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RESEARCH ARTICLE

Four-and-a-half LIM domains protein 2 (FHL2) is associated with the development of craniofacial musculature in the teleost fish *Sparus aurata*

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Abstract Four-and-a-half LIM domains protein 2 (FHL2) is involved in major cellular mechanisms such as regulation of gene transcription and cytoskeleton modulation, participating in physiological control of cardiogenesis and osteogenesis. Knowledge on underlying mechanisms is, however, limited. We present here new data on FHL2 protein and its role during vertebrate development using a marine teleost fish, the gilthead seabream (*Sparus aurata* L.). In silico comparison of vertebrate protein sequences and prediction of LIM domain three-dimensional structure revealed a high degree of conservation, suggesting a conserved function throughout evolution. Determination of sites and levels of FHL2 gene expression in seabream indicated a central role for FHL2 in the development of heart and craniofacial musculature, and a potential role in tissue calcification. Our data confirmed the key role of FHL2 protein during vertebrate development and gave new insights into its particular involvement in craniofacial muscle development and specificity for slow fibers.

Keywords
Four-and-a-half LIM domains protein 2, FHL2 · Teleost fish · Gene expression patterns · Muscle development · 3D protein structure

Abbreviations

CDS	Coding sequence
DPF	Days post fertilization
EST	Expressed sequence tag
FHL2	Four-and-a-half LIM domains protein 2
HPF	Hours post fertilization
LIM	Lin11, Isl-1 and Mec-3 proteins
WGS	Whole genome shotgun

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Introduction

Protein LIM domain is a cysteine-rich motif that forms two zinc fingers responsible for protein–protein interactions. LIM-containing proteins play critical roles in vertebrate development and in cellular differentiation, usually acting as transcription regulators involved in cell lineage determination and pattern formation, or interacting with cytoskeleton proteins [1–4]. Four-and-a-half LIM domains protein 2 (FHL2), a central member of the LIM-only protein subfamily, is involved in multiple cellular activities including regulation of gene transcription, cytoarchitecture, signal transduction and cell adhesion and mobility [5–8]. FHL2 is structurally characterized by the presence of four-and-a-half repeats of the LIM domain [9]. Acting as a co-repressor or co-activator of gene transcription, FHL2

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has been reported to bind nuclear receptors and transcription factors (e.g., androgen receptor, activator protein 1, cyclin D1, β-catenin and serum response factor), which are involved in distinct physiological processes such as regulation of cell cycle, skeletal muscle growth or cancer development [5, 10–13]. In human and mouse, expression of FHL2 gene was initially observed in cardiomyocytes and myoblasts, being down-regulated in rhabdomyosarcoma, which indicates a relevant role in heart and skeletal muscle development and regulation [10, 14]. Importantly, regulation of FHL2 gene expression and protein accumulation has been repeatedly associated with cancer diagnosis (e.g., prostate, colorectal, ovarian and breast cancers), emphasizing the need for a better understanding of FHL2-related cellular mechanisms [7, 15–18]. Non-mammalian vertebrates, and teleost fish in particular, have been recognized as suitable organisms to investigate vertebrate physiology, exhibiting unequivocal advantages to study skeleton and muscle development [19, 20]. The gilthead seabream (*Sparus aurata* L.), a marine teleost of high relevance for European aquaculture, has been recently used to study the molecular mechanisms involved in the development and homeostasis of muscle, determining flesh quality [21], and skeleton, which determines general appearance and survival of the fish [22]). Given the availability of various molecular and cellular tools [23–25], gilthead seabream was used in this study to characterize the role of FHL2 during vertebrate development. We also investigated FHL2 protein primary and three-dimensional structures across vertebrate species evidencing a remarkable conservation while validating teleost fish as a suitable model to further study FHL2 biological role.

Materials and methods

Eggs, larvae, juvenile and adult fish

Gilthead seabream eggs were collected from natural spawning and further cultured as previously described by Tiago and coworkers [26]. Eggs, embryos, larvae, juvenile and adult fish were sampled for RNA preparation, in situ hybridization and histology.

RNA and DNA preparation

Unfertilized eggs, embryos of eight and 512 cells and at 10, 14, 18 and 24 h post fertilization (HPF) larvae at 5, 7, 12, 22, 39 and 50 days post fertilization (DPF, hatching occurs approximately at 48 HPF) and juveniles at 69, 84 and 132 DPF, were anesthetized with 2-phenoxyethanol (Sigma-Aldrich, St. Louis, MO, USA) at 1:10,000 in seawater then washed twice in phosphate-buffered saline (PBS) solution and stored in Trizol reagent (Invitrogen, Carlsbad, CA, USA) at −80°C until further processed. Eggs, embryos and larvae were collected in pools of at least ten. Adult tissues for RNA preparation were removed from fish previously anesthetized as described above and then decapitated. Tissues were stored in Trizol reagent at −80°C until further processed. Total RNA was extracted from all samples following manufacturer's instructions. RNA concentration was determined by spectrophotometry at 260 nm and its quality was assessed by electrophoresis into 1% (w/v) agarose-formaldehyde gels. Genomic DNA was prepared from a single juvenile specimen using QIAGEN DNeasy Tissue kit (Hilden, Germany).

Molecular cloning of FHL2 cDNA and gene

Total RNA (1 μg) extracted from adult seabream heart and treated with RQ1 RNase-free DNase I (Promega, Madison, WI, USA) was reverse-transcribed at 37°C for 1 h using the Moloney-murine leukemia virus (M-MLV) reverse transcriptase, RNaseOUT (both from Invitrogen) and an oligo(dT)-adapter primer [5′-ACGCGTCGACCTCGAGATCGATG(T)$_{13}$-3′]. PCR amplification of FHL2 cDNA fragments were achieved in a Perkin-Elmer GeneAmp 2700 thermal cycler (Waltham, MA, USA) using 5 μl of reverse-transcribed RNA, gene-specific primers SaFHL2-01F and -02R (Table 1) and FideliTaq DNA polymerase (USB Corporation, Cleveland, OH, USA). PCR reactions were performed as follows: an initial denaturation step at 94°C for 4 min, 30 cycles of amplification (one cycle is 30 s at 94°C, 30 s at 68°C and 1 min at 68°C) and a final elongation step at 68°C for 5 min. All PCR products were size-separated on agarose gel and purified using the GFX Gel Band Purification kit (Amersham Biosciences, Piscataway, NJ, USA). Purified PCR products were cloned into pCRII-TOPO (Invitrogen) and subsequently sequenced. FHL2 gene promoter and intron 1 were amplified by PCR from a seabream GenomeWalker genomic DNA library (Clontech, Palo Alto, CA, USA) using gene-specific primers SaFHL2-10R and -06R in combination with adaptor primer AP2. Different combinations of exon-specific primers flanking predicted intron insertion sites were used to amplify introns 2–5 from seabream genomic DNA. All PCR amplifications were performed using 100 ng of genomic DNA and JumpStart *Taq* DNA polymerase (Sigma-Aldrich). PCR products were purified, cloned as described above, and sequenced.

