1 relative to β-galactosidase (internal control of

transfection) and human HPRT were determined by qRT-PCR. Normalised mRNA levels in the cells transfected in the absence of pCpG-si1sh1 were set at 1. The data represent the mean ± S.D. (n=7). Statistical significance related to cells transfected in the absence of pCpG-si1sh1 is indicated as follows: $^{**}P < 0.01$.

Fig. 3. Atomic force microscopy images of chitosan-TPP-pCpG-siRNA nanoparticles and expression of *Escherichia coli* β-galactosidase alpha peptide in liver of *S. aurata* administered with chitosan-TPP-pCpG-siRNA. **a** Atomic force microscopy images of chitosan-TPP (panel a) and chitosan-TPP-pCpG-siRNA (panel b) nanoparticles. Average particle size for chitosan-TPP and chitosan-TPP-pCpG-siRNA were measured on three samples of each nanoparticle preparation. **b** Two groups of five fish fed 20 d at a daily ration of 2 % BW with Microbaq diet were intraperitoneally injected with chitosan-TPP or chitosan-TPP-pCpG-siRNA (10 µg of plasmid per gram BW). The mRNA levels of *E. coli* β-galactosidase alpha peptide were analysed by semi-quantitative RT-PCR in the *S. aurata* liver 24 h following treatment. PCR products corresponding to individual samples were size-fractionated by electrophoresis on a 1% agarose gel and visualised by ethidium bromide staining.

Fig. 4. Effect of cALT silencing on the expression of ALT isoforms in liver of *S. aurata*. Three groups of fish fed 20 d at a daily ration of 2 % BW with Microbaq diet were intraperitoneally injected with chitosan-TPP (–), chitosan-TPP-pCpG-siRNA-Scramble (Scramble; 10 µg of plasmid per gram BW) or chitosan-TPP-pCpG-si1sh1 (si1sh1; 10 µg of plasmid per gram BW). **a** Immunodetection of ALT protein in liver crude extracts, and analysis of hepatic cALT1 and cALT2 mRNA levels relative to *S. aurata* β-actin by qRT-

PCR were performed 72 h following treatment. Normalised mRNA levels in the fish administered with chitosan-TPP were set at 1. Expression data represent the mean ± S.D. values (n=6). **b** ALT and AST specific activity values were determined in the fish liver 72 h following treatment. The data represent the mean ± S.D. values (n=4). Statistical significance related to the fish administered with chitosan-TPP is indicated as follows: *$P < 0.05$, **$P < 0.01$.

Fig. 5. Effect of cALT silencing on the expression of ALT isoforms in liver of *S. aurata* fed diets differing in nutrient composition. Three groups of fish fed 25 d at a daily ration of 2 % BW with either diet P60 or diet P45 were intraperitoneally injected with chitosan-TPP (–), chitosan-TPP-pCpG-siRNA-Scramble (Scramble; 10 μg of plasmid per gram of BW) or chitosan-TPP-pCpG-si1sh1 (si1sh1; 10 μg of plasmid per gram of BW). (Analyses were performed on liver samples obtained 72 h following treatment. **a** The mRNA levels of cALT1 relative to *S. aurata* β-actin were determined by qRT-PCR. **b** The mRNA levels of cALT2 relative to *S. aurata* β-actin were determined by qRT-PCR. **c** The mRNA levels of mALT relative to *S. aurata* β-actin were determined by qRT-PCR. **d** ALT activity was assayed in liver extracts. The data represent the mean ± S.D. values (n=7-11). Statistical significance for independent variables (Diet and Treatment) and the interaction between independent variables are indicated as follows: **$P < 0.01$; ***$P < 0.001$; NS, not significant. Homogeneous subsets for the independent variable involving three groups or more (Treatment) is indicated with different letters ($P < 0.05$).

Fig. 6. Effect of cALT silencing on the activity of key enzymes in glycolysis-gluconeogenesis in liver of *S. aurata*. Three groups of fish fed 25 d at a daily ration of 2 %

BW with either diet P60 or diet P45 were intraperitoneally injected with chitosan-TPP (–), chitosan-TPP-pCpG-siRNA-Scramble (Scramble; 10 μg of plasmid per gram BW) or chitosan-TPP-pCpG-si1sh1 (si1sh1; 10 μg of plasmid per gram BW). Enzyme activities were assayed on liver extracts from tissue samples obtained 72 h following treatment. **a** PFK-1 activity. **b** PK activity. **c** FBPase-1 activity. The data represent the mean ± S.D. values (n=7-13). Statistical significance for independent variables (Diet and Treatment) and the interaction between independent variables are indicated as follows: $^{**}P < 0.01$; $^{***}P < 0.001$; NS, not significant. Homogeneous subsets for the independent variable involving three groups or more (Treatment) are indicated with different letters ($P < 0.05$).

