Fatty acid-binding proteins have the potential to channel dietary fatty acids into enterocyte nuclei

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Abbreviations: CEY, chicken egg yolk; dpf, days post-fertilization; FA, fatty acid; FABP, fatty acid-binding protein; FD, formulated diet; iLBP, intracellular lipid binding protein; LCFA, long-chain fatty acid; OO, olive oil; qRT-PCR, real-time quantitative RT-PCR. TP3, To-Pro 3 iodide; DAPI, 4',6'-diamidino-2-phenylindole.
Abstract

Intracellular-lipid-binding proteins, including fatty acid-binding proteins 1 (FABP1) and 2 (FABP2), are highly expressed in tissues involved in the active lipid metabolism. A zebrafish model was used to demonstrate differential expression levels of fabp1b.1, fabp1b.2, and fabp2 transcripts in liver, anterior intestine, and brain. Transcription levels of fabp1b.1 and fabp2 in the anterior intestine were upregulated after feeding and modulated according to diet formulation. Immunofluorescence and electron microscopy immunodetection with gold particles localized these FABPs in the microvilli, cytosol, and nuclei of most enterocytes in the anterior intestinal mucosa. Nuclear localization was mostly in the interchromatin space outside the condensed chromatin clusters. Native PAGE-binding assay of BODIPY-FL labeled fatty acids (FAs) demonstrated binding of BODIPY-FLC_{12} but not BODIPY-FLC_{5} to recombinant Fabp1b.1 and Fabp2. The binding of BODIPY-FLC_{12} to Fabp1b.1 was fully displaced by oleic acid. In vivo experiments demonstrated, for the first time, that intestinal absorption of dietary BODIPY-FLC_{12} was followed by co-localization of the labeled FA with Fabp1b and Fabp2 in the nuclei. These data suggest that dietary FAs complexed with FABPs are able to reach the enterocyte nucleus with the potential to modulate nuclear activity.

Supplementary key words BODIPY-labeled fatty acids, diet and dietary lipids, electron microscopy, FABP1, FABP2, fluorescence microscopy, gene expression, intestine, nucleus, zebrafish, *Danio rerio*. 
Fatty acid binding proteins (FABPs) were initially described as abundant intracellular cytosolic proteins with relatively low molecular masses (14-15 kDa) able to bind organic anions (1) and radioactively-labeled fatty acids (FAs) (2, 3). Together with cellular retinol- and retinoic acid-binding proteins, these abundant chaperone proteins are members of an ancient conserved multigene family of intracellular lipid binding proteins (iLBPs) (4-6). The evolutionary relationships of vertebrate FABPs were clarified using phylogenetic and conserved synteny analyses (7, 8). They bind long-chain FAs (LCFAs) and other lipophilic compounds (9-12) and are believed to be implicated in FA intracellular uptake and transport, lipid metabolism regulation, protection from the harmful effects of non-esterified LCFAs, and modulation of cell growth and proliferation (13-17). Despite a low amino acid sequence identity among the various FABPs, these proteins fold in a highly conserved tertiary structure. A typical member of the family consists of 127-134 amino acid residues with ten or eleven antiparallel β-strands folded into a barrel capped by two short α-helices (18). The ligand binding site is within the large water-filled interior (19, 20) but binding sites and interaction modes with some other lipophilic compounds may vary (12).

Duplication of genes and entire genomes is a major evolutionary force driving the increasing complexity of organisms (21) and the history of the FABP family has been shaped by duplication events (8). The molecular diversity of FABPs, together with overlapping and tissue-specific expression patterns, may facilitate redundancy, neo-functionalization, and sub-functionalization of biochemical properties. The precise in vivo function of each FABP is not yet clearly understood. Distinct developmental and tissue-specific expression patterns support the hypothesis of a sub-specialization in ligand preferences and/or suggest diversity in uncharacterized functions.

It was demonstrated, using cell cultures, that FABPs may channel unesterified FAs and other lipophilic ligands into nuclei, potentially targeting them to transcription factors, and initiate nuclear receptor transcriptional activity (11, 22-33). To our knowledge, no in vivo data are currently available to support the hypothesis that exogenous FAs enter cell nuclei via their binding to FABPs. The dietary
Triacylglycerols are the major source of lipids in the intestinal lumen. Their hydrolysis releases large quantities of medium to LCFAs absorbed by the enterocytes via complex mechanisms, involving both passive diffusion and protein-mediated transport (34-38). Once inside the cell, dietary FAs are reversibly bound to lipid binding proteins involved in the transport of FAs from the plasma membrane to cellular compartments, e.g. FABPs and acyl CoA binding proteins. The intestinal mucosa and its enterocytes provide a very attractive system for evaluating the fate of exogenous FAs inside cells. FABP1 and FABP2 are the most strongly expressed FABP family members in the human small intestine (13, 14) and these proteins are found in abundance in absorptive cells (39, 40). In zebrafish, the anterior intestine is the major site of fat absorption (41-43) and different fabp genes were found expressed in the intestine including fabp1a, fabp1b.1, fabp1b.2, fabp2, fabp3, fabp6, fabp7a/fabp7b, fabp10a/fabp10b, and fabp11a (41, 44-47). The teleost ancestor experienced a whole-genome duplication event at the base of the teleost radiation (48) leading, in some cases, to the retention of pairs of duplicate genes, e.g. fabp1a/fabp1b. The resulting ohnologs may have retained their initial sub-functions, as well as acquiring new functions, i.e. neo-functionalization, and/or duplicate genes may be preserved by partitioning ancestral sub-functions between them, i.e. sub-functionalization. In addition, tandem gene duplication may arise as demonstrated with fabp1b.1 and fabp1b.2 found adjacent in the zebrafish genome (44).

Zebrafish were used to study the expression pattern and dietary regulation of homologs to human FABP1 and FABP2, which are significantly expressed in the anterior intestine of this vertebrate model. The next step was cellular and sub-cellular immuno-localization of Fabp1b and Fabp2 along the intestinal villi. Thirdly, an assessment of the fate of dietary fluorescent (BODIPY) labeled analogs of FAs revealed that dietary BODIPY-FLC12 (medium-chain) but not BODIPY-FLC5 (short-chain) was co-localized with Fabp1b and Fabp2 in enterocyte nuclei.
MATERIALS AND METHODS

Animals

Wild-type zebrafish (*Danio rerio*) were produced in our facilities according to the rules approved by the Ministère de l’Agriculture, de l’Agroalimentaire et de la Forêt, France, under permit number A33-522-6. All experiments were conducted in conformity with the 2010/63/EU directive on the protection of animals used for scientific purposes. Larvae were obtained by natural mating and raised in embryo water (90 µg/ml Instant Ocean [Aquarium Systems, Sarrebourg, France], 0.58 mM CaSO₄, 2H₂O, dissolved in reverse-osmosis purified water) at 28.5°C with an 11L:13D photoperiod.

Feeding experiments and tissue sampling

Zebrafish adults were fed *ad libitum* with TetraMin flakes (Tetra GmbH, Melle, Germany) containing 48% protein, 8% lipid, 11% ash, 2% fibers, and 6% moisture. Larvae were fed on ZF Biolabs formulated diet (FD) flakes (Tres Cantos, Spain), dried chicken egg-yolk (CEY) powder, or olive oil (OO).

Zebrafish eleutheroembryos at 5 days post-fertilization (dpf) and larvae at 15 dpf, as well as adult males, were sampled for real-time quantitative PCR (qRT-PCR). On the day preceding the nutritional trials, larvae and adults were divided into two groups and transferred into containers with embryo water but no food. After approximately 18 h (larvae) or 22 h (adults) food deprivation, one group of larvae or adults was fed for 2–2.5 h, while the second group continued to fast. After feeding, the eleutheroembryos/larvae were transferred into clean embryo water to eliminate any remaining food, and six pools of 25 eleutheroembryos (5 dpf) or 20 larvae (15 dpf) were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. Two separate batches were used as sources of larval experiments. The adult males were dissected and six individual samples of liver and anterior intestine were isolated. The ventral white adipose tissue was carefully removed, as it was closely associated with...
the digestive tract on the ventral side and also with liver lobes at some points (49). Samples were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction for the RT-PCR and qRT-PCR experiments. Larvae and adult animals from the fasted group were sampled and treated in the same way after the fed animals and were. In addition to the liver and anterior intestine, six separate brain samples were collected, one ovarian and one testicular sample, as well as one heart sample from a pool of six animals and one kidney sample from a pool of four animals. All samples, except the heart, kidney, and ovarian tissues, were collected from male organisms.