Genomic Southern analysis

Genomic DNA (10 μg) was digested with 25 units of endonuclease *Bgl*I, *Bgl*II, *Eco*RI, *Hin*dIII, *Pst*I or *Sca*I (Takara, Saint-Germain-en-Laye, France). DNA fragments

Table 1 Seabream gene-specific primers used in this study

Gene[a]	Primer	Sequence (5′–3′)
FHL2	SaFHL2-01F	AGGAAGCTGAGTGACTCTGGGTCAAGATG
FHL2	SaFHL2-02R	AAGACGATTTTCCTGCCTCGGCTGG
FHL2	SaFHL2-03F	AAGGACCGTCACTGGCACGAGGA
FHL2	SaFHL2-04R	GTGCCAGGGCTGGTCATGATAGGTC
FHL2	SaFHL2-05R	GGAGCCTGGCATGATGGTTTTCTTG
FHL2	SaFHL2-06R	CCTCTCTCAGGACATACTTCTTCCCAAA
FHL2	SaFHL2-07F	AGTGCGGCAAGGACATCTGATTCGT
FHL2	SaFHL2-08R	GTCTGAAAACATGTTGCTGTTTCATAAGCA
FHL2	SaFHL2-09F	GCCATGAAACGGAGACTAATGATAGTGTTA
FHL2	SaFHL2-10R	AGGAGTGTGTGCCTGTGCGGAGT
FHL2	SaFHL2-17F	AGTGCGGCAAGGACATCTGATTCGT
FHL2	SaFHL2-18R	AGGTTGTAAAGATGCTGTGCTCTGGTAGT
β-actin	SaActin288FW	CTTCCTCGGTATGGAGTCCTGCGG
β-actin	SaActin288RV	TCCTGCTTGCTGATCCACATCTGCT
RPL27a	SaRPL27a-01F	AAGAGGAACACAACTCACTGCCCCAC
RPL27a	SaRPL27a-02R	GCTTGCCTTTGCCCAGAACTTTGTAG

[a] GenBank accession numbers for seabream genes used in this study are EF639861 (FHL2 gene), DQ225183 (FHL2 cDNA), X89920 (*β*-actin) and AY188520 (ribosomal protein L27a)

were size-separated on a 0.8% (w/v) agarose gel for 10 h at 50 V, then transferred onto a Hybond-XL nylon membrane (Amersham Biosciences) by capillarity blotting with 10× standard saline citrate buffer (SSC; 1× SSC is 0.15 M NaCl and 15 mM sodium citrate, pH 7.0). FHL2 cDNA probe (168 bp corresponding to cDNA positions 225–392) was radiolabeled with [α-^{32}P]dCTP (3,000 Ci/ml; Amersham Biosciences) using the Rediprime II kit and purified from unincorporated nucleotides using MicroSpin G-50 columns (Amersham Biosciences). Hybridization was performed overnight at 42°C in ULTRAhyb buffer (Ambion, Austin, TX, USA). Blots were washed 2 × 5 min in low stringency solution (2× SSC, 0.1% SDS) and 3 × 15 min in high stringency solution (0.1× SSC, 0.1% SDS) at 60°C, then autoradiographed.

Quantitative real-time PCR (qPCR)

Total RNA (1 μg) from selected embryos, larvae, juvenile stages and adult tissues was treated with RQ1 RNase-free DNase I and reverse-transcribed, as described above, but using reverse primers SaFHL2-05R, SaRPL27a-02R or SaActin288FW (Table 1) specific for FHL2, ribosomal protein L27a (RPL27a) and *β*-actin cDNAs, respectively, instead of oligo(dT)-adapter primer. qPCR reactions were performed using iCycler PCR system (Bio-Rad, Richmond, CA, USA), 1× iQ SYBR Green I mix (Bio-Rad), 0.4 μM of forward and reverse gene-specific primers (Table 1) and a 1:100 dilution of reverse-transcribed RNA solution. The following PCR conditions were used: an initial denaturation/enzyme activation step at 95°C for 15 min, then 40–50 cycles of amplification (one cycle is 30 s at 95°C and 30 s at 68°C). RPL27a and *β*-actin were used to normalize FHL2 gene expression in adult tissues and developmental samples, respectively. All qPCR experiments were repeated at least three times.

Whole mount in situ hybridization of seabream larvae

Sense and antisense RNA probes were generated from 1 μg of linearized plasmid containing FHL2 cDNA (778 bp fragment corresponding to cDNA positions 225–1002) then labeled with digoxigenin-dUTP (DIG RNA labeling kit, Roche Diagnostics, Mannheim, Germany). Seabream larvae collected at 4, 10 and 20 DPF were used for whole mount in situ hybridization (ISH) according to the protocol described by Thisse and coworkers [27]. Briefly, larvae were digested for 30 min at room temperature with increasing concentrations of proteinase K (10, 15 and 20 μg/ml for larvae at 4, 10 and 20 DPF, respectively). Larvae were hybridized at 65°C for 12–16 h with 100–200 ng of RNA probe in hybridization buffer [27], then incubated with a 1:2,000 dilution of anti-digoxigenin-alkaline phosphatase antibody (Roche Diagnostics). Signal was revealed using NBT/BCIP (nitroblue tetrazolium salt/5-bromo-4-chloro-3-indolyl-phosphate, Roche Diagnostics) as substrate for alkaline phosphatase. All reagents were purchased from Sigma-Aldrich unless otherwise stated.

Glycol-methacrylate infiltration

Larvae previously fixed in 4% (w/v) paraformaldehyde (PFA) and preserved in methanol were dehydrated through an acetone gradient then infiltrated with glycol-methacrylate (GMA, Sigma-Aldrich) as described by Witten and

colleagues [28]. Whole mount ISH larvae were also embedded into GMA after two washes with PBS and then dehydrated through an acetone gradient. Sections of 5–7 μm thick were prepared, floated on demineralized water, mounted on uncoated slides, dried at 25°C and finally mounted with Eukitt (Merck, Whitehouse Station, NJ, USA).

Histological sample preparation and immunohistochemistry

A juvenile of 4–5 months was fixed in 4% (w/v) PFA then decalcified in a 10% (w/v) EDTA-2% (w/v) PFA pH 7.0 solution during 6 weeks. Adult heart tissue was also collected and fixed in PFA. Both samples were defatted in xylene and embedded in paraffin following standard procedures. Sections of 6–8 μm thick were prepared and collected on TESPA (3-aminopropyltriethoxysilane, Sigma-Aldrich) coated slides. Routine histological staining was performed using azan combination solution (Chroma-Waldeck, Münster, Germany) to determine cellular structures and tissues. Immunolocalization of FHL2 was performed as previously described [29] using a 1:300 dilution of human polyclonal FHL2 antibody [7], previously validated for seabream protein (supplementary Fig. S1).

Sequence reconstruction

Sequence databases at NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) were searched for genomic and cDNA sequences related to FHL2 gene using BLAST tool and seabream FHL2 as query sequence. Species-specific sequences were aligned then assembled using ContigExpress module of Vector NTI Advance software (Invitrogen) to generate highly accurate consensus sequences. Virtual transcripts were deduced by comparative analysis from joined consensus sequences using stringent overlap criteria.

Multiple sequence alignment

FHL2 sequences were fed to T-Coffee multiple sequence alignment software with parameters set to default [30] to produce, after manual adjustment, a high quality alignment. Sequence logos were then created from multiple alignments using WebLogo facilities at http://weblogo.berkeley.edu [31].

Protein structure prediction

Three-dimensional structure of the LIM domain of seabream FHL2 protein was determined by comparison with available templates—solution structure of LIM domain of human FHL2 (DOI:10.2210/pdb1x4l/pdb) and FHL3 (DOI: 10.2210/pdb1x4k/pdb) proteins—using Modweb facilities at http://modbase.compbio.ucsf.edu. Different models were obtained and the best one was chosen according to the dope (discrete optimized protein energy) score method [32]. Three-dimensional illustrations and overlapping were achieved using Chimera v1.5 (http://www.cgl.ucsf.edu/chimera, [33]).

