Role of upstream stimulatory factor 2 in glutamate dehydrogenase gene transcription

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Abstract

Glutamate dehydrogenase (Gdh) plays a central role in ammonia detoxification by catalysing reversible oxidative deamination of L-glutamate into α-ketoglutarate using NAD^+ or $NADP^+$ as cofactor. To gain insight into transcriptional regulation of *glud,* the gene that codes for Gdh, we isolated and characterised the 5' flanking region of *glud* from gilthead sea bream (*Sparus aurata*). In addition, tissue distribution, the effect of starvation as well as short- and long-term refeeding on Gdh mRNA levels in the liver of *S. aurata* were also addressed. 5'-deletion analysis of *glud* promoter in transiently transfected HepG2 cells, electrophoretic mobility shift assays, chromatin immunoprecipitation (ChIP) and site-directed mutagenesis allowed us to identify upstream stimulatory factor 2 (Usf2) as a novel factor involved in the transcriptional regulation of *glud*. Analysis of tissue distribution of Gdh and Usf2 mRNA levels by reverse transcriptase-coupled quantitative real-time PCR (RT-qPCR) showed that Gdh is mainly expressed in the liver of *S. aurata*, while Usf2 displayed ubiquitous distribution. RT-qPCR and ChIP assays revealed that long-term starvation down-regulated the hepatic expression of Gdh and Usf2 to similar levels and reduced Usf2 binding to *glud* promoter, while refeeding resulted in a slow but gradial restoration of both Gdh and Usf2 mRNA abundance. Herein, we demonstrate that Usf2 transactivates *S. aurata glud* by binding to an E-box located in the proximal region of *glud* promoter. In addition, our findings provide evidence for a new regulatory mechanism involving Usf2 as a key factor in the nutritional regulation of *glud* transcription in the fish liver.

Introduction

Glutamate dehydrogenase (Gdh) catalyses reversible oxidative deamination of glutamate to form α-ketoglutarate and ammonia while reducing $NAD(P)^+$ to NAD(P)H. Encoded by the *glud* gene, Gdh plays a major role in ammonia detoxification in the liver, acid excretion by providing urinary ammonia in the kidneys, amplification of glucose-stimulated insulin secretion in pancreatic β-cells, cycling of the neurotransmitter glutamate between neurons and astrocytes, and gluthatione synthesis, among others (Newsholme *et al*. 2003; Karaca *et al*. 2011; Göhring & Mulder 2012; Treberg *et al*. 2014; Bunik *et al*. 2016). Located in the mitocondrial matrix, Gdh activity is subjected to a complex regulation. Gdh is strongly inhibited by GTP and activated by ADP. GTP binding is antagonised by phosphate and ADP, but is synergistic with NADH bound to a second, non-catalytic site. Gdh is also activated by leucine and other monocarboxylic acids, while it is inhibited by palmitoyl-CoA and diethylstilbestrol (Li *et al*. 2014; Plaitakis *et al*. 2017). Reversible cystein-specific ADP-ribosylation inactivates Gdh (Haigis *et al*. 2006). Gdh activity comes, at least in part, from association to a multienzyme complex in the mitochondrion, as it was deduced by the fact that short-chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD) inhibits Gdh via protein-protein interaction in the pancreas, where SCHAD is expressed at high levels (Li *et al*. 2010).

Glutamate metabolism in fish differs from that of mammals. Glutamate is primarily deaminated in the fish liver with ammonia production, while in mammals most glutamate is transaminated to aspartate (Peres & Oliva-Teles 2006). The fact that Gdh plays a major role in amino acid oxidation in the liver, led to consider Gdh a marker for protein utilisation and ammonia excretion in fish (Liu *et al*. 2012). Gdh is mainly expressed in the piscine liver, and high-protein diets usually stimulate growth and hepatic Gdh activity in fish (Bibiano Melo *et al*. 2006; Borges *et al*. 2013; Viegas *et al*. 2015; Coutinho *et al*. 2016). Dietary protein increases plasma free amino acids, which in turn enhances Gdh deamination and leads to higher rates of ammonia excretion. Dietary

supplementation of glutamate down-regulates Gdh mRNA levels and decreases reductive Gdh activity in the liver of *Sparus aurata* (Gómez-Requeni *et al*. 2003; Caballero-Solares *et al*. 2015) and reduces Gdh activity in *Pagellus bogaraveo* (Figueiredo-Silva *et al*. 2010), while it increases in *Oncorhynchus mykiss* (Moyano *et al*. 1991).

Little is known about transcriptional regulation of Gdh expression in vertebrates. *In silico* analysis allowed detection of potential binding sites for a number of transcription factors, such as Sp1, AP-1, and AP-2 in humans, and Sp1 and Zif268 in rats (Das *et al*. 1993; Michaelidis *et al*. 1993). The functionality of these sites remains unclear. A glucocorticoid-responsive region was located in the gene promoter of the C8S mouse astrocyte-derived cell line (Hardin-Pouzet *et al*. 1996). Deletion of the gene coding for the transcriptional coactivator p300 in the human colon carcinoma cell line HCT116 down-regulates Gdh expression (Bundy *et al*. 2006). Despite the important role exerted by Gdh in various tissues, to our knowledge there are no reports that have adressed isolation and molecular characterisation of *glud* gene promoter from fish.

With the aim of increasing current knowledge about the transcriptional regulation of *glud*, in the present study we characterised for the first time a piscine *glud* promoter and addressed the role of upstream stimulatory factor 2 (Usf2) on *glud* transcription in gilthead sea bream (*Sparus aurata*). In addition to report for the first time transactivation of *glud* gene promoter by Usf2, we explored changes in Gdh and Usf2 expression associated with starvation and refeeding in the liver of *S. aurata*.

Materials and methods

Experimental animals

S. aurata juveniles obtained from Tinamenor (Cantabria, Spain) were maintained at 20 ºC in 260-L aquaria supplied with running seawater as described (Fernández *et al.* 2007). Nutritional regulation of Gdh and Usf2 expression was analysed in the liver of 18-day fed fish, 19-day starved fish and fish refed for 6 hours, 24 hours, 5 days and 14 days. The diet was supplied at 25 g/kg body weight (BW) once a day (10 a.m.) and contained 46 % protein, 9.3 % carbohydrates, 22 % lipids, 10.6 % ash, 12.1 % moisture and 21.1 kJ/g gross energy. To prevent stress, fish were anesthetised with MS-222 (1:12,500) before handling. Twenty-four hours after the last meal, fish were sacrificed by cervical section and tissues were dissected out, frozen in liquid N_2 and kept at -80 °C until use. The University of Barcelona's Animal Welfare Committee approved the experimental procedures (proceeding #461/16) in compliance with local and EU legislation.

Characterization of the transcription start site

The 5' end of *S. aurata* Gdh mRNA was determined using the First Choice RLM-RACE Kit (Thermo Fisher Scientific, Waltham, MA, USA). RLM-RACE allows amplification of cDNA only from full-length, capped mRNA (Schaefer 1995). Briefly, 10 μg of total RNA from *S. aurata* liver were treated with calf intestine alkaline phosphatase and submitted to phenol-chloroform extraction and isopropanol precipitation. RNA was resuspended and treated with tobacco acid pyrophosphatase to remove the cap structure from full-length mRNAs, while leaving a 5'-monophosphate required for further ligation of the 5' RACE adapter oligonucleotide provided with the kit. Following random-primed reverse transcription, a nested PCR amplified the 5' end of *S. aurata* Gdh mRNA using gene-specific primers CG1307 and CG1308, designed from the *S. aurata* partial cDNA sequence with GenBank no. JX073708 (Table 1). The single 942-bp amplicon generated was ligated into pGEM-T easy (Promega, Madison, WI, USA). Identical nucleotide sequence was obtained by sequence analysis of three independent clones.

112

113 **Isolation of the 5′-flanking region of *S. aurata glud* by chromosome walking**

114 The 5′-flanking region of *glud* was isolated using the Universal GenomeWalker Kit (Clontech, Palo
115 Alto, CA, USA) and gene-specific primers CG1315 and CG1316 (Table 1), which were designed
116 from the 5' end of *S. aurata* Gdh cDNA. Blunt-end digestion of S. *aurata* genomic DNA with *Dra*I,
117 *Eco*RV, *Pvu*II, and *Stu*I generated four libraries that were ligated to the GenomeWalker adaptor as
118 described (Metón *et al.* 2006). Two PCR rounds were performed on each library with gene-specific
119 primers CG1316 and CG1315 for the primary and nested PCR, respectively. The longer amplicon
120 (~2.1-kb) was isolated from the *Pvu*II library and ligated into pGEM-T Easy (Promega, Madison,
121 WI, USA) to generate pGEM-GDH2057. Two independent clones were fully sequenced on both
122 strands following the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit
123 instructions (Applied Biosystems, Foster City, CA, USA).