For whole-mount in situ hybridization, 15 dpf zebrafish larvae, i.e. at a fully exotrophic nutritional stage, fasted for 24 h and were then fed for 3h with FD, dried CEY, or OO. About 15 larvae per treatment or sampling point were fixed in 4% paraformaldehyde (PFA) at 4°C overnight, rinsed three times in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 0.02 M PO₄), and dehydrated by successive washes with methanol (25%, 50%, 75%, 95%, and 100%) and stored at -20°C prior to analysis.

Dietary experiments with the green fluorescent BODIPY-FA analogs were performed using adult zebrafish. BODIPY [boron dipyrromethene (4,4-diXuoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene)] and BODIPY-FL analogs (Life Technologies) were resuspended in methanol (1 µg/µl) and mixed well with a CEY emulsion (10 µl/ml), prepared from dried CEY mixed in water using an IKA Ultra-Turrax Homogenizer. The BODIPY₄₉₃/₅₀₃, BODIPY-FLC₅ (D3834), or BODIPY-FLC₁₂ (D3822)-CEY emulsion was maintained at 37°C for 10 min for the solvent to evaporate. BODIPY₄₉₃/₅₀₃ (D3922) was used as a negative control. Animals previously starved for five days were fed ad libitum (labeled emulsion/fish water = 1/600, v/v) and their food intake was checked visually. Samples were taken 3 h after feeding. Quantitative analysis of dietary BODIPY-FA analog fluorescence signal after intestinal absorption in zebrafish intestinal villi was determined using ImageJ free-processing software (National Institutes of Health (NIH), http://rsb.info.nih.gov/ij/) as previously described (50).
Extraction of total RNA, reverse transcription, and PCR analyses

Total zebrafish RNA was extracted using the NucleoSpin® RNA II extraction kit (Macherey-Nagel, Hoerdt, France) according to the manufacturer’s instructions. Total RNA was checked for integrity by ethidium bromide staining in agarose borax gel and quantified using a NanoDrop ND-1000 spectrophotometer (Nyxor Biotech, Paris, France). Reverse transcriptions were performed with 1 µg total RNAs using the StrataScript™ qRT-PCR cDNA synthesis kit (Stratagene, Amsterdam, The Netherlands), with an oligo(dT) primer, according to the manufacturer’s instructions. The reaction mixture (20 µL) was incubated at 37°C for 30 min, 42°C for 1 h 30 min, and then 70°C for 10 min. Two RTs and one RT negative control, i.e. without reverse transcriptase, were performed on each sample. Qualitative PCR was also carried out on 2 µL RT reaction product diluted 1/40 in a final volume of 20 µL containing 10 µL Brilliant® SYBR® Green qRT-PCR Master Mix and 0.25 µM of each primer. An aliquot of the PCR reactions was electrophoresed on 1.5% agarose gel containing ethidium bromide and PCR products were visualized and photographed.

Real-time quantitative RT-PCR (qRT-PCR)

Transcripts were amplified using the MX 3000P qRT-PCR thermal cycler instrument (Stratagene, Amsterdam, Netherlands). qRT-PCR amplification was carried out in a final volume of 20 µL, using 10 µL Brilliant® SYBR® Green qRT-PCR master mix (Stratagene), 0.25 µM oligodeoxyribonucleotide primers, and 3 µL diluted cDNA: 5 and 15 dpf zebrafish eleutheroembryos/larvae (1/160), anterior intestine (1/160), liver (1/80), and various adult tissues (1/40). Each run included a standard curve made up of 7-8 serial dilution points of a pool of cDNA from the samples to be quantified. Amplification was performed with systematic negative controls (NTC: non template control containing no cDNA and RTneg: RT performed without reverse transcriptase). Each RT from the same sample was amplified in duplicate and, when there were no significant differences in C_T, the two RT products were pooled. Elongation factor 1α (eef1a1) and peptidylprolyl isomerase aa (ppiiaa) were chosen as
normalizing genes for qRT-PCR. The reference sequence, design details, size of the fragment produced, and Tm of the primer pairs used are described in Supplementary Table 1. The qRT-PCR profiles contained an initial denaturation step at 95°C for 10 min, followed by 40 cycles: 30 sec at 95°C, 30 sec at the specific primer pair annealing Tm (see Supplementary Table 1), and 10 sec (30 sec for eef1a1) at 72°C. After the amplification phase, 1 min incubation at 95°C and 30 sec at 55°C was followed by a ramp up to 95°C, at 0.01°C/sec, where data was collected in continuum to obtain a single product dissociation curve. The qRT-PCR product sizes were checked on 2% agarose gel and all amplicons were sequenced to confirm specificity. No amplification was observed in RT negative controls and no primer-dimer formation occurred in the NTC. qRT-PCR efficiency was 100 ± 2% and the correlation coefficient was > 0.995 for each run. Six independent samples (5 or 15 dpf eleutheroembryos/larvae and liver or anterior intestine from individual adults) were tested in triplicate each time. Four independent brain samples were tested in triplicate. Zebrafish heart (pooled from six animals), kidney (pooled from four animals), ovary, and testicle samples were subjected to a single determination in triplicate.

**Whole-mount in situ hybridization**

Whole mount in situ hybridization was carried out on zebrafish larvae, sampled and fixed in 4% PFA, as described above. A clone purchased from the German Resource Center for Genome Research (RZPD), Berlin, Germany (IMAGE: 7225625), corresponding to GenBank database dbEST zebrafish clone gb BC095259.1, was used to generate the fabp1b RNA probe. The sense (ZF-fabp1b F1) 5-CAAGACTATTGTGAACAGAGA-3 and antisense (ZF-fabp1b R1) 5-TGAGATTGAGAACACTTTAATG-3 primers were designed from this clone and used for probe synthesis, as previously described (43), except that the primer annealing temperature (Tm) in the thermal profile was 55°C for fabp1b in the first PCR amplification. For the fabp2 RNA probe, the ZF-FB clone (41) was used, corresponding to a 203 bp PCR product of GenBank database dbEST
zebrafish clone gbAJ132590 after amplification with the sense (oligo ZFA1) 5'-CTGTCATCATGACCTTCAACGG-3 and antisense (oligo ZF A3) 5'-CCGCACACTGAAATTAACCT-3 primers, subcloned using the pGEM-T Easy vector kit (Promega, France). The second PCR was performed with the two pGEM-T Easy vector cDNAs, using the T7 and SP6 universal primers, as previously described (43). As the fragments were sense-oriented in the vector, the PCR template for the fabp1b.1 sense probe was produced using T7 universal primer, while the PCR template for the antisense probe was produced using SP6 universal primer and the inverse for the fabp2 probes. Both antisense and sense digoxigenin (DIG)-labeled RNA probes were synthesized using the DIG RNA labeling kit (SP6/T7) (Roche Diagnostics, Meylan, France), following the manufacturer’s instructions. The 211 bp fabp1b probe was able to hybridize to fabp1b.1 and fabp1b.2 transcript variants. The fabp2 probe was 203 bp long. The protocol for in situ hybridization was as previously described (51, 52), except that prehybridization and hybridization were conducted at 60°C. The post-hybridization stringent baths were at hybridization temperature, except for the last two baths in 0.2X SSC Tween at 57 °C (30 min each). In contrast, the hybridization buffer contained 50% formamide and the animals were incubated in pre-absorbed sheep anti-DIG-AP Fab (Roche Diagnostics, Meylan, France) fragments at 1:5,000 dilution at 4°C overnight. The antibody was rinsed in six PBS-Tween baths for 30 min each time.

**Amino acid sequence analyses**

Deduced protein sequences were extracted from the UniProt (53) database. Sequences were aligned (Fig. S1) using the ClustalW2 program (54).