Results

Molecular structure of seabream FHL2 cDNA and gene

A DNA fragment of approximately 1.3 kb, corresponding to the partial cDNA of seabream FHL2 gene, was amplified from total RNA of adult heart tissue using gene-specific primers SaFHL2-01F and -02R (primers designed from available seabream EST sequences; Table 1). Full-length cDNA was reconstructed using additional PCR fragments and EST sequences accessible through Marine Genomics Europe (MGE) network of excellence (supplementary Fig. S2; GenBank accession number DQ225183). Deduced protein is 279-aa long, contains four-and-a-half LIM domains (Fig. 1 and supplementary Fig. S2), and exhibits high similarity with FHL2 protein from other species (e.g., 82, 86 and 96% identity with human, chicken and zebrafish proteins, respectively). Sequence of seabream FHL2 gene was also determined using exon-specific primers (Table 1; position of intron insertions in seabream FHL2 cDNA were determined by comparison with available gene structures, e.g., human, mouse, chicken). Various genomic DNA fragments were amplified by PCR using seabream GenomeWalker libraries, then sequenced and assembled to reconstruct seabream FHL2 gene (supplementary Fig. S2, GenBank accession number EF639861). Gene structure (6 exons, 5 introns and a 1,936-bp 5′-flanking region) was determined using Spidey mRNA-to-genomic alignment tool available at NCBI (Fig. 1 and supplementary Fig. S2) and found to be similar to the FHL2 genomic structures already available from other species.

Evidence for the presence of a single copy of FHL2 gene in seabream

Although our PCR data suggested the presence of only one copy of FHL2 gene in seabream genome, a Southern hybridization of genomic DNA was performed to further investigate the presence of additional gene copies, as seen in zebrafish (our unpublished results, GenBank accession numbers DQ118096 and DQ118097). A single band was observed in each lane of the autoradiogram (probe

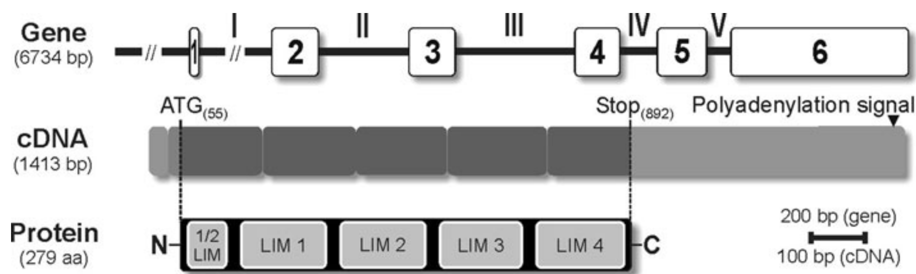

Fig. 1 Schematic representation of seabream FHL2 gene, cDNA and protein structures (GenBank accession numbers EF639861 and DQ225183). In gene structure, exons are represented by *white boxes/arabic numbers* and introns by *black lines/roman numbers*; sequence upstream exon 1 corresponds to gene promoter region. In cDNA structure, *dark grey* represents the coding sequence and *light grey* the 5′ and 3′ untranslated regions; *each box* represents an exon; first nucleotide of exon 1 is numbered 1 and initiation codon is positioned at position 55 in exon 2. In protein structure, LIM domains are represented by *grey boxes*

sequence has no cleavage sites for selected endonucleases), further supporting the idea that FHL2 gene is a single-copy gene in seabream (Fig. 2).

Early onset of FHL2 gene expression during seabream development

Onset of FHL2 gene expression during seabream development was investigated by qPCR. FHL2 transcript was first detected in 24 HPF embryos and expression levels strongly increased after hatching (~60 folds; hatching occurs at approximately 48 HPF), peaking in 7-DPF larvae and then returning to intermediate levels in all later stages analyzed (Fig. 3). Expression patterns of seabream FHL2 gene suggest an important role of the protein in fish larval development but also, to a lesser extent, throughout the entire developmental phase of seabream.

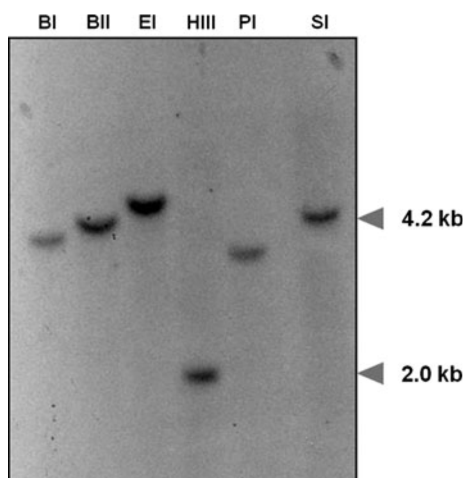

Fig. 2 Southern-blot analysis of seabream genomic DNA. DNA was digested with indicated endonucleases and hybridized with a radio-labeled probe specific for exon 3 of FHL2 gene (none of the enzymes had a restriction site within the probe sequence). *BI BglI*, *BII Bgl*II, *EI Eco*RI, *HIII Hin*dIII, *PI Pst*I, *SI Sca*I

FHL2 is associated with the development of craniofacial musculature in teleost fish

Spatial distribution of FHL2 gene expression was then assessed by in situ hybridization in three stages of early development (i.e., in 4, 10 and 20 DPF larvae of 3.9, 4.1 and 5.3 mm, respectively) and in a juvenile fish (~50 mm) using whole mount and tissue sections preparations, respectively. Independently of the developmental stage used, FHL2-specific signal was consistently observed in seabream heart, in particular in the myocardium, and at sites of craniofacial muscle formation (Figs. 4, 5, 6). During early fish development (4 DPF larvae), extraocular (e.g., superior oblique and medial rectus) and mandibular muscles (adductor operculi and levator arcus palatine) were positive for FHL2 transcript (Fig. 4a). Since cartilage and muscle precursor cells—chondroblasts and myoblasts—lie closely together, exhibiting a coordinated developmental pattern, it is usually difficult to distinguish them on the basis of their morphology only, especially during these early stages when cranial muscle derives from mesoderm and cartilage from neural crest cells [32]. For this reason, GMA-sections of 4, 10 and 20 DPF larvae were stained with azan combination, which specifically colors muscle in orange. Presumptive muscle precursor cells and developing muscular structures identities were then confirmed and co-localized with FHL2 transcript (Fig. 4h, j, l). Later during development (at 10 and 20 DPF), interhyoideus and hyohyoideus, two hyoid muscles, were also positive for FHL2 transcript. The former is attached to ceratobranchials and involved in jaw opening, while the latter is inserted at the most anterior basibranchial and the posterior ends of the ceratohyals and is involved in jaw closure (Fig. 4). Mandibular muscles intermandibularis anterior and posterior, sternohyoideus and adductor mandibulae, also exhibited a strong signal for FHL2 mRNA across all developmental stages analyzed. Finally, FHL2 transcript was detected in larval trunk, in a region specific for red

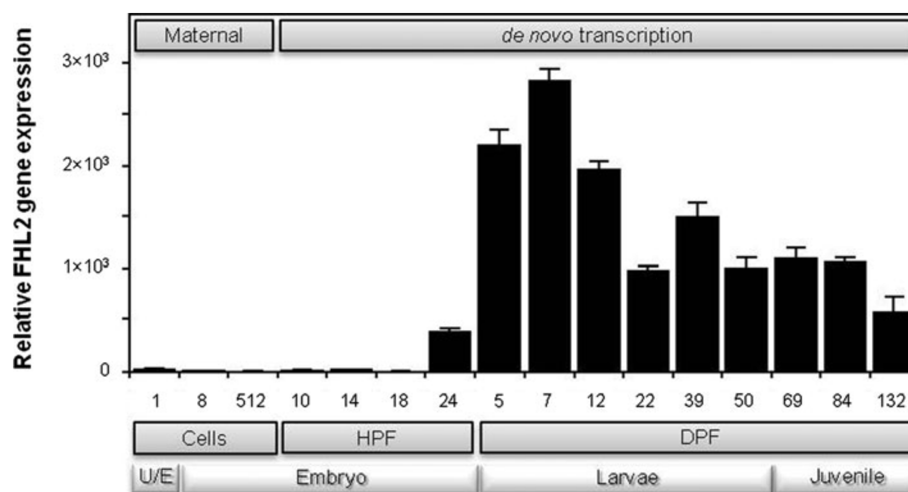

Fig. 3 Levels of FHL2 gene expression during seabream development. Values are the mean of four independent qPCR experiments. FHL2 gene expression was normalized with β-actin and set to 1 in unfertilized egg (U/E) sample (reference sample); HPF and DPF are hours and days post fertilization, respectively