124

125 **Reporter and shRNA expression constructs**

126 To generate pGDH1286, the S. *aurata glud* promoter fragment spanning positions −1286 to +70
127 relative to the transcriptional start was obtained by digestion of pGEM-GDH2057 with *Not*I,
128 followed by fill-in and *Nhe*I digestion. The product was subcloned into the *Sma*I/*Nhe*I site of the
129 promoterless luciferase reporter plasmid pGL3-Basic (Promega, Madison, WI, USA). To obtain
130 pGDH982, the promoter fragment resulting from digestion of pGDH1286 with *Pst*I followed by
131 chew back, fill-in and *Hind*III digestion, was subcloned into pGL3-Basic, previously digested with
132 *Mlu*I, filled-in and *Hind*III digested. pGDH+19, pGDH85 and pGDH128 were generated by PCR
133 using pGDH1286 as template and primer pairs CG1344 (with a 5′-anchor sequence containing a
134 *Sma*I site; Table 1)/RVprimer3 (Promega, Madison, WI, USA), CG1345 (with a 5′-anchor sequence
135 containing a *BsrB*I site; Table 1)/RVprimer3, and CG1342 (with a 5′-anchor sequence containing a

*Pvu*II site; Table 1)/RVprimer3, respectively. PCR products were digested with *Sma*I, *BsrB*I and *Pvu*II, respectively, isolated and subcloned into pGL3-Basic, previously digested with *Mlu*I, filled-in and *Hind*III digested. pGDH413 was produced by *Nde*I/*Mlu*I digestion, filling-in and self-ligation of pGDH1286. pGDH982Δ-44/+70 was obtained by *Cfr*42I digestion of pGDH982, isolation of the longest product and self-ligation. pGDH982mutUsf2 was generated by PCR using pGDH1286 as template and primer pair CG1552 (harbouring a mutated E-box and a 5′-anchor sequence with a *Cfr*42I site; Table 1)/GLprimer2 (Promega, Madison, WI, USA), and subcloning of the resulting amplicon into pGDH982, previous *Cfr*42I digestion of amplicon and plasmid. To generate pCpG-sh1Usf2, the double-stranded product obtained by hybridisation of oligonucleotides JS1711 and JS1712 (Table 1; designed using siRNA Wizard software, InvivoGen, San Diego, CA, USA), was ligated into the *Hin*dIII/*Acc*65I site of pCpG-siRNA (InvivoGen, San Diego, CA, USA). All constructs were verified by cycle sequencing.

Cell transfection and luciferase assay

Human hepatoma-derived HepG2 cells (ATCC HB 8065) were cultured at 37 ºC and 5 % CO_2 in DMEM supplemented with 2 mM glutamine, 110 mg/l sodium pyruvate, 10 % foetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin. HepG2 cells at 45–50 % confluence were transiently transfected in six-well plates using the calcium phosphate coprecipitation method. Transfection mixture included 4 µg of reporter construct and 500 ng of CMV-*β* plasmid (*lac*Z) to correct for variations in transfection efficiency, and was prepared with or without 400 ng of the Usf2 expression vector. Up to 400 ng of pCpGsh1Usf2 were added to perform shRNA-mediated silencing assays. Empty plasmids were added to ensure equal DNA amounts. Cells were harvested 16 h later, washed in PBS and incubated for 15 min in 300 µl of Cell Culture Lysis Reagent (Promega, Madison, WI, USA). Cell debris was pelleted, and luciferase activity in the supernatant

was assayed in a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA) after addition of Luciferase Assay Reagent (Promega, Madison, WI, USA). β-Galactosidase activity of the clear lysate was assayed as described (Metón *et al.* 2006). The Usf2 expression plasmid was kindly provided by Dr. B. Viollet (Viollet *et al.* 1996).

Electrophoretic mobility shift assay

Double-stranded oligonucleotides GDH-22/+9, GDH-22/+9mutUSF (carrying a mutated E-box) and USF2-cons (with a consensus Usf2 binding box) were obtained by hybridisation of oligonucleotide pairs CG1563/CG1564, CG1565/CG1566 and CG1561/CG1562 (Table 1), respectively. Two hundred pmol of double-stranded oligonucleotides were 3´-end labelled with digoxigenin-11-ddUTP in a 20-µl reaction for 30 min at 37 ºC using terminal transferase (Hoffman-La Roche, Basel, Switzerland). EDTA (18 mM) was added to stop the reaction. Binding reactions contained 100 mM HEPES, pH 7.6, 5 mM EDTA, 50 mM $(NH_4)_2SO_4$, 5 mM dithiothreitol, 1 % Tween 20, 150 mM KCl, 0.05 µg/µl non-specific competitor poly [d(I-C)], nuclear extracts of HepG2 cells overexpressing Usf2, and labelled probe. DNA-protein complexes were electrophoresed at 4 ºC on a 5 % polyacrylamide gel using 0.5 x Tris-borate-EDTA buffer. DNA was transferred by contact blotting to Nytran membranes and cross-linked by UV irradiation for 3 min. Labelled probes were immunodetected with antidigoxigenin conjugated to alkaline phosphatase using CDP-Star (Hoffman-La Roche, Basel, Switzerland) as chemiluminescent substrate. Digital imaging of membranes was performed using ImageQuant LAS 4000 mini (GE Healthcare, Little Chalfont, UK). For competition experiments, HepG2 extracts were preincubated for 30 min with 200- and 1000-fold molar excess of unlabelled double-stranded USF2-cons.

Nuclear extracts

184 Nuclear extracts were prepared from HepG2 cells as described (Andrews & Faller 1991) with minor
185 modifications. Cells grown at ~80 % confluency were washed, scraped into 1.5 ml of cold PBS,
186 pelleted by centrifugation for 10 sec at 1000 *g* and resuspended in 0.4 ml of buffer A (10 mM
187 HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl
188 fluoride). Following 10 min of incubation, cells were vortexed for 10 sec and centrifuged. The
189 pellet was resuspended in 20 µl of ice-cold buffer C (20 mM HEPES, pH 7.9, 25 % glycerol, 420
190 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl
191 fluoride) and incubated 20 min for high-salt extraction. After pelleting cell debris, the supernatant
192 was kept at -80 ºC until use.
193
194 **Western blot**
195 Proteins in cell extracts were subjected to 10 % PAGE-SDS electrophoresis, transferred to a
196 polyvinylidene fluoride membrane and immunoblotted with mouse anti-Usf2 (Santa Cruz
197 Biotechnology, Dallas, TX, USA) and rabbit anti-actin **(**Sigma-Aldrich, Saint Louis, MO, USA)
198 antibodies. Chemiluminescent detection proceeded using alkaline phosphatase-conjugated
199 secondary antibodies and the Clarity Western ECL Substrate Kit (Bio-Rad, Hercules, CA, USA).
200
201 **Quantitative real-time RT-PCR analysis**
202 Total RNA (1 µg) isolated from tissue samples was retrotranscribed 1 h at 37 °C with M-MLV RT
203 (Promega, Madison, WI, USA). Gdh and Usf2 mRNA levels were determined in a Step One Plus
204 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) using 0.4 µM of each primer
205 (CG1543/CG1544 and CG1557/CG1558 for Gdh and Usf2, respectively; Table 1), 10 µl of SYBR
206 Green (Applied Biosystems, Foster City, CA, USA), and 1.6 µl of 1:10 diluted cDNA in a 20 µl-

reaction. The temperature cycle protocol for amplification was 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 sec and 62 °C for 1 min. A dissociation curve was run after each experiment to ensure amplification of single products. Gdh and Usf2 mRNA levels were normalised with the C_T geometric mean value of ribosomal subunit 18s, beta-actin and elongation factor 1-alpha (EEF1A1), which were amplified using primer pairs JDRT18S/JDRT18AS, QBACTINF/QBACTINR and AS-EF1Fw/AS-EF1Rv, respectively (Table 1). Variations in gene expression were calculated by the standard $\Delta\Delta C_T$ method (Pfaffl 2001).

Chromatin immunoprecipitation

Chromatin from *S. aurata* liver was isolated, cross-linked and sonicated to an average size of 100-600 bp as described (Metón *et al.* 2006). Following preclearing with 15 µl of protein A/G-agarose beads, immunoprecipitation of chromatin (100 µg) proceeded overnight at 4 ºC with or without 2 µg of antibody (anti-Usf2 or anti-Srebp1; Santa Cruz Biotechnology, Dallas, TX, USA). Immune complexes were incubated with protein A/G-agarose beads, washed, eluted and reverse cross-linked with 0.4 mg/ml proteinase K for 2 h at 37 ºC and overnight at 65 ºC. Purified DNA was subjected to PCR using primer pairs CG1701/CG1702 and CG1703/CG1705 (Table 1), which amplify *glud* sequences -1766 to -1535 and -133 to +41, respectively. Nutritional regulation of Usf2 binding to *glud* promoter was determined by qPCR using primer pair CG1703/CG1705 (Table 1), Usf2-immunoprecipitated chromatin or input chromatin, and the protocol described in the previous section. Twenty-five µg of non-immunoprecipitated chromatin was reverse cross-linked and retained as a positive control and to normalise qPCR results (input).