**Recombinant Fabp1b.1 and Fabp2 production and purification**

Zebrash fish fabp1b.1 and fabp2 full coding cDNA sequences (Supplementary Table 1) were cloned in the pET5a vector (Promega). pET5a-Fabp1b.1 and pET5a-Fabp2 constructs were transformed into
BL21 (DE3) Star E. coli strain (Novagen). Bacterial cells containing the relevant expression plasmid were cultured in 2X yeast extract and tryptone media at 30°C for Fabp1b.1 and 37°C for Fabp2 for approximately 4 h before Fabp synthesis was induced by adding 0.4 mM isopropyl β-D-1-thiogalactopyranoside and incubating for a further 4 h. After centrifugation at 5,000 x g for 10 min, the cells were collected and re-suspended in cell lysis buffer (30 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 8.3) and ruptured by sonication. Cell debris were removed by centrifugation at 15,000 x g at 4°C for 30 min and the supernatant was processed in two ammonium sulfate precipitation steps. (NH₄)₂SO₄ was first added to a final concentration of 30% with stirring at room temperature for 2 h, followed by centrifugation at 15,000 x g for 15 min. The supernatant fraction was treated with (NH₄)₂SO₄ to a final concentration of 50% and centrifuged at 15,000 x g for 15 min. The latter supernatant fraction (approximately 40 ml) was dialyzed against 4 liters of 30 mM Tris-HCl pH 8.3 at room temperature for 12 h, changing the buffer every 4 h, and finally concentrated by ultrafiltration. The concentrated fraction (approximately 10 ml) was loaded onto a SephadexG50 fine gel filtration column (141 cm x 1.9 cm). Chromatography was performed at 4°C, using 30 mM Tris-HCl pH 8.3 as the mobile phase at a flow rate of 15 ml/h. Fabp fractions were analyzed by SDS-PAGE, MALDI-TOF and MALDI-TOF-TOF mass spectrometry to determine protein purity.

**Antibody production and characterization**

Polyclonal antisera against recombinant zebrafish Fabp1b.1 and Fabp2 were obtained by injecting New Zealand white rabbits with 0.8 mg purified recombinant proteins dissolved in 0.4 ml 30 mM Tris-HCl, pH 8.3, together with an equal amount of Freund's complete adjuvant. One month later, a booster injection contained 0.4 mg purified protein in Freund’s incomplete adjuvant. Bleed tests were carried out at days 0, 20, and 40, for antibody title response measurement, and a final blood sample was collected at day 68. Blood was allowed to clot and retract at 37°C for 1 h, followed by 16 h at 4°C, and the collected serum was stored at -20°C. Crude polyclonal antisera were purified by affinity column
chromatography at room temperature after linking highly purified Fabp1b or Fabp2 to cyanogen bromide-activated-Sepharose (Sigma-Aldrich), according to the manufacturer’s instructions. The eluate was washed by adding 0.1 M glycine and 0.15 M NaCl, pH 8.2, until its A280 nm reached zero. The antibodies were desorbed using 0.1M glycine and 0.5M NaCl, pH 2.6. The fractions collected were immediately spiked with 1:10 (v/v) Tris-HCl 1 M, pH 8.3, to neutralize the pH and stored at -20°C until use. Anti-serum titration was performed by ELISA. Plates were coated with 100 ng highly-purified Fabp1b.1 or Fabp2. All the following steps were conducted in PBS-Tween buffer. Plates were blocked with 5% BSA at room temperature for 1 h. Serial dilutions (1/200 to 1/102,400) of each anti-serum were incubated for 2 h at room temperature. Plates were extensively washed, incubated with Alexa-488 goat anti-rabbit IgG antibody (Molecular Probes) and, finally, extensively re-washed. The signal was measured in a Varioskan Flash spectrofluorometer (Thermo Scientific). Antibody specificity was assayed by Western immunoblotting. Following SDS-PAGE, total proteins extracted from 5 dpf zebrafish eleutheroembryos were transferred to Hybond-C membrane (GE Healthcare Amersham). Membranes were blocked with 0.1% BSA and 2% glycine in PBS-Tween, and then incubated in the presence of crude anti-Fabp1b.1 (1/10,000) or anti-Fabp2 (1/1,000) anti-sera or purified polyclonal antibodies at room temperature for 2 h. Membranes were washed and incubated with affinity purified anti-rabbit IgG-alkaline phosphatase produced in goat (1/30,000, Sigma-Aldrich), at room temperature for 1 h and then washed again. Alkaline phosphatase activity was developed with 0.33 mg/ml nitro-blue tetrazolium and 0.165 mg/ml 5-bromo-4-chloro-3’-indolyphosphate in developing buffer (100 mM Tris-HCl, pH 9.5, 0.1 M NaCl, and 5 mM MgCl2). Proteins extracted from 5 dpf zebrafish eleutheroembryos exhibited one immuno-reactive band at the right predicted relative molecular mass of Fabp1b.1 and Fabp2, thus demonstrating the mono-specificity of each antibody (Fig. S2).

Immunofluorescence labeling analyses

Immunofluorescence was measured on dissected zebrafish digestive cavity and anterior intestinal
fragments. Tissues were fixed with 4% paraformaldehyde in PBS at 4°C overnight and embedded in graded alcohol/paraplast and toluene/paraplast series, and then, finally, in paraplast. Sections 10-µm thick were then used for immunodetection and confocal microscopy. The sections were blocked in 5% normal goat serum, 0.1% BSA, and 0.05% Tween-20 in PBS (PBST) at room temperature for 20 min. Sections were incubated with rabbit pre-immune serum, or purified polyclonal anti-Fabp1b.1 (1/2,000), or anti-Fabp2 (1/200) primary antibodies in 1% normal goat serum and 0.1% BSA in PBST at 37°C for 2 h. After extensive washing, 4 x 5min with PBS, the sections were incubated with secondary Alexa 488- or Alexa 594-conjugated goat anti-rabbit IgG (H+L) (Life Technology) at 1/1000 dilution in PBS at room temperature for 1 h. The slides were then immersed in a solution of To-Pro 3 iodide (TP3) or 4',6'-diamidino-2-phenylindole (DAPI) (1/4,000) to label the nuclei red or blue. Following several washes in PBS, samples were mounted with Gold ProLong anti-fade mounting medium (Life Technology), sealed with nail polish and stored in a dark place at 4°C prior to analysis. Control sections were treated without the primary antibody. Sections were viewed using an Olympus FV-300 scanning laser confocal microscope.

**Electron microscope immunocytochemistry**

Fragments of anterior intestine were fixed in 1% glutaraldehyde and 4% formaldehyde in 0.1 M PBS, pH 7.4, for 12 h, changing the solution three times. After washing in the same buffer, the samples were embedded in white LR resin. Ultra-thin sections were treated successively with glycine 0.02 M and PBS, blocked with 1% BSA for 5 min, treated with primary antibody diluted in PBS containing 1% BSA for 1 h, washed with PBS, and then incubated with the 10 nm protein A-colloidal-gold complex (Sigma-Aldrich) in PBS containing 1% BSA for 1 h. All the incubation samples consisted of a 30 µL drop of solution at 25°C. Controls were prepared by floating sections on PBS to replace primary antibodies. Slices were contrasted with 5% uranyl acetate for 5 min and examined under a Zeiss 6EM902 electron microscope. Sub-nuclear immunogold particle density was determined using the area
and particle tools in ImageJ free-processing software.