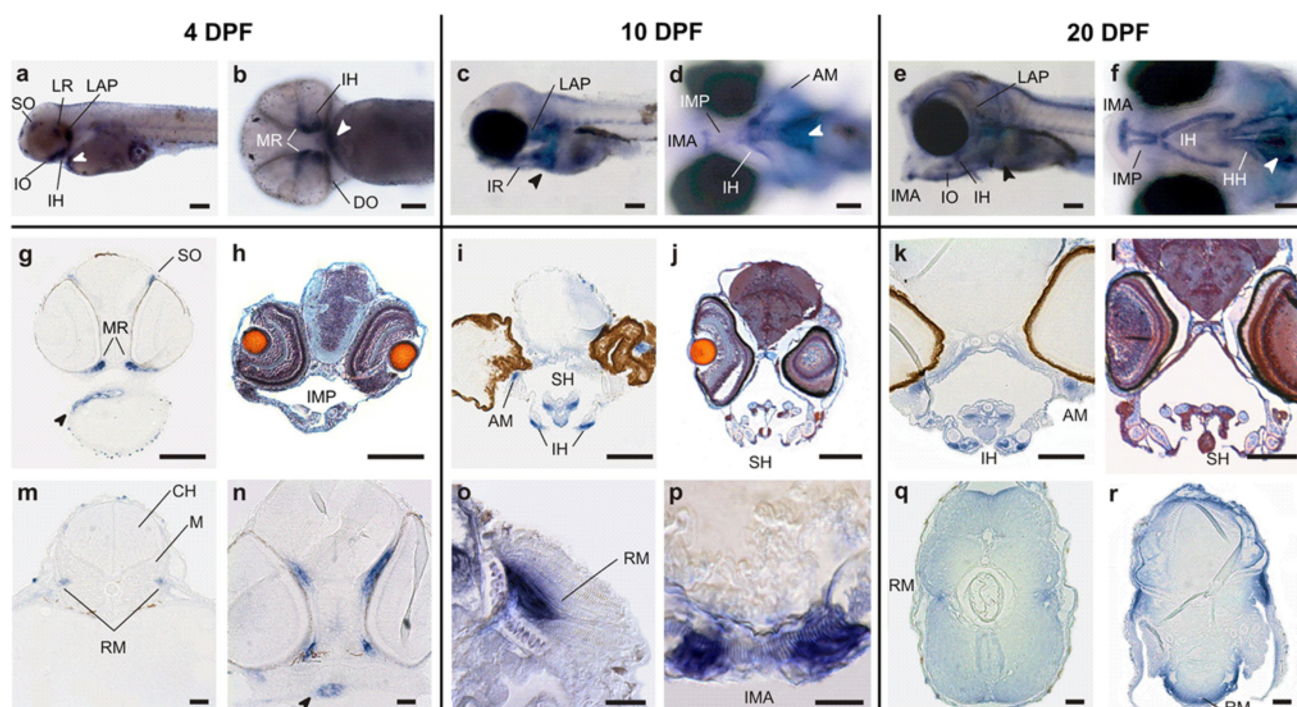

Fig. 4 In situ localization of FHL2 transcript in seabream larvae at 4, 10 and 20 days post fertilization (DPF). **a–f** Whole mount ISH of seabream larvae then sectioned **g–r** except for **h**, **j** and **l** which correspond to azan histological staining. *AM* adductor mandibulae, *CH* caudal hindbrain, *DO* dilator operculi, *HH* hyohyoideus, *IH* interhyoideus, *IMA* intermandibularis anterior, *IMP* intermandibularis posterior, *IO* inferior oblique, *IR* inferior rectus, *LAP* levator arcus palatini, *LR* lateral rectus, *M* myotome, *MR* medial rectus, *RM* red muscle, *SH* sternohyoideus, *SO* superior oblique, *arrow head* indicates heart. *Bar* 100 μm for **a–l** and 20 μm for **m–r**

muscle fibers (Fig. 4) [24, 34]. No signal was observed in white muscle fibers of the trunk.

Cross-sections of a juvenile fish were hybridized in situ to further explore FHL2 gene expression during late development (4–5 months), when all organs are fully formed. Signal was observed in hyperplasic craniofacial muscle cells where two different types of muscle fibers can be clearly distinguished by azan staining and ISH (Fig. 5): (1) proliferative, small and collagen-rich fibers and (2) differentiated and large fibers with low collagen content. FHL2 transcript was only detected in the proliferative muscle fibers (Fig. 5). Sternohyoideus has been consistently positive for FHL2 transcripts throughout development. This mandibular muscle, positioned on the anterior region of the cleithrum, is initially formed as left and right regions (Fig. 4i, j), that fuse together forming a

round structure already observed at 20 DPF (Fig. 4l). Later in development, it appears as a triangular structure in a cross section (Fig. 5a, b), as previously described for zebrafish [35]. No signal was observed in the smooth muscle cells of other soft tissues or in skeletal muscle white fibers of juvenile fish.

FHL2 tissue patterning in seabream

FHL2 gene expression was also determined by qPCR in a variety of adult tissues (collected from fish approximately 2 years old). While highest expression levels were measured in heart tissue, as expected from expression data from mouse and human, FHL2 transcript was also detected, although to a much lower extent (ten times less), in most mineralized tissues analyzed (skull, dentary, operculum, branchial arches and cartilage) and in skin (collected from the trunk region), the only other soft tissue, in addition to heart, expressing FHL2 (Fig. 6a). No expression of FHL2 gene was detected in the remaining tissues analyzed (i.e., soft and spiny fins, adipose tissue, aorta and aortic bulb, brain, gill filaments, intestine, kidney, liver, white muscle, ovary, pancreas, scales, spleen, teeth, testis and vertebra; results not shown). Sites of FHL2 expression in mineralized tissues was then investigated by ISH in a juvenile fish of 4–5 months with, however, no success. Absence of ISH signal in juvenile mineralized tissues could be related either to the lower sensitivity of the technique when compared to qPCR (PCR amplifies a DNA molecule into more than a billion of copies) or to the lower rate of mineralization in tissues used for ISH (qPCR was performed using RNA from adult tissues while ISH was performed in tissues from a juvenile specimen). Cardiac muscle was the only adult tissue found to simultaneously express FHL2 gene and accumulate corresponding protein, as determined by ISH and immunohistochemistry, respectively (Fig. 6b, c), in the conditions and samples we have used. Lack of expression in adult white muscle is in agreement with ISH results during development, which showed expression in hyperplasic red muscle cells but not in white muscle fibers.