Statistics

SPSS Version 22 (IBM, Armonk, NY, USA) was used to analyse data with Student's two-tailed unpaired *t*-test or one-way ANOVA when comparing more than two groups. For ANOVA, significant differences were determined with the Bonferroni post hoc test.

Results

Cloning of the 5′-flanking region of *S. aurata glud*

A 5' RLM RACE was performed on total RNA isolated from *S. aurata* liver to determine the nucleotide sequence at the 5' end and the transcription start site of *S. aurata* Gdh mRNA. Analysis of the single fragment obtained indicated that *S. aurata* Gdh mRNA initiates 140 nucleotides upstream from the translation start codon. Availability of the 5' end of Gdh mRNA enabled to design gene-specific primers to isolate a 2057 bp fragment upstream from the translation start codon of *S. aurata glud* by chromosome walking. Sequence analysis of the 1286-bp 5′-flanking region with JASPAR (Sandelin *et al.* 2004) revealed the presence of a TATA box at positions -32 to -18 relative to the transcription start site. Potential transcription factor sites included binding boxes for Usf2, Cebp and Hnf1, among others (Fig. 1). The nucleotide sequence of *S. aurata* Gdh mRNA and *glud* promoter were submitted to the GenBank database under accession numbers MF459045 and MF459046, respectively.

Functionality of *S. aurata glud* promoter

To assess whether the 5'-flanking region of *S. aurata glud* encompasses a functional promoter, nucleotide positions -1286 to +70 relative to the transcriptional start were subcloned into the promoterless pGL3-Basic plasmid upstream from the luciferase reporter gene. Consistent with the presence of a functional promoter, transient transfection of HepG2 cells with the resulting construct (pGDH1286; -1286 to +70) resulted in a 45-fold increase of luciferase activity relative to pGL3-

Basic (Fig. 2). To identify functional regions involved in modulation of basal Gdh expression in *S. aurata*, sequential 5′- deletion of the isolated promoter was performed. HepG2 cells were transfected with pGL3-Basic constructs harbouring deletion fragments of *glud* promoter (5′ ends ranging from -1286 to +19 and 3′ ends at -70) fused to the luciferase reporter gene. The longer 5′ constructs (pGDH1286 and pGDH982; -982 to +70) yielded a 45-fold increase in luciferase activity relative to the empty vector. The reporter constructs pGDH413 (-413 to +70), pGDH128 (-128 to +70) and pGDH85 (-85 to +70) exhibited a 25- to 30-fold increase of promoter activity compared to pGL3-Basic. A significant drop of activity was observed using the smallest construct (pGDH+19; +19 to +70) or pGDH982Δ-44/+70, which is a deleted construct that encompasses promoter sequences located at positions -982 to +70, but lacks the region comprised between positions -44 and +70 (Fig. 2). Therefore, the core promoter of *S. aurata glud* localises within 85 bp upstream from the transcriptional start, suggesting the presence of *cis*-acting elements in this region.

Transactivation of *S. aurata glud* promoter by Usf2

Analysis with JASPAR indicated the presence of an E-box that could function as a putative Usf2 binding site in the proximal promoter region of *S. aurata glud* (Fig. 1). Usf proteins regulate the transcription of essential gene networks. The fact that USF2 null-mutant mice are small and exhibit decreased fertility and reduced lifespan, while USF1 null mice present a rather normal phenotype, highlights Usf2 as the more important Usf variant (Sirito *et al.* 1998; Horbach *et al.* 2014). To study the role of Usf2 in Gdh expression we performed transfection experiments on HepG2 cells in the presence and absence of an expression plasmid encoding Usf2. Cotransfection with the Usf2 expression plasmid together with pGDH85 or longer 5′ constructs increased ~4-fold *glud* promoter activity compared to the basal activity of the corresponding promoter constructs. No Usf2-dependent enhancement of *glud* transcription was observed when using the shortest construct

278 (pGDH+19). Altogether, these results suggest that a functional Usf2 binding site may be located
279 within 85 pb upstream from the transcriptional start. Consistently, cotransfection of HepG2 cells
280 with the Usf2 expression plasmid and pGDH982Δ-44/+70, which lacks the region between
281 positions -44 to +70, did not transactivate *glud* (Fig. 3).
282
283 **Usf2 binds to *glud* promoter**
284 The shorter reporter construct that exhibited Usf2-mediated transactivation (pGDH85) contains an
285 E-box at positions -10 to -5 relative to the transcriptional start. Bearing in mind that Usf2
286 transactivates numerous genes by binding to E-boxes (Corre & Galibert 2005), electrophoretic
287 mobility shift assays (EMSA) were performed with nuclear extracts obtained from HepG2 cells
288 overexpressing Usf2. Probes GDH-22/+9 (with the putative E-box at positions -10 to -5) and USF2-
289 cons (containing a consensus Usf2 binding site) generated one major shifted band with the same
290 mobility. The shifted DNA-protein complex disappeared by competition with 200- to 1000-fold
291 molar excess of unlabelled USF2-cons. These results confirmed binding of Usf2 to a response
292 element at positions -22 to +9 of *S. aurata glud*. Bandshift experiments performed using nuclear
293 extracts of HepG2 cells overexpressing Usf2 and a labelled probe harbouring positions -22 to +9 of
294 *glud* but with a mutated E-box element (GDH-22/+9mutUSF) completely prevented the formation
295 of DNA-protein complexes (Fig. 4A).
296 Chromatin immunoprecipitation (ChIP) was performed to study association of Usf2 with *S. aurata*
297 *glud* promoter *in vivo*. Following ChIP with anti-Usf2, PCR analysis on purified DNA using primer
298 pairs to amplify *glud* promoter positions -133 to +41 confirmed that the E-box at positions -10 to -5
299 contains a functional Usf2 binding site *in vivo*. No binding was observed with a different antibody
300 (anti-Srebp1), no antibody or using oligonucleotides to amplify an upstream region (-1766 to -1535)
301 (Fig. 4B).

Mutating the E-box abolishes transactivation by Usf2

To analyse the effect of mutating the E-box located at positions -10 to -5 on Usf2-dependent transactivation of *glud*, we generated a reporter construct containing the same mutations introduced in the double-stranded oligonucleotide GDH-22/+9mutUSF used for bandshift assays. Usf2 failed to enhance transcriptional activity of the resulting construct (pGDH982mutUSF2; -982 to +70 with a mutated E-box). Indeed, Western blot analysis revealed immunodetection of endogenous Usf2 in HepG2 cells, while confirmed overexpression of Usf2 after cotransfection with the Usf2 expression plasmid (Fig. 5A). Furthermore, cotransfection of HepG2 cells with pGDH982 and a construct expressing an shRNA to knock-down Usf2 (pCpGsh1Usf2) abolished Usf2-dependent transactivation of *glud* promoter (Fig. 5B). Therefore, the E-box located at positions -10 to -5 relative to the major transcriptional start of the S. *aurata glud* is responsible for transactivation by Usf2.

Tissue distribution of Gdh and Usf2 expression in *S. aurata*

To study tissue specificity of Gdh and Usf2 expression in *S. aurata*, reverse transcriptase-coupled quantitative real-time PCR (RT-qPCR) was performed in tissue samples of fed *S. aurata*. The highest mRNA levels of Gdh were found in the liver, followed by the intestine, heart and kidney. Gdh expression was barely detectable in other tissues. Usf2 was ubiquitously expressed, albeit higher Usf2 mRNA abundance was exhibited by the brain and spleen, followed by the heart, gill, kidney and liver (Fig. 6).

Effect of starvation and refeeding on the hepatic expression of Gdh and Usf2

Having concluded that Usf2 can bind and transactivate *glud* promoter in the liver, we addressed the role that Usf2 may exert in the nutritional regulation of hepatic *glud* transcription in *S. aurata*. Gdh and Usf2 mRNA levels were determined by RT-qPCR in liver samples of 18-day fed fish, 19-day starved fish and fish refed up to 14 days. Nutritional changes affected similarly Gdh and Usf2 expression. Starvation significantly decreased 1.7-fold mRNA abundance of both Gdh and Usf2. Remarkably, a trend to present lower expression levels of Gdh and Usf2 than starved fish was observed 6 hours after refeeding. Thereafter, Gdh and Usf2 gradually recovered their mRNA levels until reaching total restoration after 14 days of refeeding (Fig. 7A). ChIP assays showed that starvation decreased Usf2 binding to *glud* promoter, while a trend to recover the values observed in fed fish was observed after 14 days of refeeding (Fig. 7B).