**BODIPY-FL FAs binding to recombinant Fabps**

Stock 2 mM solutions of BODIPY-FLC$_5$ or BODIPY-FLC$_{12}$ in ethanol were diluted in PBS prior to mixing and incubating with purified proteins for a few minutes. Prior binding assays, the proteins were delipidated in batch using hydroxyalkoxypropyl-dextran (Sigma-Aldrich) chromatography, equilibrated with 50 mM Tris-HCl, pH 7.4, at 37°C for 45 min. The proteins were then diluted at the working concentration with 30 mM Tris HCl, pH 8.3 sample buffer. 5 µL 20 µM BODIPY-FLC$_5$ or BODIPY-FLC$_{12}$ were mixed with 20 µL 5.6 µM recombinant zebrafish Fabp1b.1, 12 µL 4.3 µM tropomyosin A (TPMA) from *Echinococcus granulosus* (GenBank: AAB65799.4), or 6.6 µL 7.5 µM BSA. A 12 µL sample of 20 µM BODIPY-FLC$_5$ or BODIPY-FLC$_{12}$ was mixed with 20 µL 30 µM recombinant zebrafish Fabp2. The binding of fluorescent carbon chain BODIPY FAs to recombinant Fabps was then evaluated by an in-gel fluorescence imaging method derived from the one previously described for visualizing BODIPY-arachidonic acid-tagged cellular proteins (55, 56). A dye-free 4X loading sample buffer containing 200 mM Tris-HCl (pH 6.8), 20% glycerol, and 8% SDS was then added to each sample. Samples were then loaded on a 1.5 mm thick, 15% native polyacrylamide gel and electrophoresed using a running buffer containing 0.05 M Tris-HCl and 2.6 M glycine. After in-gel fluorescence imaging (G:Box, Syngene), gels were stained with Coomassie blue R250 to check for protein loading and electrophoretic migration. All manipulations were done in the dark.

Fluorescence emission spectra of BODIPY-FLC$_5$ or BODIPY-FLC$_{12}$ in the presence and absence of Fabp1b.1 were determined at 25°C using a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Santa Clara). BODIPY-FLC$_5$ or BODIPY-FLC$_{12}$ 2 mM stock solutions in ethanol were diluted in PBS prior to mixing and incubating for 2 minutes with purified FABP1b.1. The protein sample was previously dissolved with 30 mM Tris HCl, pH 8.3 buffer. The emission spectrum was measured from 500 to 600 nm while the excitation wavelength was set at 488 nm.
Displacement experiments

Fabp1b.1 was dissolved in 30 mM Tris HCl, pH 8.3 sample buffer (5.6 µM, 22.5 µL) and incubated with BODIPY-FLC₁₂ (20 µM, 7.5 µL) at room temperature for 10 min. The solution was then mixed with 3 µL unlabeled FA taken from a 5 mM stock solution in ethanol and supplemented with 10 µL loading sample buffer used for loading on the native polyacrylamide gel. Under these conditions, the unlabeled FAs were in around 100-fold molar excess, i.e. 0.35 mM, over the labeled FA and protein used. The following competitor ligands assayed were oleic acid (C₁₈:₁w₉), linoleic acid (C₁₈:₂w₆), and DHA (C₂₂:₆w₃). The samples were mixed and allowed to equilibrate for 20 min at room temperature before loading 29 µL into the native polyacrylamide gel.

Tandem mass spectrometry

Stained PAGE bands were faded, excised, and in-gel digested with trypsin in the appropriate buffer. The peptides resulting from the proteolytic digestion were identified by peptide mass fingerprinting and collision-induced dissociation using a 4800 MALDI-TOF-TOF mass spectrometer (Applied Biosystems). The MS data were analyzed using Mascot software (Matrix Science LtD, London, UK) and searching the NCBI/rr and ZFIN zebrafish databases.

Statistics

Variations in transcription levels (normalized by reference genes) were analyzed for statistical significance using REST-384-version 2 software for multiple reference genes (http://rest.gene-quantification.info/) and/or a nonparametric Mann-Whitney test (GraphPad InStat v3.5, GraphPad Software). The significance level was set at 0.05. Data are presented as mean ± standard deviation (SD). The same test was used to check for variations in normalized ef1a/ppiaa values.
RESULTS

**FABP1 and FABP2 homologs in zebrafish**

Tandem duplicated *fabp1b.1* and *fabp1b.2* on zebrafish chromosome 8 and *fabp2* on chromosome 1 [(http://zfin.org/)](http://zfin.org/) generated proteins of the predicted molecular mass of 14,115 Da and 128 amino acids long, 14,461 Da and 128 amino acids long, and 15,090 Da and 132 amino acids long, respectively. Human FABP1 exhibited 54.33% and 41.73% identity with Fabp1b.1 and Fabp1b.2, respectively (Fig. S1). Zebrafish Fabp2 was identical in size and exhibited 66.67% identity with its human homolog (Fig. S1). Zebrafish Fabp1b.1 and Fabp1b.2 exhibited 21.09% and 15.62% identity with zebrafish Fabp2, while these two proteins showed 50.78% identity between them.

**Tissue-specific distribution of zebrafish fabp1b.1, fabp1b.2, and fabp2 transcripts**

Qualitative RT-PCR detected *fabp1b.1* and *fabp1b.2* transcripts in total RNA extracted from the anterior intestine in adult zebrafish, irrespective of the animals' nutritional status (Fig. 1A). qRT-PCR indicated similarly high levels of transcripts for *fabp1b.1* and *fabp2* in the anterior intestine, by comparison with normalizing genes *eef1a1* (Fig. 1B, D) and *ppiaa* (data not shown). *fabp1b.2* mRNA levels were around threefold lower (Fig. 1C, data not shown). The normalized level of *fabp1b.2* transcripts was very high in brain (Fig. 1C, data not shown). Trace amounts of *fabp2* transcripts were also expressed in adult brain while *fabp1b.1* transcripts were not detected. By contrast, *fabp1b.1* transcripts were detected in the liver, together with very small amounts of *fabp2* mRNA, while *fabp1b.2* transcripts were not detected (Fig. 1, data not shown). Zebrafish *fabp1b.2* transcripts were also detected in ovaries, testicles, kidneys, and heart, and trace amounts of *fabp2* transcripts were found in kidneys.
Regulating *fabp1b.1* and *fabp2* transcript levels by the nutritional status and lipid composition of the diet and cephalocaudal patterning of their expression in gut

The aim of the experimental work was to determine whether the *fabp1b.1* and *fabp2* transcript levels were regulated after feeding and/or according to the lipid composition of the diet. Suitable primer sets were designed on the basis of the cDNA sequences previously obtained to be used for qRT-PCR and primers for synthesizing specific probes for whole-mount, in situ hybridization. The modulation, if any, of transcript levels after feeding and by dietary lipid composition was evaluated by using commercially and in-house formulated synthetic larval diets and the techniques described above.

The first step was to study whether regulation of *fabp1b.1* and *fabp2* transcript levels occurred in the zebrafish adult intestine and liver after feeding. As indicated in Figure 2, transcript levels of *fabp2* (*P = 0.0087*) were upregulated by 1.59 fold and *fabp1b.1* by 1.36 fold (not significant) in the anterior intestine when the reference gene used was *eef1a1*. REST software, using both *ppiaa* and *eef1a1* as reference transcripts, gave *P = 0.021* for *fabp2*. This upregulation was not observed in the liver (Fig. 2).

A similar study was then conducted at early developmental nutritional stages, i.e. first feeding at 5 dpf and at the start of the exogenous nutritional phase of larval development, i.e. 15 dpf (Fig. 3). There were no significant differences in *fabp1b.1* and *fabp2* transcript levels between nourished and unnourished 5 dpf eleutheroembryos when normalized with the two reference genes, while a slight upregulation of *fabp1b.1* was detected when using *ppiaa* only (x 1.15 fold, *P = 0.026*). Transcripts levels of *fabp1b.1* and *fabp2* were also significantly upregulated after feeding in whole 15 dpf zebrafish larvae homogenates, irrespective of the reference gene used (Fig. 3). *fabp1b.1* transcript levels were 1.41 (*P = 0.026*) and 1.54 (*P = 0.0022*) higher, respectively, when *eef1a1* and *ppiaa* were used as reference transcripts. REST software using the two reference transcripts at the same time gave a *P* value of 0.001. *fabp2* transcripts levels were 1.59 (*P = 0.026*) and 1.71 (*P = 0.0022*) higher, respectively, when *eef1a1* and *ppiaa* were used as reference transcripts. REST software using the two reference transcripts at the same time gave a *P* value of 0.004. The effect of diet composition on the
level of *fabp1b* (*fabp1b.1 + fabp1b.2*) and *fabp2* transcript levels was then studied by whole-mount *in situ* hybridization ([Fig. 4](#)). An upregulation of *fabp1b* and *fabp2* hybridization signals between starved and fed conditions was detected and the signal was apparently modulated according to the lipid composition of the diet. 15 dpf larvae nourished with FD experienced a high induction of the signal after feeding, while larvae fed with dried CEY or OO were in an intermediate state ([Fig. 4](#)).