Primary structure of FHL2 protein is highly conserved throughout vertebrate evolution

To support our hypothesis that FHL2 function may have been maintained throughout vertebrate evolution, the conservation of protein primary structure, including features such as domains and motifs, was analyzed in a variety of species. A total of 41 FHL2 sequences, representing most classes of vertebrates (38 species including mammals, sauropsids, amphibians, ray-finned fish and one cartilaginous fish) were retrieved from GenBank sequence database: 11 of those sequences were already annotated as FHL2 (including zebrafish and seabream sequences reported in this study) and the remaining 30 were reconstructed from EST and/or WGS sequences (supplementary Fig. S3). While a single ortholog of FHL2 was identified in most species, a second gene was found in three ray-finned fish species, i.e., European sea bass, guppy and zebrafish (supplementary Figs. S3 and S4). The complete set of FHL2 protein sequences collected or reconstructed within the scope of this study and including the two ray-finned fish isoforms (types a and b), was aligned using T-Coffee software (supplementary Fig. S5). Resulting alignment was displayed as logos using Weblogo online facility to highlight residues conserved throughout evolution and likely to be essential for protein function and/or structure (supplementary Fig. S6). Although this analysis was performed using sequences from a large set of organisms with diverse evolutionary pathways—last common ancestor of gnathostomes lived approximately 520 million years ago [19]—logos of full-length FHL2 revealed a remarkable conservation of the primary structure throughout evolution: 45% of the residues were totally conserved (Supplementary Fig. S6). To further pinpoint residues critical for FHL2 function, logos of the LIM domain were created by aligning the four complete domains present in FHL2: eight residues were totally conserved throughout evolution and more six residues were conserved in all sequences but one; overall, 25% of the residues of FHL2 LIM domain were totally or highly conserved. Sequence signature of FHL2 LIM domain was then redefined as C-X_2-C-X_3-I-X-G-L-X_{12}-W-H-X_2-C-F-X-C-X_2-C-X_8-F-X_8-C-X_2-C (Fig. 7a).

Three-dimensional structure of FHL2 LIM domain is maintained across vertebrate species

Three-dimensional structure modulation of the LIM domain of seabream FHL2, and comparison of this model with structures determined by multidimensional NMR spectroscopy and by X-ray crystallography [1], allowed us to illustrate the conservation of the LIM fold across species. The structure of each LIM domain from FHL2 consists of two zinc fingers, composed by two antiparallel β-hairpins, and a short α-helix. The secondary structure and the tertiary fold of the LIM domain is assured by the coordination of the zinc atom and hydrophobic residues assist in the packing of the two zinc fingers [1]. To highlight the conservation of LIM domain throughout vertebrate evolution, an overlay of a structural alignment of LIM domains from human and seabream FHL2 proteins was performed (Fig. 7b–d) then compared to the consensus sequence of vertebrate LIM domain (Fig. 7a). The high conservation of LIM domain from primary to tertiary structure and the high conservation of tertiary structure of

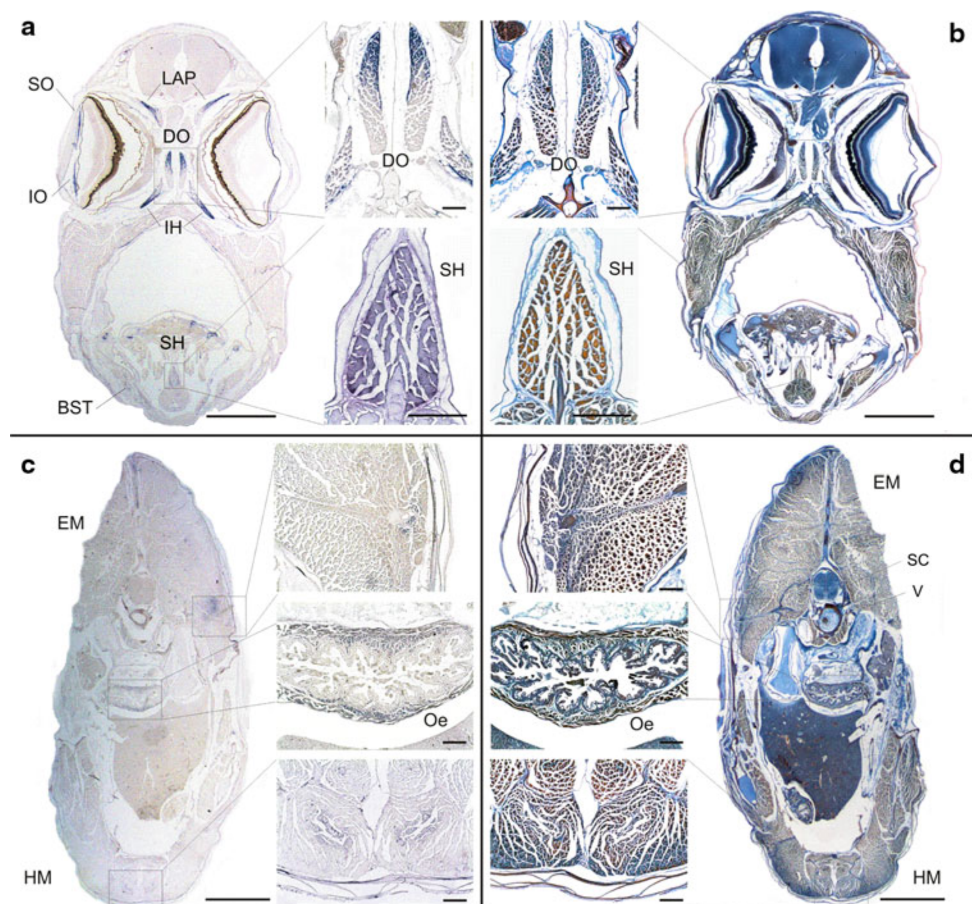

Fig. 5 In situ localization of FHL2 transcript in a juvenile seabream. **a** and **c** FHL2 localization by ISH **b** and **d** azan histological staining. *BST* branchiostegal ray muscles, *DO* dilator operculi, *EM* epaxial muscle, *Oe* esophagus, *HM* hypaxial muscle, *IH* interhyoideus, *IO* inferior oblique, *LAP* levator arcus palatini, *SC* spinal cord, *SH* sternohyoideus, *SO* superior oblique, *V* vertebra. *Bar* 200 μm

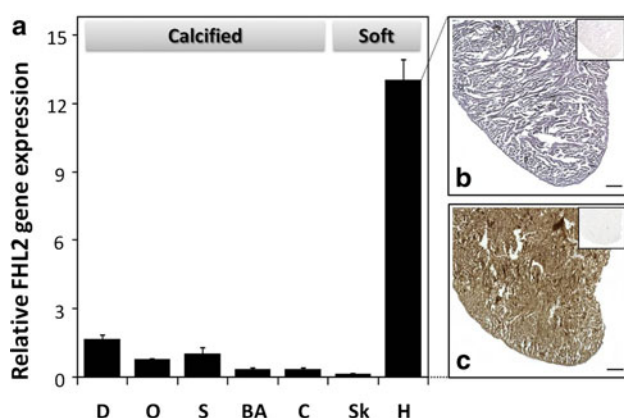

Fig. 6 **a** Levels of FHL2 gene expression in seabream adult tissues by qPCR. Values are the mean of at least three independent qPCR experiments. *D* dentary, *O* opercula, *S* skull, *V* vertebra, *BA* branchial arches, *C* cartilage, *H* heart, *S* skin. FHL2 gene expression was also analyzed, but not detected, in the following tissues: soft and spiny rays, adipose tissue, aorta and aortic bulb, brain, gill rays, intestine, kidney, liver, muscle, ovary, pancreas, scales, spleen, teeth and testis. **b** In situ hybridization and **c** immunohistochemistry of FHL2 in adult seabream heart; *bar* 100 μm; respective negative control is indicated on the *upper right corne*r of each microphotograph

LIM domain from fish to human further suggested that FHL2 function has probably been maintained throughout evolution, and evidenced the central role of protein three-dimensional structure for its functionality.