Discussion

In the liver, Gdh is essential for ammonia detoxification, nitrogen metabolism and urea synthesis (Karaca *et al*. 2011; Treberg *et al*. 2014). However, knowledge of the transcription factors involved in the regulation of *glud* gene expression is scarce. To study the transcriptional regulation of *glud*, we addressed cloning and characterisation of *S. aurata glud* promoter by chromosome walking. Functionality of *S. aurata glud* promoter was confirmed by transient transfection of HepG2 cells with fusion constructs of sequential 5´- deletions of the isolated genomic fragment to the luciferase gene. We found that the core functional promoter of *S. aurata glud* is comprised within 85 bp upstream from the transcription start site. The presence of a putative Usf2 binding box in the proximal region of *glud* gene promoter prompted us to study involvement of Usf2 in the transcriptional regulation of *glud*.

Usf proteins belong to the basic helix-loop-helix-leucine zipper (bHLHzip) transcription factor family and are encoded by two different genes: *usf1* and *usf2*. Usf proteins regulate the transcription

of a wide number of genes involved in stress and immune responses, cell cycle and proliferation, and carbohydrate and lipid metabolism, among other functions, by binding as homodimers and heterodimers to the E-box binding motif CANNTG (being NN nucleotides in most cases either GC or CG), non-canonical E-boxes and pyridine-rich initiator sites (Viollet *et al.* 1996; Corre & Galibert 2005; Pawlus *et al.* 2012). Transient transfection studies in HepG2 cells together with EMSA and ChIP assays allowed us to demonstrate that Usf2 transactivates the promoter activity of *S. aurata glud* through binding to the E-box located at positions -10 to -5 upstream from the transcriptional start. Transactivation of *glud* by Usf2 was confirmed by introducing mutations in the E-box that abolished binding of Usf2 and prevented Usf2-dependent enhancement of *glud* transcription. Indeed, transfection of HepG2 cells with an shRNA expression plasmid to knock-down Usf2 abolished Usf2-dependent transactivation of *glud* promoter.

Optimal growth of teleostean fish requires higher levels of dietary protein than other vertebrates. Fish metabolism, and more remarkably that of carnivorous fish, enables efficient use of amino acids for growth and to obtain energy (Li *et al.* 2009; Kaushik & Seiliez 2010; Liu *et al.* 2012). The fish liver is the main site for amino acid catabolism, where Gdh exerts a major role in amino acid transdeamination by catalysing oxidative deamination of glutamate and giving rise to the end product of protein catabolism, ammonia (Lushchak *et al.* 2008). As for other fish species (Liu *et al*. 2012) and similar to mammals (Plaitakis *et al*. 2017), we found that Gdh is mainly expressed in the liver of *S*. *aurata*, while high mRNA levels were also observed in the kidney, heart and intestine. In contrast to Gdh, Usf2 displayed ubiquitous expression in *S. aurata* tissues, albeit higher mRNA levels were found in the brain, spleen, heart, gill, kidney and liver. These results are consistent with the pattern of tissue distribution of Usf2 in other vertebrates (Sirito *et al.* 1994; Fujimi & Aruga 2008). Our findings suggest that in addition to Usf2-dependent transactivation of *glud* promoter, other yet unknown transcription factors may contribute to upregulation of Gdh mRNA levels in the piscine liver. Posttranslational modifications as phosphorylation or interaction with other

374 transcription factors and cofactors may also explain tissue-specific differences in Usf2 action
375 (Spohrer *et al.* 2016).

376 Since Gdh expression can be considered a significant marker for protein utilisation and ammonia
377 excretion in fish (Liu *et al.* 2012), we also adressed the effect of nutritional status on hepatic mRNA
378 levels of Gdh, and the role that Usf2 may have on *glud* transcription under starvation and during the
379 starved-to-fed transition in the liver of *S. aurata*. Long-term starvation similarly affected the hepatic
380 expression of Usf2 and Gdh, which significantly decreased to about 60 % of the values observed in
381 fed fish, and reduced Usf2 binding to *glud* promoter. Downregulation of Gdh expression in starved
382 *S. aurata* may be related with a mechanism preventing insulin secretion in β-cells. In favour of this
383 hypothesis, overexpression of Gdh in mice increases insulin secretion (Carobbio et al. 2004),
384 whereas Gdh inhibition in pancreatic β-cells decrease impairs insulin secretion (Carobbio et al.
385 2009). Furthermore, activating mutations in Gdh causes hyperinsulinemia and hyperammonemia in
386 humans (Li *et al*. 2014; Barrosse-Antle *et al*. 2017). However, the effect of nutritional status on Gdh
387 expression seems species-specific in fish. In contrast to *S. aurata*, starvation did not affect Gdh
388 activity in *Salmo gairdneri* (Tranulis *et al*. 1991), while it increased Gdh activity in the liver of
389 *Oncorhynchus mykiss*, *Protopterus dolloi* and *Dentex dentex*, and Gdh mRNA levels in *Danio rerio*
390 (Sánchez-Muros *et al*. 1998; Frick *et al*. 2008; Pérez-Jiménez *et al*. 2012; Tian *et al*. 2015). We
391 cannot discard that in addition to species-specificity, differences in the effect of starvation on the
392 expression of Gdh among experiments may result also from diet composition, ration size and
393 feeding regime. In this regard, it is well known that dietary protein greatly influences the hepatic
394 activity of Gdh in fish (Liu *et al*. 2012; Borges *et al*. 2013; Caballero-Solares *et al*. 2015; Viegas *et*
395 *al*. 2015; Coutinho *et al*. 2016). Indeed, it was reported that starvation decreased or unaffected Gdh
396 activity in the liver of starved *Dicentrarchus labrax* depending on dietary protein levels (Pérez-
397 Jiménez *et al*. 2007). As for Gdh, starvation decreased hepatic mRNA levels of Usf2 in the liver of
398 *S. aurata*. In this regard, it was previously reported that high glucose levels upregulate Usf2

399 expression in human-derived HK-2 cells and primary rat mesangial cells (Shi *et al*. 2008;
400 Visavadiya *et al*. 2011; Wang 2015). Therefore, low levels of glycemya associated to long-term
401 starvation may be critical to downregulate Usf2 expression, which in turn may lead to decreased
402 Gdh mRNA levels in the liver of *S. aurata*.

403 As for starvation, Usf2 and Gdh mRNA levels followed the same expression pattern after short- and
404 long-term refeeding in the liver of *S. aurata*: a slow but gradual recovery of the values observed in
405 fed fish. Five days of refeeding did not promote significantly higher expression levels than in
406 starved fish for both Usf2 and Gdh. However, 14 days of refeeding allowed restoration of pre-
407 starvation values. Furthermore, Usf2 and Gdh mRNA values in the liver of 14-day refed fish
408 showed a trend to present slightly higher levels than fed fish. Similarly, long-term refeeding after
409 starvation increased Gdh activity in the liver of *Dicentrarchus labrax* and *Dentex dentex* to values
410 higher than in control fed fish (Pérez-Jiménez *et al*. 2007, 2012). Conceivably, refeeding after long-
411 term starvation may require a long period of adaptation involving enhanced nutrient catabolism to
412 restore metabolic parameters, as pointed out for other key enzymes involved in the intermediary
413 metabolism (Soengas *et al.* 2006; Polakof *et al.* 2007). Therefore, an increased hepatic expression
414 of Gdh may be essential for glutamate deamination and transdeamination of dietary amino acid in
415 long-term refed fish to provide α-ketoglutarate for the Krebs cycle and supply ATP for energetic
416 demands and biosynthesis.
417 Although Gdh and Usf2 may be subjected to similar regulatory cascades, the fact that Usf2 mRNA
418 levels showed a complete correlation with Gdh expression during starvation and refeeding suggests
419 that Usf2 may have a major role in the nutritional regulation of *glud* transcription in the liver of *S.*
420 *aurata*. Involvement of Usf2 in the expression of genes encoding key enzymes in amino acid
421 metabolism, such as Gdh, is consistent with previous observations showing transcriptional control
422 of genes related to lipid, carbohydrate and energy metabolism by USF family members in mammals

(Shih & Towle 1994; Lefrançois-Martinez *et al.* 1995; Iynedjian 1998; Martin *et al.* 2003; Corre & Galibert 2005; Pawlus *et al.* 2012).

In conclusion, in the present study we report for the first time characterisation of a piscine *glud* gene promoter and provide evidence for a novel regulatory mechanism that links Usf2 to the nutritional regulation of *glud* transcription in the fish liver.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contributions

IVB and IM conceived and designed the study. CG, JIS-M and MCS performed the experiments. CG, IVB and IM analysed the data and edited the manuscript.