A differential expression pattern of *fabp* genes along the anterior-posterior axis of the gut was demonstrated by whole-mount *in situ* hybridization ([Figs. 4, Fig. S3](#)). *fabp1b* (*fabp1b.1 plus fabp1b.2*) and *fabp2* transcripts were strongly expressed in the anterior intestine and its rostral expansion and no mRNAs could be detected in the posterior intestine. A significant *fabp2* transcript level was detected in the rectum but no hybridization signal with *fabp1b*.

**Localization of Fabp1b and Fabp2 in adult zebrafish intestine**

Histological sections of the digestive cavity were stained with monospecific polyclonal antibodies raised against recombinant zebrafish Fabp1b.1 or Fabp2 ([Fig. 5](#)). It is important to notice that the antibody raised against Fabp1b.1 may also detect a Fabp1b.2 signal, due to a significant sequence identity between the two; from now on we will refer to the signal generated by Fabp1b. Fabp1b and Fabp2 were immunodetected at high levels in the anterior intestine. A lower level Fabp1b signal was detected in the liver ([Fig. 5A](#) and data not shown). No Fabp1b ([Fig. 5A](#)) or Fabp2 (data not shown) labeling signal was found in the posterior intestine, testicles, white adipose tissue, or infiltrated pancreatic islets. Higher magnification revealed homogenous cytoplasmic Fabp1b and Fabp2 expression in the intestinal absorptive cells. Expression was detected through the cytoplasm, including the microvilli, and inside the nuclei of some enterocytes mostly outside condensed chromatin clusters.

Immunogold labeling electron microscopy was used for cellular and subcellular localization of Fabp1b ([Fig. 6, Fig. S4](#)) and Fabp2 ([Fig. S5](#)) in enterocytes. Gold particles were observed inside and on the surface of the microvilli, as well as in the terminal web. Immunogold particles were also
abundant in the cytosol, outside the endoplasmic reticulum membrane, in close proximity to the plasma membrane, and inside the nucleus. No immunogold particles were detected inside oil globules, mitochondria, intercellular space, goblet cells, or lamina propria. Fabp1b nuclear immunogold particles were found in the interchromatin space with a labeling efficiency 4.34 times higher than in nuclear membrane-associated condensed chromatin and condensed chromatin clusters. The same was true of Fabp2, with 3.01-fold more labeling in the interchromatin space by comparison with the condensed chromatin areas.

**Native PAGE binding assay of BODIPY-FL labeled FAs to recombinant Fabp1b.1 and Fabp2**

A native PAGE binding assay was used to check that recombinant Fabp1b.1 or Fabp2 were able to specifically bind BODIPY-labeled FAs (Fig. 7). Tropomysin A, used as a negative control, was unable to bind the labeled FAs. We demonstrated that neither Fabp1b.1 nor Fabp2 were able to bind BODIPY-FLC₅ to any significant extend, but both of them avidly bound BODIPY-FLC₁₂. While a very heavily labeled band was observed with BODIPY-FLC₁₂ (Fig. 7, panels B, D), only a very faintly-labeled band was observed with Fabp1b.1 and BODIPY-FLC₅ (Fig. 7, panel B, lane 5). Adding SDS to a final concentration of 2% in the loaded sample improved the PAGE protein resolution without a drastic change in the quantity of BODIPY-FLC₁₂ fluorescence bound to FABPs (Fig. S6, data not shown). The inability of BODIPY-FLC₅ to bind to Fabp1b.1 was confirmed by very similar fluorescence emission spectra of this labeled compound in the presence or absence of Fabp1b.1 (Fig. S7A). The binding of BODIPY-FLC₁₂ by Fabp1b.1 was supported by the increase in fluorescence quantum yield to the quantum yield obtained with BODIPY-FLC₁₂ in buffer alone (Fig. S7B). Native PAGE of purified recombinant zebrafish Fabp2 revealed two protein bands (Fig. 7, panel C). In both bands extracted from native PAGE, MALDI-TOF-TOF mass spectrometer analysis recovered Fabp2. The lower molecular weight Fabp2 bound BODIPY-FLC₁₂, while the Fabp2 multimer upper band was unable to do so (Fig. 7, panel D). BSA was used as a positive control. Under these experimental conditions, BSA
was able to bind to both BODIPY-FL labeled FAs (Fig. 7, panel B, lanes 8 and 9). However, the intensity of the labeled bands was much lower than that observed with BODIPY-FLC\textsubscript{12} binding to Fabp1b.1 or Fabp2. By higher the amount of BSA loaded into the gel, our in-gel fluorescence imaging method confirmed that this protein was able to bind BODIPY-FLC\textsubscript{12} (Fig. S8).

We then demonstrated that oleic acid was able to fully abolish the binding of BODIPY-FL C\textsubscript{12} to Fabp1b.1 (Fig. 8). DHA had partial displacement activity under these experimental conditions, while linoleic acid was ineffective.

**Feeding with BODIPY-labeled FAs**

To investigate the involvement of Fabp1b and Fabp2 in the targeting of dietary FAs to nuclei, adult zebrafish were fed with BODIPY-FLC\textsubscript{5} (Fig. 9) or BODIPY-FLC\textsubscript{12} (Fig. 10, Fig. S9) mixed with dried CEY. Both fluorescent FAs were taken up by the enterocyte and a wide cytoplasmic distribution was observed. These experiments also showed that nuclear incorporation of FA analogs depended on the ligand carbon chain length. A strong BODIPY-FLC\textsubscript{5} signal was located in the basal region of the enterocyte and surrounding the nuclei, while a robust BODIPY-FLC\textsubscript{12} signal was also observed inside the nuclei. Fluorescence microscopy images demonstrated that the BODIPY-FLC\textsubscript{12} signal associated with the nuclear fraction was 18.05 ± 1.76% of the total fluorescence detected in the villi (7 villi analyzed which included 225 enterocyte nuclei). Some enterocyte nuclei were heavily stained while other one with more condensed chromatin had low BODIPY-FLC\textsubscript{12} intra-nuclear labeling (Fig. 10, Fig. S9). The labeled enterocyte nuclei were 13.17 ± 2.04% of the total surface area analyzed demonstrating a significant nuclear fraction enrichment of BODIPY-FLC\textsubscript{12} label (P < 0.0006). Since both fluorescent FAs and Fabp1b and Fabp2 have a homogenous cytoplasmic distribution, it was difficult to assign a co-localization. However, an accumulation of both labels was observed in the basal region of the enterocyte and co-localization of BODIPY-FLC\textsubscript{12} with Fabp1b and Fabp2 was observed inside the enterocyte nuclei, in the interchromatin space between the condensed chromatin areas (Fig. 10).
DISCUSSION

The primary role of all FABP family members is to bind FAs and other hydrophobic ligands, such as lipophilic drugs and xenobiotics, controlling their uptake and intracellular transport, i.e. FABPs act as chaperones (13-17, 57). The cephalocaudal patterning of FABP transcript expression in the mammalian intestine was conserved through evolution. In mice, FABP1 is highly expressed in the proximal two-thirds of the small intestine (58), FABP2 is expressed in the distal jejunum, decreasing gradually towards both the proximal duodenum and the mid-colon (59), while FABP6 transcripts encoding ileal lipid binding protein were located in the distal one-third of the small intestine (60). The molecular patterning of the intestine is remarkably conserved in zebrafish, where the fabp2 expression domain is restricted to the anterior intestine (41), while fabp6 transcripts are found in the posterior intestine (61). Functional cis-acting regulatory sequences may be conserved between zebrafish and mammals, driving intestine-specific FABP2/fabp2 expression (62). Axial patterning of FABP expression has been attributed to ligand and absorption specificities along the digestive track (58), but, in other cases, overlapping FABP expression sites and ligand binding specificities may represent a mechanism for ensuring functional redundancy. Studies directly comparing FABP1- and FABP2-null mice have revealed markedly different phenotypes, indicating that the encoded proteins indeed have different functions in the intestinal lipid metabolism and whole body energy homeostasis (13, 63, 64).