Fig. 7. Effect of cALT silencing on the activity of key enzymes in pentose phosphate pathway and GDH in liver of *S. aurata*. Three groups of fish fed 25 d at a daily ration of 2 % BW with either diet P60 or diet P45 were intraperitoneally injected with chitosan-TPP (–), chitosan-TPP-pCpG-siRNA-Scramble (Scramble; 10 μg of plasmid per gram BW) or chitosan-TPP-pCpG-si1sh1 (si1sh1; 10 μg of plasmid per gram BW). Enzyme activities were assayed on liver extracts from tissue samples obtained 72 h following treatment. **a** G6P-DH activity. **b** 6PG-DH activity. **c** GDH activity. The data represent the mean ± S.D. values (n=7-13). Statistical significance for independent variables (Diet and Treatment) and the interaction between independent variables are indicated as follows: $^{**}P < 0.01$; $^{***}P < 0.001$; NS, not significant. Homogeneous subsets for the independent variable involving three groups or more (Treatment) are indicated with different letters ($P < 0.05$).

Figure 1

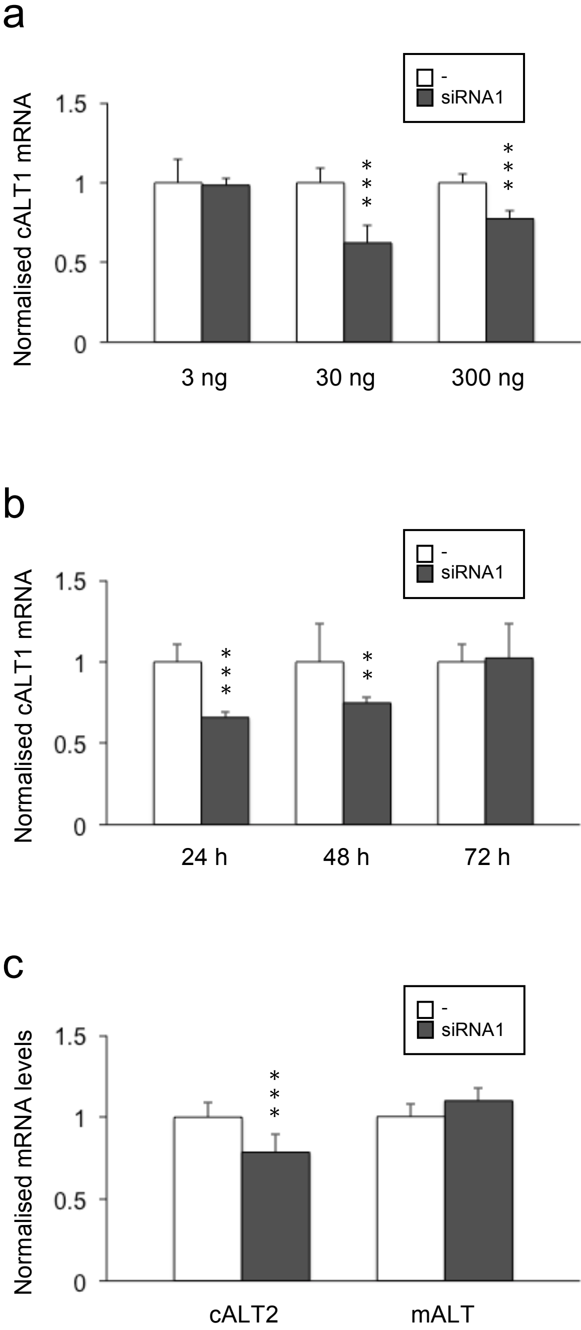


Figure 2

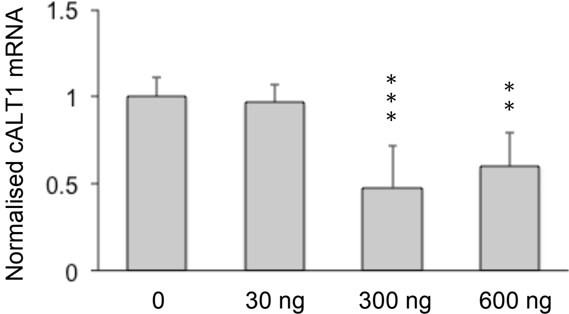
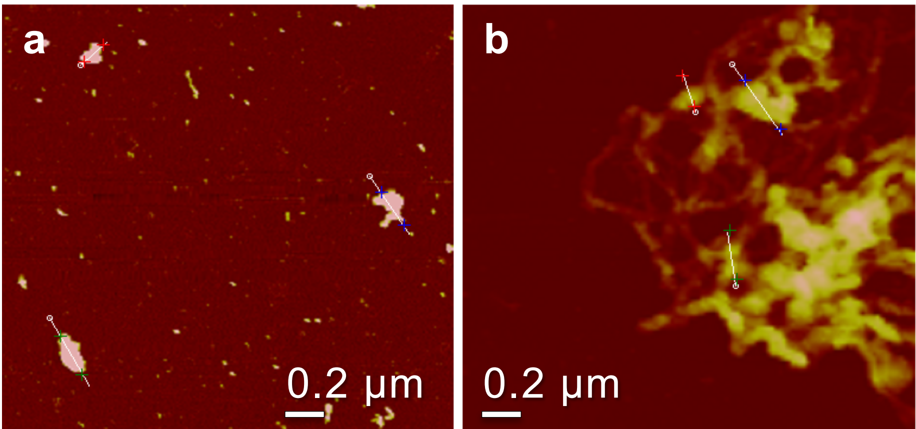


Figure 3

a



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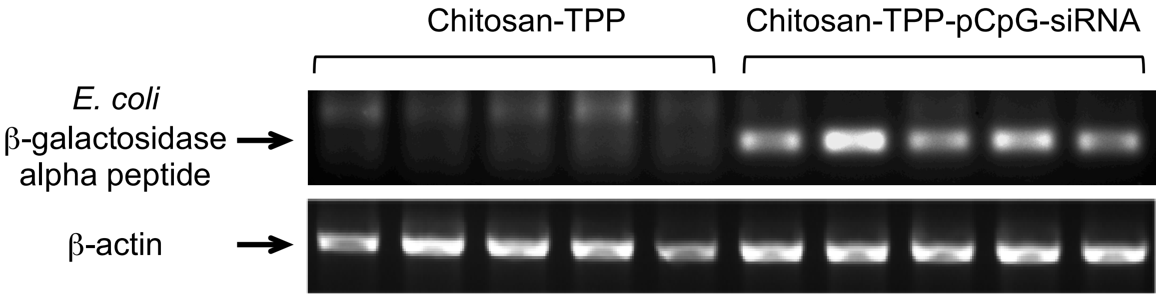

Figure 4

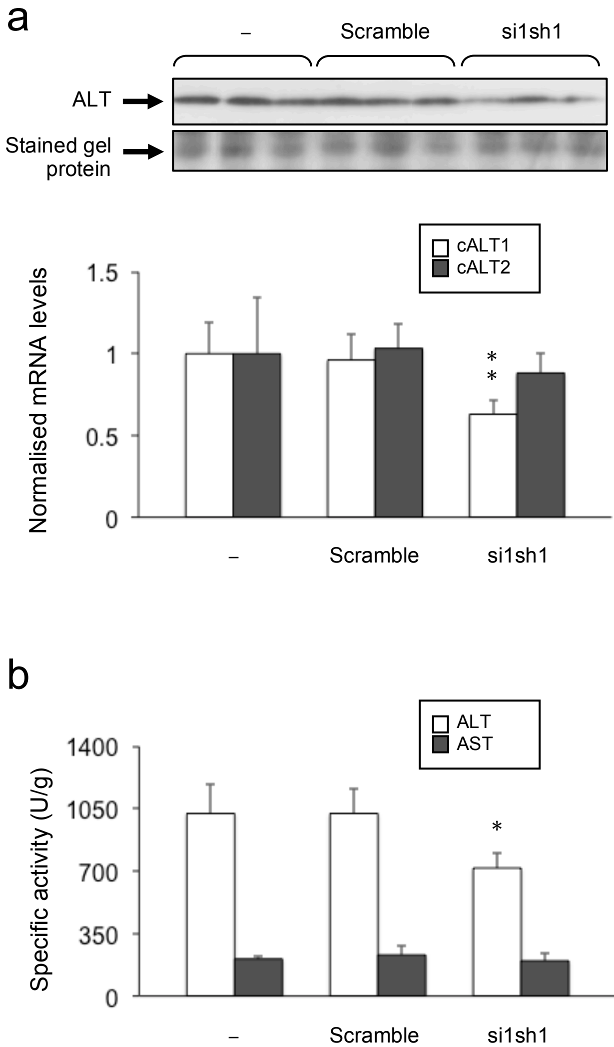

Figure 5

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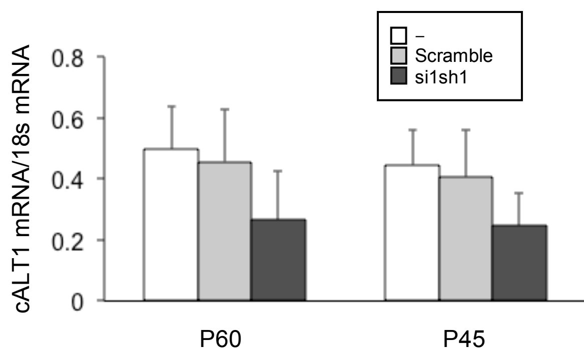