Discussion

FHL2 gene expression is specific for craniofacial muscles and slow fibers

Expression data related to the LIM-only protein FHL2 have been collected for the first time in a non-mammalian system; beyond this innovation, the use of the teleost fish gilthead seabream allowed the spatiotemporal analysis of FHL2 gene expression from larval stages throughout adulthood. Onset of FHL2 expression in seabream coincides with the initiation of de novo transcription at 24 HPF indicating that the FHL2 transcript is not maternally inherited and it is likely not to play a role during egg development and segmentation. After

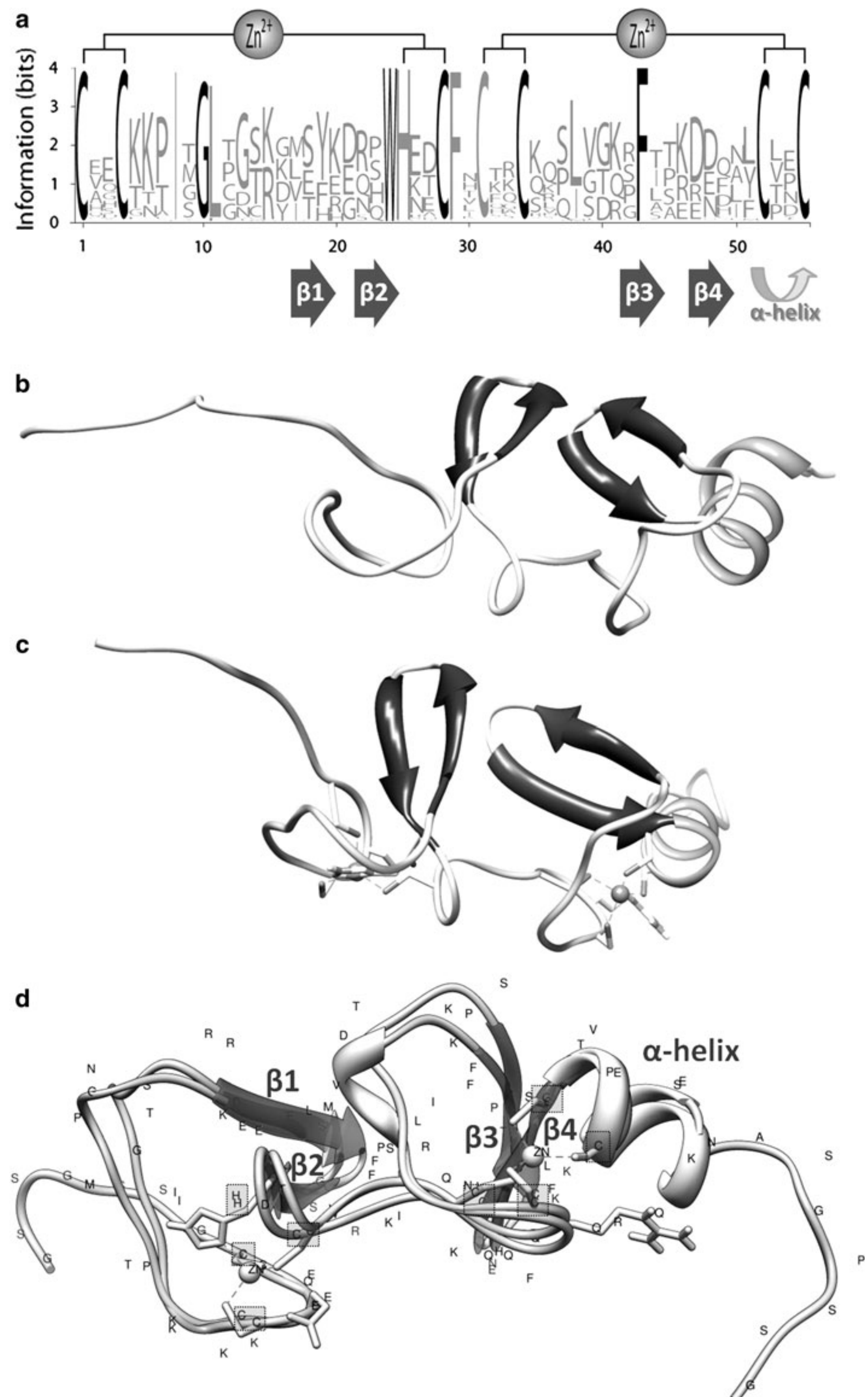

Fig. 7 **a** Consensus sequence (logos) for LIM domain of vertebrate FHL2. The *height of each lette*r is directly proportional to its frequency and 100% conserved residues are shown in black. Predicted secondary structures are indicated below consensus sequence. **b** Illustration of the three-dimensional structure of LIM domain 2 (amino acids from 90 to 162) from gilthead seabream FHL2. **c** Illustration of the three-dimensional structure of a LIM domain from human FHL2 (PDB:1XK4). **d** Three-dimensional alignment of seabream and human FHL2 LIM domain and structural pairwise of main residues. *Black boxes* represent conserved residues responsible for zinc finger structures

hatching, FHL2 has been associated with larval development, in particular the development of cardiac tissue and formation of the first craniofacial musculature structures. While FHL2 has been repeatedly associated with the formation of cardiac muscle in mammals [4, 36, 37], its association with craniofacial muscle development, as suggested in this work, has never been reported previously. Seabream FHL2 transcript was detected in muscles associated with vital functions in fish: respiration, food consumption (onset of exotrophic period is at 3–4 DPF in gilthead seabream) and eye movement [38]. Transcript was also detected specifically in red, slow muscle cells of the developing trunk; in zebrafish, these cells derive from adaxial cells that migrate from the

somite in response to notochord signaling [39]. Interestingly, in trout embryos, FHL1, which shares 47% of identity with seabream FHL2 protein, was shown to be expressed in adaxial cells while migrating toward the outermost domain of the somite and differentiating into red muscle fibers as well [40], confirming the importance of this protein for the development of this specific type of muscle fibers. In mouse, Fimia and coworkers [41] have shown that FHL1 is highly expressed in skeletal muscle, while no expression was observed for FHL2; other studies had however previously shown FHL2 expression in these tissues [14, 42], which can be related to the type of skeletal muscle sample used in each case (including or not slow muscle fibers). We propose that FHL2 may have the same behavior in seabream slow muscle tissue, as reported for FHL1 in trout [40]. FHL2 gene expression could also be detected in proliferating muscle cells of the pectoral fin at 10 and 20 DPF, which should be related to the locomotion needs of the larvae [43]. The FHL2 positive craniofacial musculature in the juvenile supports a conserved functional relevance of this gene for muscle growth and maintenance during late fish development. The fact that FHL2 is only detected in proliferative, collagen-rich fibers seems to be related to hyperplasia, when continuous proliferation of muscle cells contributes to the higher content of collagen in the matrix [44]. FHL2 transcript was not detected in large differentiated white muscle fibers, only proliferating cells, exhibiting a fiber type specific expression pattern. Skeletal muscle growth occurs in fish both by hyperplasia and by hypertrophy throughout life, in contrast with mammals where growth by hyperplasia is restricted largely to pre- and perinatal periods [21]. In mammalian skeletal muscles, FHL2 was shown to promote differentiation of myoblasts into myotubes, by repressing β-catenin target genes involved in proliferation [10]. Recently, Shi and coworkers have shown that FHL2 is required to activate the myogenic progenitor population (MPC) of cells in skeletal muscle, further supported by the fact that FHL2 deficient mice exhibit a perturbed muscle regeneration process [45]. Our results indicate a significant role of FHL2 during fish myogenesis, thus suggesting that teleost fish is a suitable model to further investigate vertebrate muscle-related function of FHL2. Traditional fish models such as zebrafish and medaka show little postlarval skeletal muscle hyperplasia and reach only relatively small adult sizes, whereas seabream exhibits extensive postlarval muscle hyperplasia, contributing for its large adult size, mechanism in which FHL2 seems to be closely related [23]. The validation of FHL2 as a marker of fish hyperplasic muscle could represent a major step toward the evaluation of flesh quality in farmed fish, such as the gilthead seabream.

Is FHL2 associated to fish tissue mineralization?