The first step in using the zebrafish model was to determine the expression pattern and dietary regulation of homologs to human FABP1 and FABP2, which were significantly expressed in their anterior intestine. Different fabp genes were expressed in the zebrafish intestine (41, 44-47), including high transcript levels of fabp1b.1 and fabp2 and trace amount of fabp1a and fabp1b.2 (44, 45, 47, 65). By carefully removing the ventral white adipose tissue closely associated with the digestive tract and dissecting the anterior intestine, which is the major site of fat absorption (41-43), we retrieved similarly
high transcript levels of *fabp1b.1* and *fabp2* and smaller amounts of *fabp1b.2*, irrespective of the animals' nutritional status. In addition, *fabp1b.1* and *fabp1b.2* transcripts were found in liver and brain, respectively, supporting the hypothesis that these tandem-duplicated genes have a differential, tissue-specific function. The relatively high levels of *fabp1b.2* in brain may be attributable to ligand preferences, since it is known that polyunsaturated FAs are highly abundant in the central nervous system (66, 67). Whole-mount in situ hybridization demonstrated high transcript levels of *fabp1b* (*fabp1b.1* plus *fabp1b.2*) and *fabp2* in the anterior intestine, but no mRNAs were detected in the posterior intestine. As previously described (41), a significant level of *fabp2* transcripts was detected in the rectum while no hybridization signal with *fabp1b*, thus highlighting the differential expression pattern of these genes. Levels of *fabp1b.1* and *fabp2* transcripts were upregulated in the anterior intestine after feeding, both at the larval stages and in adults. As previously reported (45, 47), these levels were modulated according to the lipid composition of the diet. Lipid-mediated regulation of gene transcription via the activation of nuclear receptors to modify the expression of genes coding for proteins involved in the lipid metabolism, triggered the proliferation of intestinal cells and modulated the expression of molecular signals that may affect homeostasis (26, 36).

The second step of our study was to produce monospecific polyclonal antibodies against purified recombinant proteins from the two most highly-expressed *fabps* in the zebrafish anterior intestine, i.e. *fabp1b.1* and *fabp2*. These tools were used for the cellular and sub-cellular immuno-localization of Fabp1b and Fabp2 in the digestive cavity and demonstrated a very high level of these FABPs in enterocytes in the anterior intestine. Given the very intense immune staining signal observed for both Fabps in the zebrafish intestinal villi, it is very likely that these proteins are present simultaneously in the same enterocytes. Immunofluorescence and immunocytochemical labeling methods revealed Fabps in the microvilli and cytosol, as previously demonstrated with human enterocytes from jejunal mucosa (39), as well as in the nuclei of most enterocytes in the anterior intestine. No staining signal was observed inside mitochondria or oil globules. Nuclei of cells present in the intestinal mucosa and with
fully-condensed chromatin, as well as cells from lymph and blood vessels, were devoid of any Fabp immuno-fluorescent signal. Electron microscopy immunodetection with gold particles demonstrated that enterocyte nuclear localization was mostly in the interchromatin space outside the condensed chromatin clusters. The nucleus is compartmentalized into chromosome territories and interchromatin spaces (68, 69). Zebrafish enterocyte nuclei revealed the conventional pattern with dense chromatin/heterochromatin enriched at the nuclear periphery and around the nucleoli, whereas less condensed/euchromatin regions extended toward the nuclear interior, together with dispersed interior clumps of condensed chromatin/heterochromatin. The spatial organization of eukaryotic genomes in the cell nucleus is linked to their transcriptional regulation (70, 71). However, it has been difficult to find general rules on the involvement of nuclear organization in transcriptional regulation. It remain to be determined whether Fabp1b and Fabp2 immunolabeling, found almost exclusively in less condensed/euchromatin regions, was located in transcriptionally permissive areas.

The third step was to evaluate whether fluorescent FA analogs were able to bind to recombinant zebrafish Fabp1b.1 and Fabp2. We demonstrated that neither recombinant Fabp1b.1 nor Fabp2 were able to bind significantly to BODIPY-FLC₅ (short-chain) but both of them bound avidly to BODIPY-FLC₁₂ (medium-chain). Our in gel binding assay showed that FABP2 in an oligomerization state was unable to bind to the labeled FA analog. Fluorescent short-to-long-chain BODIPY-labeled FAs were used in cell culture to assess the cellular uptake and nuclear localization of unesterified FAs (72-75). These fluorescent FAs analogs, which have a fluorophore linked to the terminal (omega) carbon atom furthest from the carboxylate moiety, were also esterified into complex lipids after adding to cells/animals. Human FABP1 had high affinity for BODIPY-FLC₁₂, in the same range as LCFAs (74). FABP1 expression significantly enhanced the uptake of medium-chain BODIPY-FLC₁₂ and differentially targeted BODIPY-FLC₁₂ into nuclei, in marked contrast to the lack of effect of FABP1 expression on the intracellular distribution of short-chain BODIPY-FLC₅ (73). The findings demonstrated that oleic acid was the only FA tested able to fully abolish the binding of BODIPY-FLC₁₂
to zebrafish Fabp1b.1. This is interesting, as oleate binding causes a conformational change in rat FABP1 (76) and increases the nuclear localization of FABP1 and FABP2 (23, 33). Oleic acid may be involved in the transcriptional regulation of gene expression (77, 78) and the presence of either FABP1 or FABP2 magnifies the transcriptional activation mediated by PPARα (33).

The last step was to assess the fate of dietary fluorescent (BODIPY) FAs at the intestinal level, as the digestive physiology of zebrafish may be addressed using these labeled analogs (79, 80). In vertebrates, the absorption and transport of short- to medium-chain FAs occurs differently in enterocytes, as LCFAs are usually re-esterified inside the cell. There is evidence indicating that both FABP1 and FABP2 participate in the cellular uptake and transport of unesterified FAs and the lipid metabolism (16, 81, 82). However, some authors report that they play a role in lipid-sensing but not in direct dietary FA absorption (13, 62, 83, 84). Cell culture models have proven very useful for mechanistic investigations into the role of FABPs in modulating nuclear receptors and gene transcription (26). For example, it was demonstrated that FABP1 interacted with PPARα (24, 27) and FABP4 with PPARγ (22), respectively, in cultured primary hepatocytes, HepG2 cells, and 3T3-L1 adipocytes. A physical contact between FABP1 and PPARα occurs during this activation, so it has been suggested that FABP1 is a co-activator in PPAR-mediated gene control (27). It has also been demonstrated that FABP5 served as co-activator in the nucleus of transfected cells for PPARβ/δ-mediated gene transcription control (25). FABP5 shuttles FAs directly to the nucleus where it may deliver the ligand to PPARβ/δ, thus enabling its activation (31, 32). In addition, DHA binding to FABP7 triggers the accumulation of FABP7 in the nucleus and modulates cell migration (28). In transfected COS-7 cells, nuclear accumulation of FABP1 and FABP2 is unlikely to be mediated by increased nuclear transport. FABP accumulation in the nucleus is due to a reduced rate of nuclear egress in the presence of the ligand and the dependent interaction of FABP with PPARα (33). The results reported in this manuscript indicate that dietary BODIPY-FLC12 but not BODIPY-FLC5 may be
detected in enterocyte nuclei after feeding and co-localized with Fabp1b and Fabp2 at the nuclear level. To our knowledge, this is the first demonstration that exogenous unesterified FAs reach the nucleus in an in vivo context. The interaction between FA-transporter FABPs and FA-activated, nuclear-receptor PPARs may promote nuclear localization of their ligands, thus constituting a mechanism whereby, following uptake by the cell, dietary FAs become signaling molecules for conveying messages to the nucleus. It cannot be completely ruled out that these FABPs may also play a role in FA retention in and/or release from the nuclei.

In summary, we demonstrated that fabp1b.1 and fabp2 transcript levels in the zebrafish anterior intestine were upregulated after feeding and modulated according to diet formulation. We provided also evidence that BODIPY-FLC12 was able to bind to recombinant Fabp1b.1 and Fabp2. The interaction of BODIPY-FLC12 with Fabp1b.1 was fully displaced by unlabeled oleic acid. In addition to their cytosolic localization, Fabp1b and Fabp2 were also found in enterocyte nuclei. In vivo experiments demonstrated that, following intestinal absorption of dietary fluorescent BODIPY-FLC12, the labeled FA analog was co-localized with Fabp1b and Fabp2 in the nucleus. It appears likely that dietary FAs complexed with FABPs may promote gene transcription regulation, either indirectly or via interaction with nuclear receptors.