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631

Figure legends

Figure 1

Sequence analysis of the 5′-flanking region comprised between positions -1316 to +143 relative to the transcriptional start of *S. aurata glud*. Chromosome walking allowed isolation of the genomic sequence upstream from the transcription start site of *S. aurata* Gdh, which is shown in capital letters. An arrow indicates the transcription start site. The translation start codon is in boldface and underlined. Putative binding sites for relevant transcription factors are boxed.

Figure 2

Functional analysis of the 5′-flanking region of *S. aurata glud* in HepG2 cells. The top left part represents the genomic organization of the 5′-flanking region of *S. aurata glud*. Relative position of relevant restriction sites and exon 1 are indicated. Nucleotide numbering starts with +1, which corresponds to the transcriptional start. Reporter constructs having varying 5′ ends and identical 3′ ends (+70), except for pGDH982Δ-44/+70, were transfected in HepG2 cells along with pCMVβ to normalise for transfection efficiency. Luciferase activity is expressed as fold increase over promoterless reporter plasmid pGL3-Basic. Results shown are the mean ± SD from three independent experiments performed in duplicate. Different letters indicate significant differences among conditions ($p < 0.05$).

Figure 3

Effect of Usf2 on the promoter activity of *S. aurata glud* gene in HepG2 cells. HepG2 cells were transfected with pGL3-Basic and promoter constructs pGDH+19, pGDH85, pGDH128, pGDH413, pGDH982 or pGDH982Δ44/+70 along with pCMVβ and with or without an expression plasmid

encoding Usf2. The promoter activity of the constructs alone was set at 1. Results are presented as mean ± SD values of three independent duplicate experiments. Statistical significance related to promoter activity of reporter constructs in absence of the Usf2 expression plasmid is indicated as follows: ***$p < 0.001$.

Figure 4

(A) Analysis of USF binding to *glud* promoter by electrophoretic mobility shift assay. To perform a competition analysis, nuclear extracts of HepG2 cells overexpressing Usf2 were incubated with labelled oligonucleotides USF2-cons (lanes 1-4), GDH-22/+9 (lanes 5-8) or GDH-22/+9mutUSF (lanes 9-10). Lanes 1, 5 and 9 contained no extract. Lanes 2 and 6 show binding of nuclear extracts to labelled probes without competitor. Lanes 3 and 7 show competition with 200-fold molar excess of unlabelled double-stranded competitor (USF2-cons). Lanes 4, 8 and 10 show competition with 1000-fold molar excess of unlabelled double-stranded competitor (USF2-cons). DNA-protein complexes are indicated by an arrow. NE, nuclear extracts. (B) *In vivo* association of Usf2 with *S. aurata glud* promoter. A ChIP assay was performed on *S. aurata* liver. The upper part of the figure shows a schematic drawing of *S. aurata glud* promoter, location of the PCR primers (arrows) and sequence of E-box at position -10 to -5 relative to the transcriptional start (underlined). After cross-linking with 1 % formaldehyde, chromatin was sheared by sonication, and immunoprecipitated in the presence of anti-Usf2 and anti-Srebp1 antibodies, or incubated without antibodies. Immune complexes were collected with protein A/G-agarose beads, and following intensive washing, bound DNA-complexes were eluted and reverse cross-linked. Analysis of purified DNA was performed by PCR with primer pairs to amplify *glud* promoter positions -1766/-1535 or -133/+41 relative to the transcription start site. The PCR products were electrophoresed on an agarose gel and visualised by means of RedSafe nucleic acids staining.

Figure 5

(A) Effect of Usf2 on the promoter activity of *glud* containing a mutated E-box. The upper part of the figure shows a representative Western blot analysis of immunodetectable Usf2 and actin proteins in extracts of HepG2 cells transfected with the promoter constructs pGDH+19, pGDH85, pGDH982 or pGDH982mutUsf2, along with pCMVβ and with or without an expression plasmid encoding Usf2. The lower part of the figure shows induction of promoter activity in HepG2 cells transfected with the promoter constructs pGDH+19, pGDH85, pGDH982 or pGDH982mutUsf2, along with pCMVβ and with or without an expression plasmid encoding Usf2. The luciferase activity of the reporter constructs alone was set at 1. Results are presented as mean ± SD values of three independent duplicate experiments. Statistical significance related to promoter activity of reporter constructs in absence of the Usf2 expression plasmid is indicated as follows: $^{**}p < 0.01$; $^{***}p < 0.001$. (B) Effect of Usf2 silencing on Usf2-dependent transactivation of *glud* promoter. HepG2 cells were transfected with the promoter construct pGDH982 along with pCMVβ, an expression plasmid encoding Usf2 and increasing amounts of pCpGsh1Usf2. The luciferase activity of pGDH982 in the absence of pCpGsh1Usf2 was set at 1. Results are presented as mean ± SD values of triplicate experiments. Different letters indicate significant differences among conditions ($p < 0.05$).

Figure 6

Tissue distribution of Usf2 and Gdh expression in *S. aurata*. RT-qPCR assays of Usf2 and Gdh mRNA levels were performed on total RNA isolated from the spleen, gill, brain, heart, fat, liver, intestine, skeletal muscle and kidney of 18-day fed fish. Expression levels for each gene were

normalised using 18S, beta-actin and EEF1A1 as housekeeping genes. Results are presented as mean ± SD (n = 4).

Figure 7

Effect of starvation and refeeding on Usf2 and Gdh mRNA levels, and Usf2 binding to *glud* promoter in the liver of *S. aurata*. (A) RT-qPCR assays of Usf2 and Gdh mRNA levels were performed on total RNA isolated from the liver of 18-day fed, 19-day starved, and refed fish for 6 hours, 24 hours, 5 days and 14 days. Expression levels for each gene were normalised using 18S, beta-actin and EEF1A1 as housekeeping genes. Results are presented as mean ± SD (n = 6). (B) ChIP analysis of Usf2 association with *glud* promoter in the liver of 18-day fed, 19-day starved, and refed fish for 6 hours and 14 days. Results are presented as mean ± SD (n = 3). Different letters indicate significant differences among conditions ($p < 0.05$).

1 **Table 1** Oligonucleotides used in the present study.

Primer	Sequence (5’ to 3’)	GenBank accession no.
CG1307	GTCTTGTCCTGGAAGCCTGGTGTCA	JX073708
CG1308	GGCTGAGATACGACCGTGGATACCTCCC	JX073708
CG1315	GACAGGAGAAGGGGGGTAGAATGAACGAC	MF459045
CG1316	AACAACAAGGACAATGGGGGTGACGACAG	MF459045
CG1342	CCCAGCTGTCAGTTGGACAGCACGG	MF459046
CG1344	CCCCCGGGACACGGTGAGGAGCTGC	MF459046
CG1345	CCCCGCTCTTCCGCGTGAGTCCCG	MF459046
CG1543	GGTATTTCGGGGAGCTGCTGAG	MF459045
CG1544	CGCATCAGGGACGAGGACA	MF459045
CG1552	CTCTCCGCGGCTCGTGCTGCCTTTTAAAGCAAA	MF459046
	CTGACACAG**TT**T**T**TCATTCCCCACTCGGCCAGA	
	GGAC	
CG1557	AGAGCTGAGGCAAAGCAACC	*
CG1558	GGGGAGGACGCATTCACTAA	*
CG1561	CACCCGGTCATGTGACCTACAC	MF459046
CG1562	TGTAGGTCACATGACCGGGTGG	MF459046
CG1563	AAACTGACACAGCATGTCATTCCCCACTCGGC	MF459046
CG1564	CCGAGTGGGGAATGACATGCTGTGTCAGTTTG	MF459046
CG1565	AAACTGACACAG**TT**T**T**TCATTCCCCACTCGGC	MF459046
CG1566	CCGAGTGGGGAATGA**A**A**AA**CTGTGTCAGTTTG	MF459046
CG1701	CCAGCACAATGACATTTCTATTG	MF459046
CG1702	GTTAAAAAACTTGTATGGTTG	MF459046

CG1703	CGCGCGCTGTCAGTTGGACAGCAC	MF459046
CG1705	ACAGCAGCTCCTCACCGTGTCC	MF459046
AS-EF1Fw	CCCGCCTCTGTTGCCTTCG	AF184170
AS-EF1Rv	CAGCAGTGTGGTTCCGTTAGC	AF184170
JDRT18S	TTACGCCCATGTTGTCCTGAG	AM490061
JDRT18AS	AGGATTCTGCATGATGGTCACC	AM490061
QBACTINF	CTGGCATCACACCTTCTACAACGAG	X89920
QBACTINR	GCGGGGGTGTTGAAGGTCTC	X89920
JS1711	GTACCTCGAGGCCAGTTCTACGTCATGATCAAG	*
	AGTCATGACGTAGAACTGGCCTCTTTTTGGAAA	
JS1712	AGCTTTTCCAAAAAGAGGCCAGTTCTACGTCAT	*
	GACTCTTGATCATGACGTAGAACTGGCCTCGAG	

The following primers contain restriction sites (*underlined*): CG1344 (*Sma*I), CG1345 (*Bsr*BI) and CG1342 (*Pvu*II). *Bold* and *double-underlined* letters indicate site-directed mutations in primers CG1552, CG1565 and CG1566. *CG1557, CG1558, JS1711 and JS1712 were designed from recent transcriptome sequencing data (Silva-Marrero *et al*. 2017).