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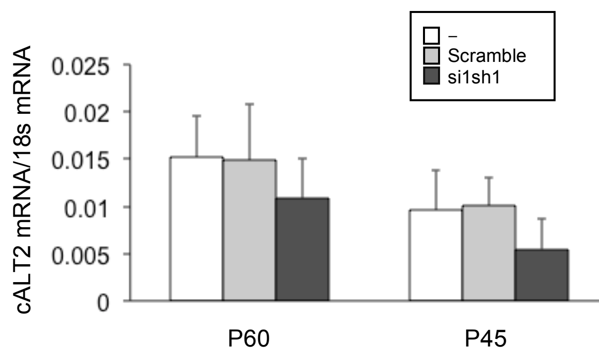

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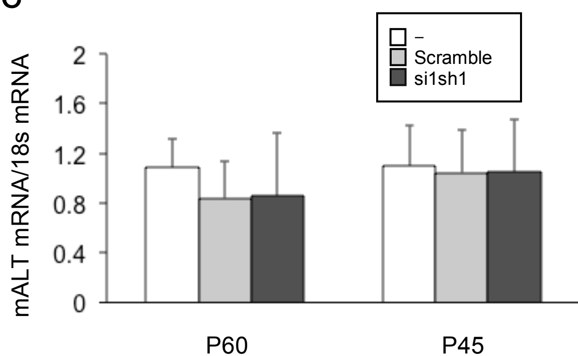

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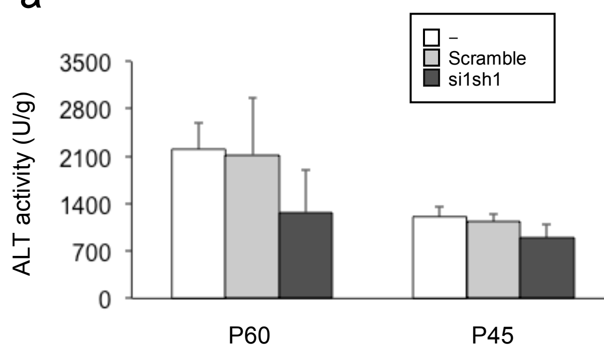
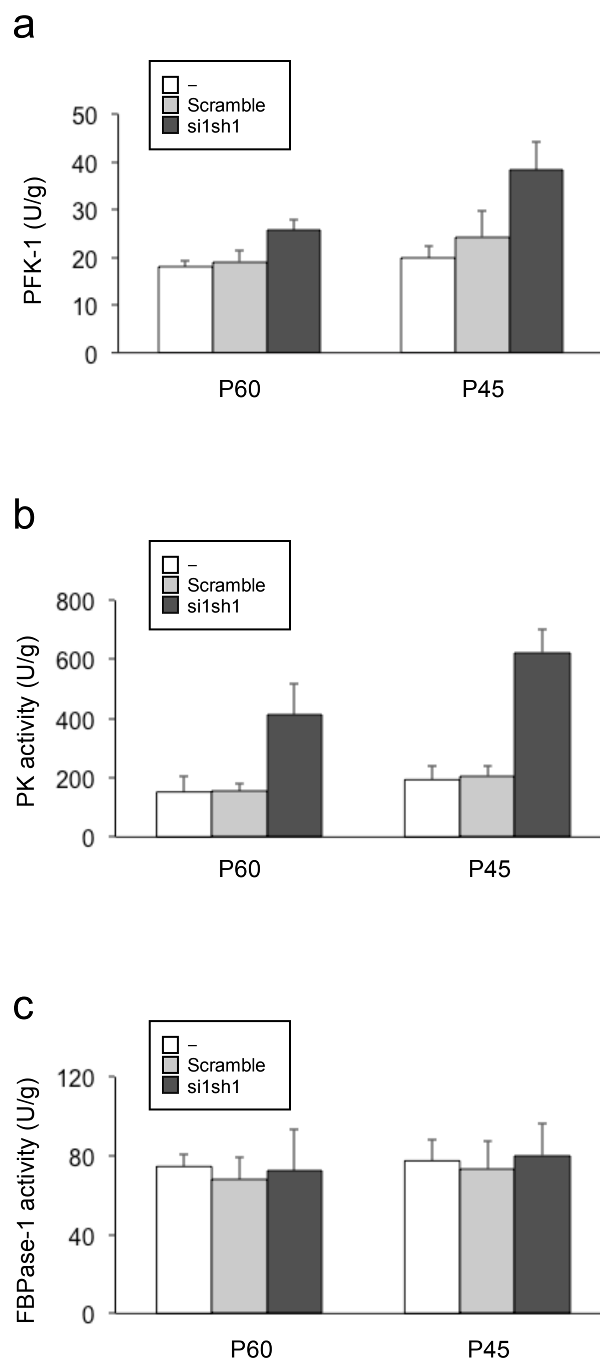