Conflict of interest

None.

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REFERENCES


**FIGURE LEGENDS**
**Fig. 1.** *fabp1b.1, fabp1b.2, and fabp2* transcript expression in various adult zebrafish tissues. A: RT-PCR products of *fabp1b.1* and *fabp1b.1* transcripts resolved by electrophoresis on agarose ethidium bromide gels. *eef1a1* transcripts were analyzed to assess qualitative integrity of mRNAs. Lane 1: size standard. Lanes 2 to 7: RT-PCR products generated from total RNAs and extracted from dissected brain (b) (lanes 2 and 3), anterior intestine (ai) (lanes 4 and 5), and liver (l) (lanes 6 and 7) after a period of over 24 h fasting (lanes 2, 4, 6) or 22 h fasting followed by 2 h feeding (lanes 3, 5, 7). Lane 8: A negative control lacking reverse transcriptase generated no RT-PCR products. B-D: qRT-PCR analysis of *fabp1b.1* (B), *fabp1b.2* (C), and *fabp2* (D) transcript expression was performed on fed animals after dissecting various tissues. Six independent liver or anterior intestine and four brain samples from individual adults were tested in triplicate in each condition. Zebrafish heart (h; pool from six animals), kidney (k; pool from four animals), ovary (o) and testicle (t) samples were subjected to a single determination in triplicate. *eef1a1* transcript levels were determined and used as a normalizing gene. The results shown are the number of copies of *fabp1b.1, fabp1b.2,* and *fabp2* transcripts divided by the number of copies of *eef1a1*. nd: no amplification product detected.

**Fig. 2.** qRT-PCR analysis of *fabp1b.1* and *fabp2* transcript expression in the dissected anterior intestine (ai) and liver (l) of adult zebrafish. A: *fabp1b.1*. B: *fabp2*. After a period of over 24 h fasting (fasted group, fa) or 22 h fasting followed by 2 h feeding (fed group, fe), organs from 6 individuals were sampled and analyzed independently, in triplicate. The amount of *eef1a1* transcripts was also quantified and used to express the results as a number of copies of *fabp1b.1* or *fabp2* divided by the number of copies of the normalizing gene. Very similar results were obtained with *ppiaa*, used as another normalizing gene (data not shown). Data are means ± SD. **Significant difference (P < 0.01) between fa and fe groups for the organ examined using the nonparametric Mann-Whitney test.
Fig. 3. qRT-PCR analysis of the expression of \( fabp1b.1 \) and \( fabp2 \) transcripts in whole, 5 dpf zebrafish eleutheroembryos and 15 dpf larvae homogenates. A, B: \( fabp1b.1 \). C, D: \( fabp2 \). Animals were sampled after a period of over 18 h fasting (fasted group, fa) or 18 h fasting followed by 2–2.5 h feeding with FD (fed group, fe). At each age, six independent pools of 25 (5 dpf) or 20 (15 dpf) animals were used to quantify \( fabp1b.1 \) and \( fabp2 \) transcript levels in triplicate. \( eef1a1 \) and \( ppiaa \) were also quantified and used as normalizing genes. The results shown are the number of copies of the \( fabp1b.1 \) or \( fabp2 \) transcripts divided by the number of copies of \( eef1a1 \) (A, C) or \( ppiaa \) (B, D). Data are means ± SD. Means were compared in reference to the fasted group using the nonparametric Mann-Whitney test. Asterisks indicate significant differences (*P<0.05; *** P<0.005) between the fasted and fed groups, for the transcript and age concerned.

Fig. 4. The effects of feeding and diet formulation on \( fabp1b \) (\( fabp1b.1 \) plus \( fabp1b.2 \)) and \( fabp2 \) mRNA transcript hybridization signal in zebrafish larvae were evaluated by whole-mount in situ hybridization. A, C, E, G, I, K: \( fabp1b \). B, D, F, H, J, L: \( fabp2 \). 8 dpf (A, B) or 15 dpf (C-L) larvae fasted for 24 h and were then fed (A-D, G-L) or not (E, F) for 3 h with FD (A-D, G, H), dried CEY (I, J), or OO (K, L). The larvae were then collected and hybridized with sense (S) (C) or antisense (AS) (A, E, G, I, K) \( fabp1b \) or with sense (D) or antisense (B, F, H, J, L) \( fabp2 \) riboprobes, respectively. The \( fabp1b \) antisense probe was able to hybridize to \( fabp1b.1 \) and \( fabp1b.2 \) transcript variants. Representative larvae are shown in lateral view with the anterior part to the left. The specificity of the riboprobes used was demonstrated by a differential expression pattern between \( fabp1b \) and \( fabp2 \) transcripts in the rectum (r) (indicated by a black arrow in panels A, B, G, H) (see also Fig. S3). High transcript levels were found for these genes in the anterior intestine (ai), while no mRNAs were detected in the posterior intestine (pi) (A, B, G, H). Under the hybridization conditions used, high level of \( fabp2 \) transcript was detected in the rectum (r) (B) at 8 dpf and, to a lesser extent, at 15 dpf (H), while no hybridization signal was retrieved with \( fabp1b \) (A, G). Inserts in A and B are enlargements of
the most posterior part of the gut including the rectal area. The *fabp1b* and *fabp2* hybridization signal in the anterior intestine rose significantly in fed larvae nourished with FD (G, H) compared with fasting larvae (E, F). A weaker signal was measured when larvae were nourished with dried CEY (I, J) or OO (K, L). The staining hybridization reaction was stopped after 2 h, i.e. before appearance of the signal in the starved animals, to facilitate comparison between starved and fed states. A longer staining hybridization reaction led to a dark saturated signal in both starved and nourished larvae (data not shown), as these genes were strongly expressed in the anterior intestine, irrespective of the animals' nutritional status. No staining signal was detected using control sense probes (C, D). Other abbreviations: e, eye; l, liver. Scale bar = 250 µm in A, B and 300 µm in C-L.

**Fig. 5.** Immunofluorescence localization of Fabp1b and Fabp2 in the digestive cavity of zebrafish. The histological sections were stained with monospecific polyclonal antibodies developed against recombinant zebrafish Fabp1b.1 (A, B, E) or Fabp2 (C, D, F) proteins, with Alexa 488-conjugated secondary antibody (green). The sections were counterstained with TP3 to label the nuclei red. A: High levels of Fabp1b were immunodetected in the anterior intestine (ai) and lower levels in the liver (l) while no signal was detected in the posterior intestine (pi), testicles (t), adipose tissue (at), or pancreatic islets (p). B: In the intestinal mucosa, Fabp1b was detected inside the intestinal villi (iv) while the lamina propria (lp) was devoid of any signal. C, D: Immuno-fluorescent staining for Fabp2 was similar to that of Fabp1b, with high levels in iv. E, F: Higher magnification revealed Fabp1b and Fabp2 expression in the enterocytes (e), including the microvilli (mv), while goblet cell (gc) and infiltrated cells from lymph and blood vessels (lbv) were devoid of any immuno-fluorescent signal. Nuclear localization of Fabp1b and Fabp2 was highlighted by the yellow coloring in the nuclei of most enterocytes in the intestinal mucosa (B, D, E, F). TP3 nuclear staining demonstrated that cells with fully-condensed chromatin appeared homogeneously red and did not exhibit any nuclear Fabp signal. The nuclei located in the supra-basal position of a large number of enterocytes exhibited
immunofluorescent labeling outside condensed chromatin clusters. White arrow: nucleus (n) with condensed chromatin clusters, immunolabeled with Fabp antibodies outside the clusters. White arrowhead: nucleus with condensed chromatin clusters, not immunolabeled with Fabp1b antibody. Inserts in E, F are enlarged views of the region surrounded by dashed lines in the main pictures. Other abbreviation: il, intestinal lumen; Scale bar = 75 µm in A, B, C, D; 20 µm in E, F.