Figure 1

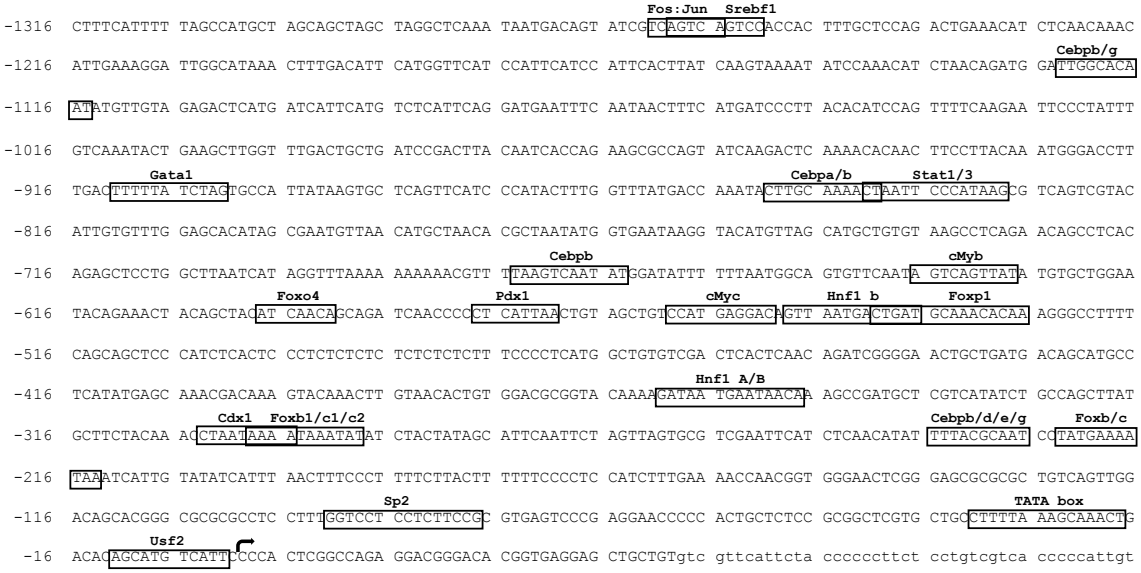

Fos:Jun
Srebf1
Cebpb/g
Gata1
Cebpa/b
Stat1/3
Cebpb
cMyb
Foxo4
Pdx1
cMyc
Hnf1 b
Foxp1
Hnf1 A/B
Cdx1
Foxb1/c1/c2
Cebpb/d/e/g
Foxb/c
Sp2
TATA box
Usf2
-1316 CTTTCATTTT TAGCCATGCT AGCAGCTAGC TAGGCTCAAA TAATGACAGT ATCGTCAGTC AGTCCACCAC TTTGCTCCAG ACTGAAACAT CTCAACAAAC
-1216 ATTGAAAGGA TTGGCATAAA CTTTGACATT CATGGTTCAT CCATTCATCC ATTCACTTAT CAAGTAAAAT ATCCAAACAT CTAACAGATG GATTGGCACA
-1116 ATATGTTGTA GAGACTCATG ATCATTCATG TCTCATTCAG GATGAATTTC AATAACTTTC ATGATCCCTT ACACATCCAG TTTTCAAGAA TTCCCTATTT
-1016 GTCAAATACT GAAGCTTGGT TTGACTGCTG ATCCGACTTA CAATCACCAG AAGCGCCAGT ATCAAGACTC AAAACACAAC TTCCTTACAA ATGGGACCTT
-916 TGACTTTTTA TCTAGTGCCA TTATAAGTGC TCAGTTCATC CCATACTTTG GTTTATGACC AAATACTTGC AAAACTAATT CCCATAAGCG TCAGTCGTAC
-816 ATTGTGTTTG GAGCACATAG CGAATGTTAA CATGCTAACA CGCTAATATG GTGAATAAGG TACATGTTAG CATGCTGTGT AAGCCTCAGA ACAGCCTCAC
-716 AGAGCTCCTG GCTTAATCAT AGGTTTAAAA AAAAAACGTT TTAAGTCAAT ATGGATATTT TTTAATGGCA GTGTTCAATA GTCAGTTATA TGTGCTGGAA
-616 TACAGAAACT ACAGCTACAT CAACAGCAGA TCAACCCCCT CATTAACTGT AGCTGTCCAT GAGGACAGTT AATGACTGAT GCAAACACAA AGGGCCTTTT
-516 CAGCAGCTCC CATCTCACTC CCTCTCTCTC TCTCTCTCTT TCCCCTCATG GCTGTGTCGA CTCACTCAAC AGATCGGGGA ACTGCTGATG ACAGCATGCC
-416 TCATATGAGC AAACGACAAA GTACAAACTT GTAACACTGT GGACGCGGTA CAAAAGATAA TGAATAACAA AGCCGATGCT CGTCATATCT GCCAGCTTAT
-316 GCTTCTACAA ACCTAATAAA ATAAATATAT CTACTATAGC ATTCAATTCT AGTTAGTGCG TCGAATTCAT CTCAACATAT TTTACGCAAT CCTATGAAAA
-216 TAAATCATTG TATATCATTT AACTTTCCCT TTTCTTACTT TTTTCCCCTC CATCTTTGAA AACCAACGGT GGGAACTCGG GAGCGCGCGC TGTCAGTTGG
-116 ACAGCACGGG CGCGCGCCTC CTTTGGTCCT CCTCTTCCGC GTGAGTCCCG AGGAACCCCC ACTGCTCTCC GCGGCTCGTG CTGCCTTTTA AAGCAAACTG
-16 ACACAGCATG TCATTCCCCA CTCGGCCAGA GGACGGGACA CGGTGAGGAG CTGCTGTgtc gttcattcta ccccccttct cctgtcgtca cccccattgt
+85 ccttgttgtt tagttgcctt ttattagttc gtttacacgg ttagctagtt ttagtcatg

Figure 2

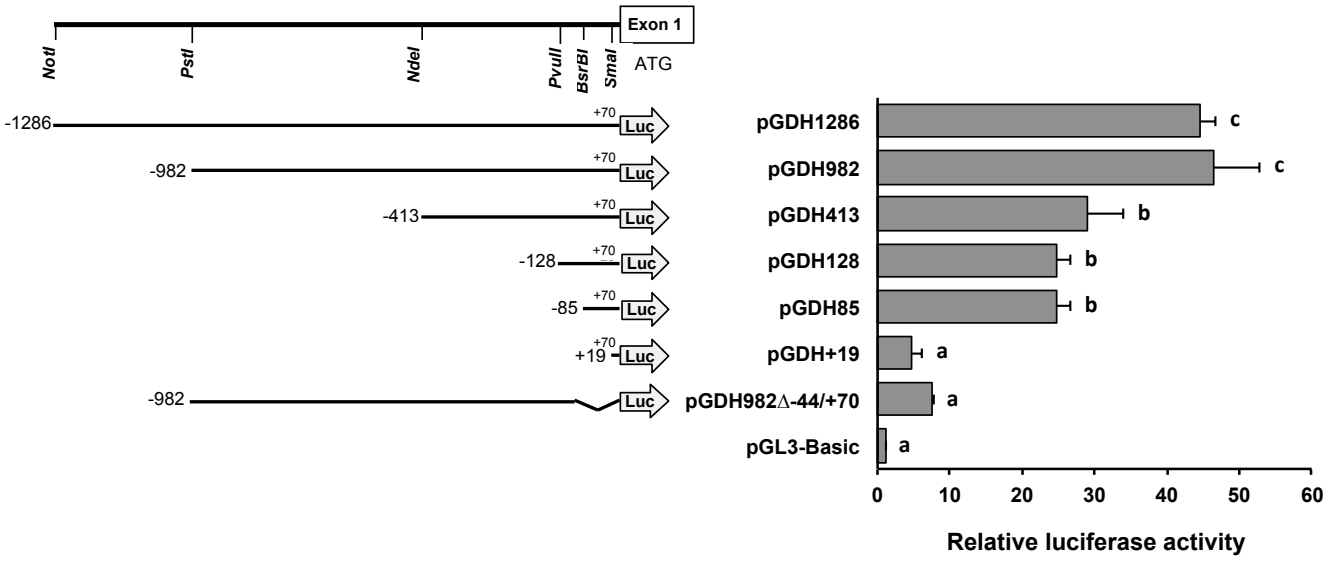

Exon 1
NotI
PstI
NdeI
PvuII
BsrBI
SmaI
ATG
-1286
-982
-413
-128
-85
+19
-982
+70
Luc
pGDH1286
pGDH982
pGDH413
pGDH128
pGDH85
pGDH+19
pGDH982Δ-44/+70
pGL3-Basic
c
c
b
b
b
a
a
a
0
10
20
30
40
50
60
Relative luciferase activity