Seabream FHL2 transcript was also detected by qPCR in adult mineralized tissues, although at significantly lower levels than in cardiac muscle (ten times less). While it was not confirmed by ISH in the samples analyzed, the presence of FHL2 transcript in fish mineralized tissues is in agreement with what has been described for mammals: mRNA is expressed in osteoblasts [46] and protein stimulates osteoblastic activity [11, 46]. Importantly, FHL2 deficiency was associated in mice with a mild and age-dependent osteopenia (low bone mineral density) phenotype, further demonstrating FHL2 action on skeleton metabolism [11]. It has been proposed that osteoblast-specific role of FHL2 is associated with its ability to bind to regulators of bone such as insulin growth factor binding protein 5 (IGFBP5), androgen receptor, β-catenin and Runx2, acting as transcription co-activators of target bone-related genes [5, 11, 46–48]. Based on these data and in our preliminary results, showing expression of FHL2 gene in seabream VSa16 pre-osteoblastic cell line (unpublished data), we propose that FHL2 may also have a role in skeleton homeostasis in teleost fish, although we could not clearly evidence it. FHL2 transcript was also detected in seabream skin that might be related to cytoskeleton and extracellular matrix organizations, which recent work has revealed to be important for mammalian skin metabolism through the transcriptional regulation of α-smooth muscle actin and subsequent collagen contraction defects [49, 50].

FHL2 protein is highly conserved among vertebrates supporting an ancient and preserved role

The similarity in FHL2 expression patterns of phylogenetically distant species would indicate that protein function is ancient and has been maintained from the last common ancestor of sarcopterygians (tetrapods) and actinopterygians (ray-finned fish such as seabream) throughout approximately 520 million years of evolution [51]. To support this hypothesis, we evidenced the remarkable conservation of vertebrate FHL2 protein, in particular the core LIM domain. It is therefore likely that FHL2 has the same molecular function and physiological role in teleost fish and mammals. LIM domains have been described to function as a modular protein-binding interface to mediate protein–protein interactions, providing a stable structure where many kinds of molecules could attach. In fact, the two zinc fingers present on a treble-clef fold format in each LIM domain can also mediate interactions with nucleic acids and proteins [1, 12].

In conclusion, we present here novel data on the expression and structure of the LIM-only protein FHL2 in a non-mammalian model organism. A teleost fish was used to study the role of FHL2 during development; we have

clearly associated FHL2 gene expression with the formation of craniofacial musculature structures and proposed FHL2 as a suitable marker for early development of fish slow muscles. Comparative analysis of protein primary and tertiary structures in evolutionary distant species have revealed a striking similarity that suggests the conservation of protein function throughout 520 million years of vertebrate evolution and validates the use of teleost fish systems to investigate FHL2 function. Cellular and molecular tools that have recently been developed for *S. aurata*, including (1) bone and cartilage-derived cell lines, VSa13, VSa16 and ABSa15 [52], (2) characterization of various bone and muscle-related genes, [53–55] and (3) Agilent oligo-based microarray [56] will certainly give new insights into seabream FHL2 function and its specific role during in vitro mineralization and bone cell differentiation.

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References

1. Kadrmas JL, Beckerle MC (2004) The LIM domain: from the cytoskeleton to the nucleus. Nat Rev Mol Cell Biol 5:920–931
2. Lumsden A (1995) Neural development. A ‘LIM code’ for motor neurons? Curr Biol 5:491–495
3. Weiskirchen R, Pino JD, Macalma T, Bister K, Beckerle MC (1995) The cysteine-rich protein family of highly related LIM domain proteins. J Biol Chem 270:28946–28954
4. Chu P-H, Bardwell WM, Gu Y, Ross J Jr, Chen J (2000) FHL2 (SLIM3) is not essential for cardiac development and function. Mol Cell Biol 20:7460–7462
5. Müller JM, Isele U, Metzger E, Rempel A, Moser M, Pscherer A, Breyer T, Holubarsch C, Buettner R, Schüle R (2000) FHL2, a novel tissue-specific coactivator of the androgen receptor. EMBO J 19:359–369
6. Canault M, Tellier E, Bonardo B, Mas E, Aumailley M, Juhan-Vague I, Nalbone G, Peiretti F (2006) FHL2 interacts with both ADAM-17 and the cytoskeleton and regulates ADAM-17 localization and activity. J Cell Physiol 208:363–372
7. Müller JM, Metzger E, Greschik H, Bosserhoff A-K, Mercep L, Buettner R, Schüle R (2002) The transcriptional coactivator FHL2 transmits Rho signals from the cell membrane into the nucleus. EMBO J 21:736–748
8. Johannessen M, Moller S, Hansen T, Moens U, Ghelue MV (2006) The multifunctional roles of the four-and-a-half-LIM only protein FHL2. Cell Mol Life Sci 63:268–284
9. Bach I (2000) The LIM domain: regulation by association. Mech Dev 91:5–17
10. Martin B, Schneider R, Janetzky S, Waibler Z, Pandur P, Kuhl M, Behrens J, von der Mark K, Starzinski-Powitz A, Wixler V (2002) The LIM-only protein FHL2 interacts with β-catenin and promotes differentiation of mouse myoblasts. J Cell Biol 159: 113–122
11. Günther T, Poli C, Müller JM, Catala-Lehnen P, Schinke T, Yin N, Vomstein S, Amling M, Schüle R (2005) Fhl2 deficiency results in osteopenia due to decreased activity of osteoblasts. EMBO J 24:3049–3056
12. Labalette C, Nouet Y, Sobczak-Thepot J, Armengol C, Levillayer F, Gendron M-C, Renard C-A, Regnault B, Chen J, Buendia M-A, Wei Y (2008) The LIM-only protein FHL2 regulates cyclin D1 expression and cell proliferation. J Biol Chem 238:15201–15208
13. Morlon A, Sassone-Corsi P (2003) The LIM-only protein FHL2 is a serum-inducible transcriptional coactivator of AP-1. Proc Natl Acad Sci USA 100:3977–3982
14. Genini M, Schwalbe P, Scholl FA, Remppis A, Mattei MG, Schafer BW (1997) Subtractive cloning and characterization of DRAL, a novel LIM-domain protein down-regulated in rhabdomyosarcoma. DNA Cell Biol 16:433–442
15. Gabriel B, Fischer DC, Orlowska-Volk M, Hausen A, Schüle R, Müller JM, Hasenburg A (2006) Expression of the transcriptional coregulator FHL2 in human breast cancer: a clinicopathologic study. J Soc Gynecol Investig 13:69–75
16. Gullotti L, Hirner S, Merckelback-Bruse S, Fishel R, Schüle R, Buettner R (2004) LIM only protein FHL2 expression in colorectal cancer. AACR Meeting Abstracts 45:213
17. Heemers HV, Regan KM, Dehm SM, Tindall DJ (2007) Androgen induction of the androgen receptor coactivator four-and-a-half LIM domain protein-2: evidence for a role for serum response factor in prostate cancer. Cancer Res 67:10592–10599
18. Gabriel B, Mildenberger S, Weisser CW, Metzger E, Gitsch G, Schüle R, Müller JM (2004) Focal adhesion kinase interacts with the transcriptional coactivator FHL2 and both are overexpressed in epithelial ovarian cancer. Anticancer Res 24:921–927
19. Roest Crollius H, Weissenbach J (2005) Fish genomics and biology. Genome Res 15:1675–1682
20. Bassett DI, Currie PD (2003) The zebrafish as a model for muscular dystrophy and congenital myopathy. Hum Mol Genet 12:R265–R270
21. Johnston IA (1999) Muscle development and growth: potential implications for flesh quality in fish. Aquaculture 177:99–115
22. Andrades JA, Becerra J, Fernández-Llebrez P (1996) Skeletal deformities in larval juvenile and adult stages of cultured gilthead sea bream (*Sparus aurata* L.). Aquaculture 141:1–11
23. Rowlerson A, Radaelli G, Mascarello F, Veggetti A (1997) Regeneration of skeletal muscle in two teleost fish: *Sparus aurata* and *Brachydanio rerio*. Cell Tissue Res 289:311–322
24. Ramírez-Zarzosa G, Gil F, Latorre R, Ortega A, García-Alcaráz A, Abellán E, Vázquez JM, López-Albors O, Arencibia A, Moreno F (1995) The larval development of lateral musculature in gilthead sea bream *Sparus aurata* and sea bass *Dicentrarchus labrax*. Cell Tissue Res 280:217–224
25. Boukouvala E, Leaver MJ, Favre-Krey L, Theodoridou M, Krey G (2010) Molecular characterization of a gilthead sea bream (*Sparus aurata*) muscle tissue cDNA for carnitine palmitoyltransferase 1B (CPT1B). Comp Biochem Physiol Part B Biochem Mol Biol 157:189–197
26. Tiago DM, Laizé V, Cancela ML (2008) Alternatively spliced transcripts of *Sparus aurata* insulin-like growth factor 1 are differentially expressed in adult tissues and during early development. Gen Comp Endocrinol 157:107–115