Fig. 6. Immunocytochemical labeling of Fabp1b in zebrafish enterocytes. Fabp1b antigenic sites, revealed using anti-Fabp1b/secondary IgG coupled to 10 nm protein A-colloidal-gold complex, registered as dark spots on the micrographs. A-C: Localization of antigen sites in the microvilli (mv), cytosol, and nucleus (n), respectively. Inserts in A and C are controls without the primary antibody. Nuclear immunogold particles were found in the interchromatin space, i.e. euchromatin, with a labeling efficiency (gold particle number/ arbitrary surface area unit) of 2.04 while membrane-associated condensed chromatin (mc) and condensed chromatin clusters (ccc), i.e. heterochromatin, were mostly unlabeled, with a labeling efficiency of 0.47 (ratio = 4.34). Other abbreviations: c, cytosol; er, endoplasmic reticulum; gc, goblet cell; ics, interchromatin space; il, intestinal lumen; is, intercellular space; nm, nuclear membrane; np, nuclear pore (indicated by a black arrow); m, mitochondria; og, oil globule; pm, plasma membrane; tw, terminal web. Scale bar = 0.5 µm.

Fig. 7. Native PAGE binding assay of BODIPY-FL labeled FAs to recombinant Fabp1b.1 and Fabp2. Representative native PAGE of purified proteins, labeled with BODIPY-FL FAs or unlabeled, was stained with Coomassie blue (A, C) and detected for fluorescence (B, D). Only parts of the gels stained with Coomassie blue and contained the migrated proteins have been shown. A, B: Lane 1: 41.8 ng BODIPY-FLC12; Lane 2: 1.66 µg Tropomysin A (TPMA) plus 32 ng BODIPY-FLC5; Lane 3: 1.66 µg TPMA plus 41.8 ng BODIPY-FLC12; Lane 4: 1.66 µg TPMA; Lane 5: 1.58 µg Fabp1b.1 plus 32 ng BODIPY-FLC5; Lane 6: 1.58 µg Fabp1b.1 plus 41.8 ng BODIPY-FLC12; Lane 7: 1.58 µg Fabp1b.1; 38
Lane 8: 3.28 µg BSA plus 32 ng BODIPY FL-C5; Lane 9: 3.28 µg BSA plus 41.8 ng BODIPY-FLC12.
Lane 10: 3.28 µg BSA; Lane 11: Fabp2 plus 76.8 ng BODIPY-FLC5; Lane 12: Fabp2 plus 100 ng BODIPY-FLC12; Lane 13: Fabp2. Tropomysin A (TPMA) from *Echinococcus granulosus* (GenBank: AAB65799.4) and BSA fraction V (Sigma-Aldrich) were used for comparison. C, D: Lane 1: 9.05 µg Fabp2 + 76.8 ng BODIPY-FLC5. Lane 2: 9.05 µg Fabp2 + 57.8 ng BODIPY-FLC5; Lane 3: 9.05 µg Fabp2; Lane 4: 9.05 µg Fabp2 + 146.5 ng BODIPY-FLC12. Lane 5: No sample; Lane 6: 9.05 µg Fabp2 + 100 ng BODIPY-FLC12; Lane 7: 9.05 µg Fabp2 + 43.8 ng BODIPY-FLC12; Lane 8: 4.52 µg Fabp2 + 146.5 ng BODIPY-FLC12; Lane 9: 4.52 µg Fabp2 + 119.2 ng BODIPY-FLC12; Lane 10: 4.52 µg Fabp2 + 75.3 ng BODIPY-FLC12; Lane 11: No sample; Lane 12: 9.05 µg Fabp2. Lane 13: 4.52 µg Fabp2. Labeled BODIPY-FLC12 proteins observed in (B, D) are Fabp1b.1, Fabp2 and, to a lesser extent, BSA (see also Fig. S8 for a higher amount of BSA loaded). Fabp2* is a Fabp multimer able to enter the polyacrylamide gel but unable to bind BODIPY-FL labeled FAs.

**Fig. 8.** Displacement of BODIPY-FLC12 to recombinant Fabp1b.1. A: Representative native PAGE of purified recombinant Fabp1b.1 labeled with BODIPY-FLC12 and displaced by 100-fold molar excess of unlabeled FAs or not. Lane 1: Fabp1b; Lane 2: Fabp1b.1 plus BODIPY-FLC12; Lane 3: Fabp1b.1 plus BODIPY-FLC12 and oleic acid (C18:1w9); Lane 4: Fabp1b.1 plus BODIPY-FLC12 and linoleic acid (C18:2w6); Lane 5. Fabp1b.1 plus BODIPY-FLC12 and DHA (C22:6w3). B: Labeled Fabp1b.1 bands were detected for fluorescence and full displacement of BODIPY-FLC12 to Fabp1b.1 was observed in the presence of oleic acid (lane 3). DHA exhibited partial displacement activity (lane 5), while linoleic acid was ineffective (lane 4). Lanes 3 to 5 come from the same gel with the lanes moved.

**Fig. 9.** The intestinal absorption of dietary BODIPY-FLC5 and simultaneous localization of Fabp1b and Fabp2 in zebrafish intestinal villi. Histological sections were counterstained with DAPI to label the nuclei blue (A-D). The sections were stained with monospecific polyclonal antibodies developed
against recombinant zebrafish Fabp1b.1 (E-G) or Fabp2 (H) proteins with Alexa 594-conjugated secondary antibody (red). Adult animals were previously fed with BODIPY\textsubscript{493/503} mixed with CEY powder and used as a negative control (see the Material and Methods section for details) (I) or BODIPY-FLC\textsubscript{5} mixed with CEY powder (J-L) and fluorescence was detected on histological sections in GFP channel (green). (N-P) Merging between nuclei labeling, immunofluorescence signal of Fabps, and BODIPY\textsubscript{493/503} or BODIPY-FLC\textsubscript{5} signals are presented for each corresponding column of the Figure. The apex of the villi is positioned at the bottom of each image. C, G, K, and O are enlargements of the indicated area on B, F, J, and M, respectively. No BODIPY-FLC\textsubscript{5} signal was detected inside the nuclei and the intra-nuclear overlap between Fabp and DAPI labels is shown as pink spots (white arrows in N, O, P). Abbreviations: lbv, lymph and blood vessels; e, enterocyte, mv, microvilli. Scale bar = 20 µm, except 5 µm in C, G, K, O.

**Fig. 10.** Intestinal absorption of dietary BODIPY-FLC\textsubscript{12} and its co-localization with Fabp1b and Fabp2 in zebrafish enterocytes. A-C: Histological sections were counterstained with DAPI to label the nuclei blue. D, E: The sections were stained with monospecific polyclonal antibodies developed against recombinant zebrafish Fabp1b.1 or Fabp2 proteins with Alexa 594-conjugated secondary antibody (red). F, H: Adult animals were previously fed with BODIPY-FLC\textsubscript{12} mixed with CEY powder (see the Material and Methods section for details) and fluorescence was detected in the GFP channel (green). I, J: Merged view of labeled nuclei, Fabp immunofluorescence signal, and BODIPY-FLC\textsubscript{12} signals are presented in the left and right columns of the Figure. The apex of the villi is positioned at the bottom in each image. B, G are enlargements of the indicated large area from A, I, respectively. Inserts in A, F are enlargements of the indicated small area focused on an enterocyte nucleus with a strong BODIPY-FLC\textsubscript{12} signal. The selected nucleus has been surrounded by a yellow line after using the threshold and wand tool options of ImageJ software (see Fig. S9 to see an enlarged view of these panels). Yellow intra-cytoplasmic granulation indicates an overlap between Fabp1b and BODIPY-FLC\textsubscript{12} (I, J, red arrow.
in G). Some nuclei with more condensed chromatin had low BODIPY-FLC$_{12}$ intra-nuclear labeling while the pink granules indicate intra-nuclear Fabp1b (white arrowhead in G). White and red asterisks indicate goblet cell and putative red blood cell nuclei, respectively, free of any Fabp1b and BODIPY-FLC$_{12}$ labeling (B, G). Intra-nuclear overlapping was observed between Fabp, BODIPY-FLC$_{12}$, and nucleus labels (white arrows in G, J). Abbreviations: lbv, lymph and blood vessels; e, enterocyte; mv, microvilli. Scale bar = 20 µm.
Figure 2
Figure 3
Figure 4
Figure 5
Figure 7
Figure 10