Two-way ANOVA

Dependent variable	Interaction	Diet	Treatment	Treatment		
				–	Scramble	si1sh1
cALT1 mRNA	NS	NS	***	b	b	a
cALT2 mRNA	NS	***	**	b	b	a
mALT mRNA	NS	NS	NS	-	-	-
ALT activity	NS	***	**	b	b	a

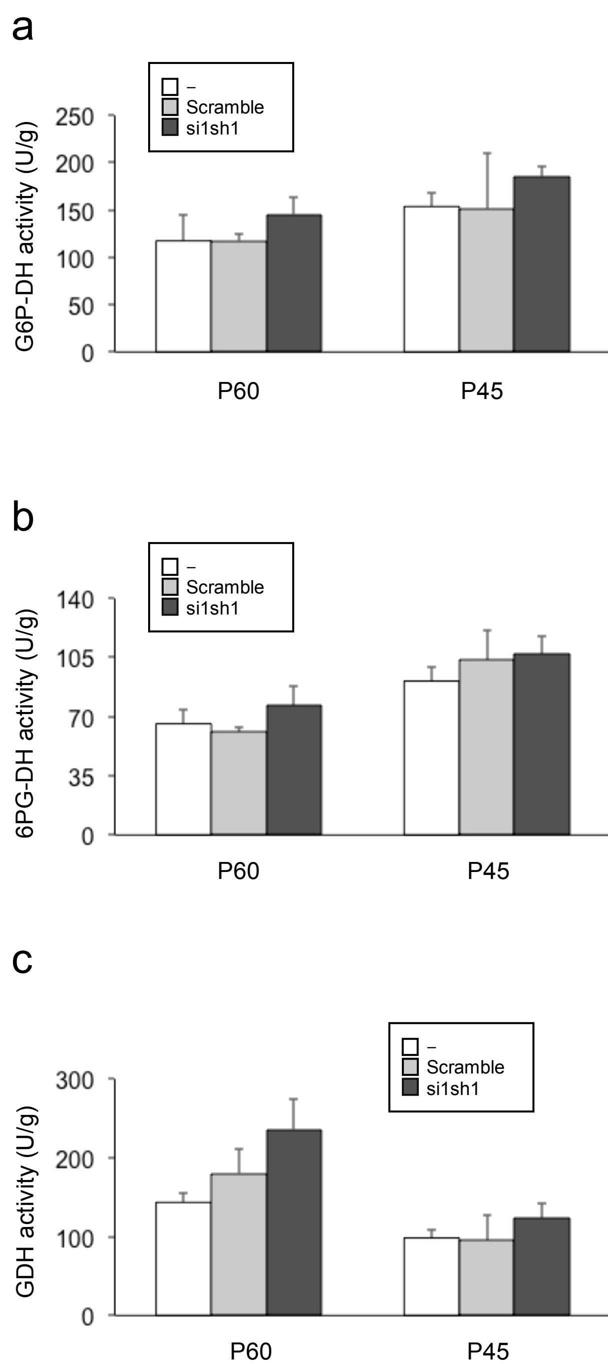
Figure 6



Two-way ANOVA

Dependent variable	Interaction	Diet	Treatment	Treatment		
				–	Scramble	si1sh1
PFK-1 activity	**	***	***	a	a	b
PK activity	***	***	***	a	a	b
FBPase-1 act.	NS	NS	NS	-	-	-

Figure 7



Two-way ANOVA

Dependent variable	Interaction	Diet	Treatment	Treatment		
				–	Scramble	si1sh1
G6P-DH activ.	NS	*	NS	-	-	-
6PG-DH activ.	NS	***	NS	-	-	-
GDH activity	**	***	***	a	a	b