27. Thisse C, Thisse B, Schilling TF, Postlethwait JH (1993) Structure of the zebrafish *snail1* gene and its expression in wild-type, *spadetail* and *no tail* mutant embryos. Development 119:1203–1215
28. Witten PE, Hansen A, Hall BK (2001) Features of mono- and multinucleated bone resorbing cells of the zebrafish *Danio rerio* and their contribution to skeletal development, remodeling, and growth. J Morphol 250:197–207
29. Simes DC, Williamson MK, Ortiz-Delgado JB, Viegas CSB, Price PA, Cancela ML (2003) Purification of matrix Gla protein from a marine teleost fish, *Argyrosomus regius*: calcified cartilage and not bone as the primary site of MGP accumulation in fish. J Bone Miner Res 18:244–259
30. Notredame C, Higgins DG, Heringa J (2000) T-Coffee: a novel method for fast and accurate multiple sequence alignment. J Mol Biol 302:205–217
31. Schneider TD, Stephens RM (1990) Sequence logos—a new way to display consensus sequences. Nucleic Acids Res 18:6097–6100
32. Shen M-y, Sali A (2006) Statistical potential for assessment and prediction of protein structures. Protein Sci 15:2507–2524
33. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE (2004) UCSF Chimera—a visualization system for exploratory research and analysis. J Comput Chem 25:1605–1612
34. Silva P, Rowlerson AM, Valente LM, Olmedo M, Monteiro RA, Rocha E (2008) Muscle differentiation in blackspot seabream (*Pagellus bogaraveo*, Brunnich): histochemical and immunohistochemical study of the fibre types. Tissue Cell 40:447–458
35. Hernandez LP, Patterson SE, Devoto SH (2005) The development of muscle fiber type identity in zebrafish cranial muscles. Anat Embryol (Berl) 209:323–334
36. Chu P-H, Ruiz-Lozano P, Zhou Q, Cai C, Chen J (2000) Expression patterns of FHL/SLIM family members suggest important functional roles in skeletal muscle and cardiovascular system. Mech Dev 95:259–265
37. Kong Y, Shelton JM, Rothermel B, Li X, Richardson JA, Bassel-Duby R, Williams RS (2001) Cardiac-specific LIM protein FHL2 modifies the hypertrophic response to β-adrenergic stimulation. Circulation 103:2731–2738
38. Moretti A, Fernandez-Criado MP, Cittolin G, Guidastri R (1999) Manual on hatchery production of seabass and gilthead seabream. Food and Agriculture Organization of the United Nations, Rome
39. Devoto SH, Melancon E, Eisen JS, Westerfield M (1996) Identification of separate slow and fast muscle precursor cells in vivo, prior to somite formation. Development 122:3371–3380
40. Chauvigné F, Cauty C, Rallière C, Rescan PY (2005) Muscle fiber differentiation in fish embryos as shown by in situ hybridization of a large repertoire of muscle-specific transcripts. Dev Dyn 233:659–666
41. Fimia GM, De Cesare D, Sassone-Corsi P (2000) A family of LIM-only transcriptional coactivators: tissue-specific expression and selective activation of CREB and CREM. Mol Cell Biol 20:8613–8622
42. Chan KK, Tsui SKW, Lee SMY, Luk SCW, Liew CC, Fung KP, Waye MMY, Lee CY (1998) Molecular cloning and characterization of FHL2, a novel LIM domain protein preferentially expressed in human heart. Gene 210:345–350
43. Patterson SE, Mook LB, Devoto SH (2008) Growth in the larval zebrafish pectoral fin and trunk musculature. Dev Dyn 237:307–315
44. Amann K, Wolf B, Nichols C, Tornig J, Schwarz U, Zeier M, Mall G, Ritz E (1997) Aortic changes in experimental renal failure: hyperplasia or hypertrophy of smooth muscle cells? Hypertension 29:770–775
45. Shi X, Bowlin KM, Garry DJ (2010) Fhl2 interacts with Foxk1 and corepresses Foxo4 activity in myogenic progenitors. Stem Cells 28:462–469
46. Amaar YG, Thompson GR, Linkhart TA, Chen S-T, Baylink DJ, Mohan S (2002) Insulin-like growth factor-binding protein 5 (IGFBP-5) interacts with a four-and-a-half LIM protein 2 (FHL2). J Biol Chem 277:12053–12060
47. Wei Y, Renard C-A, Labalette C, Wu Y, Levy L, Neuveut C, Prieur X, Flajolet M, Prigent S, Buendia M-A (2003) Identification of the LIM protein FHL2 as a coactivator of β-catenin. J Biol Chem 278:5188–5194
48. Hamidouche Z, Hay E, Vaudin P, Charbord P, Schüle R, Marie PJ, Fromigué O (2008) FHL2 mediates dexamethasone-induced mesenchymal cell differentiation into osteoblasts by activating Wnt/β-catenin signaling-dependent Runx2 expression. FASEB J 22:3813–3822
49. Wixler V, Hirner S, Müller JM, Gullotti L, Will C, Kirfel J, Günther T, Schneider H, Bosserhoff A, Schorle H, Park J, Schüle R, Buettner R (2007) Deficiency in the LIM-only protein Fhl2 impairs skin wound healing. J Cell Biol 177:163–172
50. Park J, Will C, Martin B, Gullotti L, Friedrichs N, Buettner R, Schneider H, Ludwig S, Wixler V (2008) Deficiency in the LIM-only protein FHL2 impairs assembly of extracellular matrix proteins. FASEB J 22:2508–2520
51. Blair JE, Hedges SB (2005) Molecular phylogeny and divergence times of deuterostome animals. Mol Biol Evol 22:2275–2284
52. Marques CL, Rafael MS, Cancela ML, Laizé V (2007) Establishment of primary cell cultures from fish calcified tissues. Cytotechnology 55:9–13
53. Conceição N, Laizé V, Simões B, Pombinho AR, Cancela ML (2008) Retinoic acid is a negative regulator of matrix Gla protein gene expression in teleost fish *Sparus aurata*. Biochim Biophys Acta 1779:28–39
54. Pinto JP, Ohresser MCP, Cancela ML (2001) Cloning of the Bone Gla Protein gene from the teleost fish *Sparus aurata*. Evidence for overall conservation in gene organization and bone-specific expression from fish to man. Gene 270:77–91
55. Pinto JP, Conceição N, Gavaia PJ, Cancela ML (2003) Matrix Gla protein gene expression and protein accumulation colocalize with cartilage distribution during development of the teleost fish *Sparus aurata*. Bone 32:201–210
56. Ferraresso S, Vitulo N, Mininni AN, Romualdi C, Cardazzo B, Negrisolo E, Reinhardt R, Canário AV, Patarnello T, Bargelloni L (2008) Development and validation of a gene expression oligo microarray for the gilthead sea bream (*Sparus aurata*). BMC Genomics 9:580