Figure 3

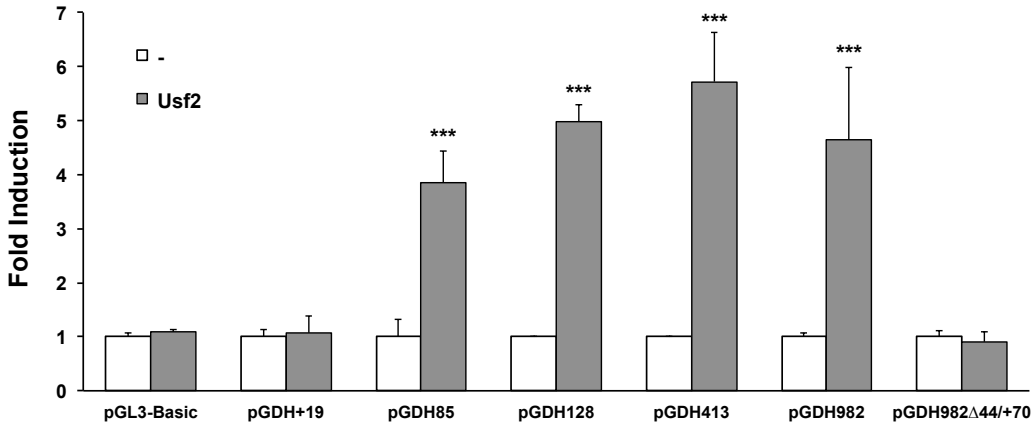
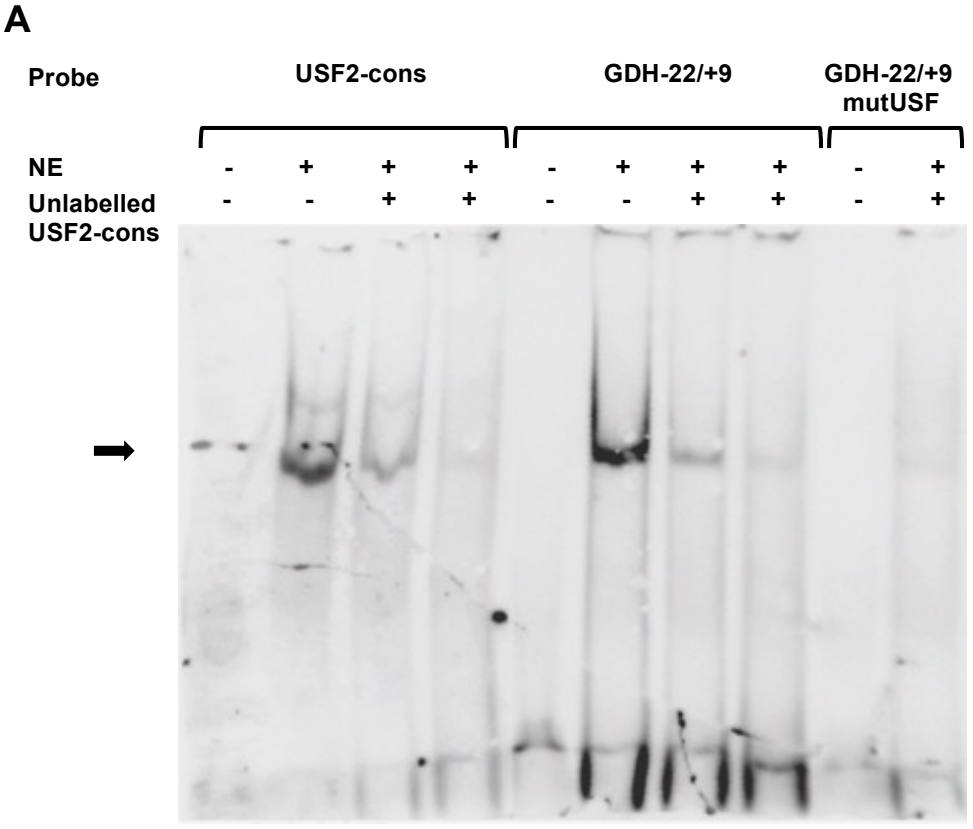

Fold Induction
7
6
5
4
3
2
1
0
-
Usf2
***
***
***
***
pGL3-Basic
pGDH+19
pGDH85
pGDH128
pGDH413
pGDH982
pGDH982Δ44/+70

Figure 4


A
Probe
USF2-cons
GDH-22/+9
GDH-22/+9 mutUSF
NE
Unlabelled USF2-cons
- + + + - + + + - +
- - + + - - + + - +

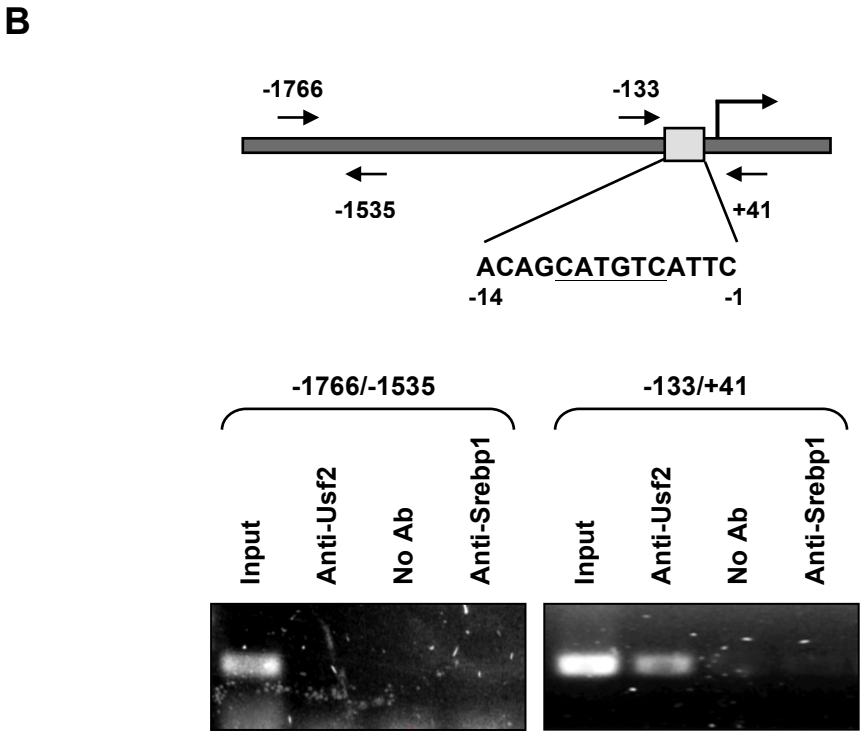

B
-1766
-133
-1535
+41
ACAGCATGTCATTC
-14
-1
-1766/-1535
-133/+41
Input
Anti-Usf2
No Ab
Anti-Srebp1
Input
Anti-Usf2
No Ab
Anti-Srebp1

Figure 5

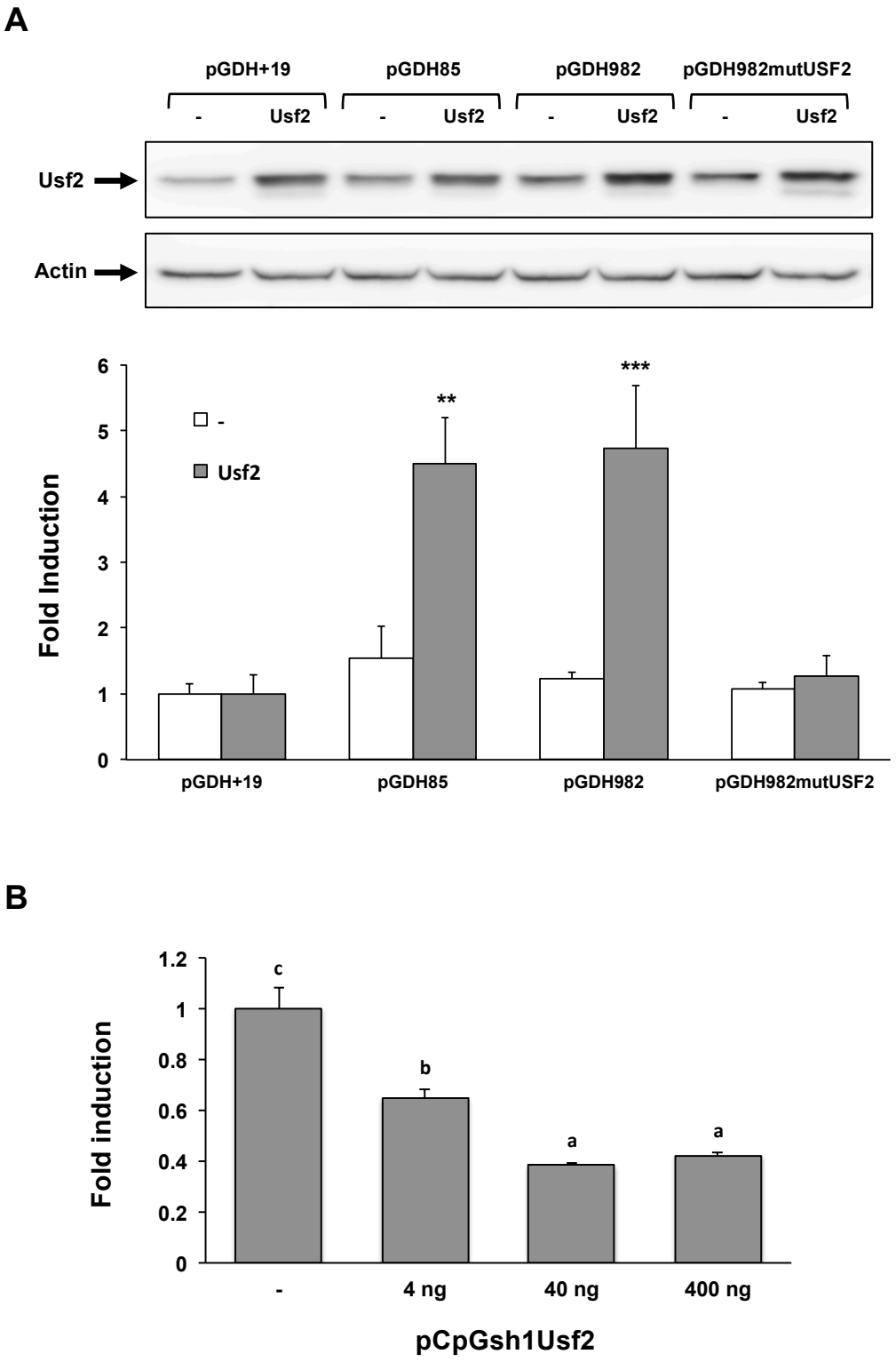

A
pGDH+19
pGDH85
pGDH982
pGDH982mutUSF2
- Usf2 - Usf2 - Usf2 - Usf2
Usf2
Actin
Fold Induction
- Usf2
**
***
pGDH+19
pGDH85
pGDH982
pGDH982mutUSF2
B
Fold induction
c
b
a
a
- 4 ng 40 ng 400 ng
pCpGsh1Usf2

Figure 6

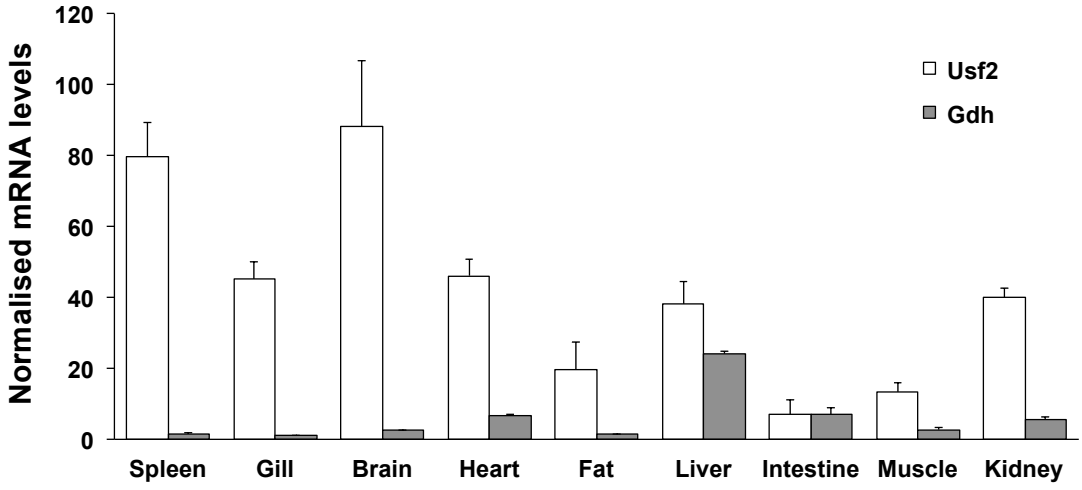
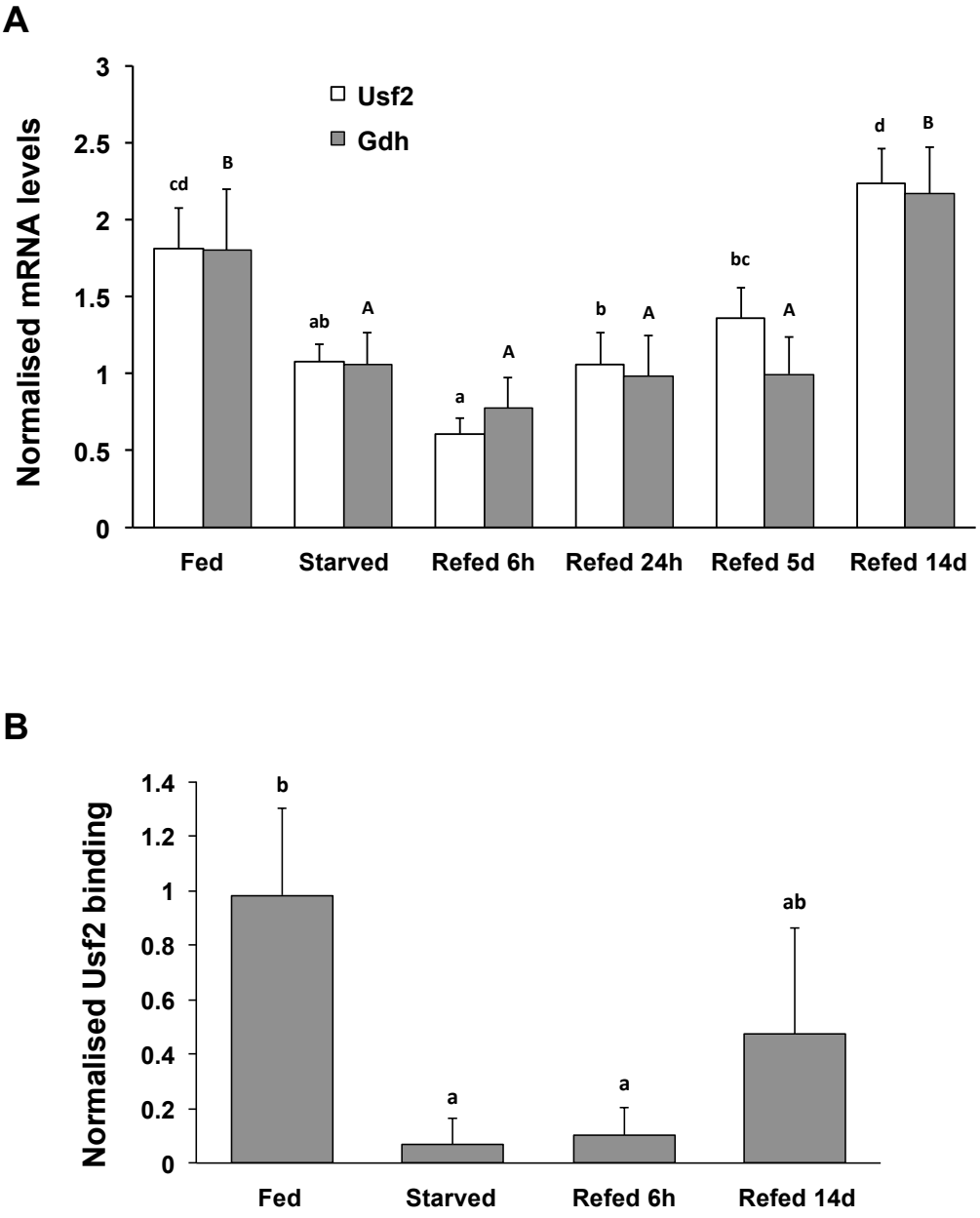

Normalised mRNA levels
120
100
80
60
40
20
0
Usf2
Gdh
Spleen
Gill
Brain
Heart
Fat
Liver
Intestine
Muscle
Kidney

Figure 7


A
Normalised mRNA levels
3
2.5
2
1.5
1
0.5
0
Usf2
Gdh
cd
B
ab
A
a
A
b
A
bc
A
d
B
Fed
Starved
Refed 6h
Refed 24h
Refed 5d
Refed 14d
B
Normalised Usf2 binding
1.4
1.2
1
0.8
0.6
0.4
0.2
0
b
a
a
ab
Fed
Starved
Refed 6h
Refed 14d