

Analysis of the *Ush2a* Gene in Medaka Fish (*Oryzias latipes*)

Elena Aller^{1,2*}, Ana V. Sánchez-Sánchez^{2,7}, Javier U. Chicote³, Gema García-García², Patricia Udaondo^{2,4}, Laura Cavallé^{2,5}, Marina Piquer-Gil², Antonio García-España³, Manuel Díaz-Llopis^{2,4}, José M. Millán^{1,2,6}, José L. Mullor^{2,7*}

1 CIBER de Enfermedades Raras (CIBERER), Valencia, Spain, **2** Grupo de Investigación en Enfermedades Neurosensoriales, IIS-La Fe, Valencia, Spain, **3** Unitat de Recerca, Hospital Joan XXIII, Institut de Investigació Sanitària Rovira i Virgili (IISPV), Universitat Rovira i Virgili, Tarragona, Spain, **4** Servicio de Oftalmología, Hospital Universitario La Fe, Valencia, Spain, **5** Servicio de Otorrinolaringología, Hospital Universitario La Fe, Valencia, Spain, **6** Unidad de Genética y Diagnóstico Prenatal, Hospital Universitario La Fe, Valencia, Spain, **7** Bionos, SL, Biopolo La Fe – Hospital La Fe, Valencia, Spain

Abstract

Patients suffering from Usher syndrome (USH) exhibit sensorineural hearing loss, retinitis pigmentosa (RP) and, in some cases, vestibular dysfunction. USH is the most common genetic disorder affecting hearing and vision and is included in a group of hereditary pathologies associated with defects in ciliary function known as ciliopathies. This syndrome is clinically classified into three types: USH1, USH2 and USH3. USH2 accounts for well over one-half of all Usher cases and mutations in the *USH2A* gene are responsible for the majority of USH2 cases, but also for atypical Usher syndrome and recessive non-syndromic RP. Because medaka fish (*Oryzias latipes*) is an attractive model organism for genetic-based studies in biomedical research, we investigated the expression and function of the *USH2A* ortholog in this teleost species. *Ol-Ush2a* encodes a protein of 5.445 aa codons, containing the same motif arrangement as the human USH2A. *Ol-Ush2a* is expressed during early stages of medaka fish development and persists into adulthood. Temporal *Ol-Ush2a* expression analysis using whole mount in situ hybridization (WMISH) on embryos at different embryonic stages showed restricted expression to otoliths and retina, suggesting that *Ol-Ush2a* might play a conserved role in the development and/or maintenance of retinal photoreceptors and cochlear hair cells. Knockdown of *Ol-Ush2a* in medaka fish caused embryonic developmental defects (small eyes and heads, otolith malformations and shortened bodies with curved tails) resulting in late embryo lethality. These embryonic defects, observed in our study and in other ciliary disorders, are associated with defective cell movement specifically implicated in left-right (LR) axis determination and planar cell polarity (PCP).

Citation: Aller E, Sánchez-Sánchez AV, Chicote JU, García-García G, Udaondo P, et al. (2013) Analysis of the *Ush2a* Gene in Medaka Fish (*Oryzias latipes*). PLoS ONE 8(9): e74995. doi:10.1371/journal.pone.0074995

Editor: Filippo Del Bene, Institut Curie, France

Received: November 16, 2012; **Accepted:** August 13, 2013; **Published:** September 23, 2013

Copyright: © 2013 Aller et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by grants from the “Conselleria de Sanitat de la Comunitat Valenciana” (GE-011/10) and from the “Fondo de Investigaciones Sanitarias, FIS” (P110/01825) and Ministerio de Economía y Competitividad BFU2009-10808. The authors would like to thank MICINN (Spain) for financial support (Projects CONSOLIDER INGENIO CSD-2010-00065). AGE is supported by the Research Stabilization Program of the Instituto de Salud Carlos III-Institut Catala de la Salut in Catalonia. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: jlmullor@iislafe.es (JLM); elenaller@yahoo.es (EA)

Introduction

Usher syndrome (USH) is the most frequent genetic cause of combined deafness and blindness causing hearing loss, retinitis pigmentosa (RP) and, in some cases, vestibular dysfunction. USH is associated with defects in ciliary function like Bardet-Biedl syndrome, Joubert syndrome, Senior-Loken syndrome and some forms of non-syndromic RP [1,2]. Based on clinical features of the hearing impairment, Usher syndrome is classified into three types: I, II, and III (USH1, USH2, USH3). Usher syndrome type I is the most severe form with profound congenital deafness and vestibular dysfunction. USH2 is characterized by moderate non-progressive hearing loss without vestibular dysfunction and USH3 is distinguished from USH1 and USH2 by the progressive nature of its hearing loss [3].

USH2 accounts for well over one-half of all Usher cases and up to date, 3 genes are known to be involved in the pathogenesis of this clinical form: *USH2A*, *GPR98* and *DFNB31* [4,5,6,7]. Mutations in the *USH2A* gene are responsible for the majority of

USH2 cases [8,9,10] and are also responsible for atypical Usher syndrome and recessive non-syndromic RP [11,12]. Two main isoforms have been described for this gene. The short isoform_a, reported to be 5 kb, encoding a protein of 170 kDa; and the long isoform_b, that expands the length of coding sequence to 15 kb, encoding a 600 kDa protein [5].

USH2A_isoform_b protein is mostly extracellular except for a membrane-spanning segment followed by an intracellular PDZ-binding domain at the C-terminus. Its function has been related to the true cilia of the retinal photoreceptors and the microvilli of cochlear hair cells. In the inner ear, USH2A_iso_b contributes to the conformation of ankle links, necessary for the development and maintenance of stereocilia cohesion [13]. In the retina, USH2A_iso_b would be part of the complex participating in the delivery of cargo to the outer segment of vertebrate photoreceptor cells [14,15].

The majority of USH genes have been knocked out in mice. All mutant mice suffer from inner ear defects, but only the *USH2A*

knockout mice develop a detectable retinal degeneration [14,16,17]. In addition to mouse knock out mutants, zebrafish mutants have also been described for some genes causing Usher syndrome. Zebrafish mutants have been described for 4 USH1 genes: *MYO7A* (*mariner*) [18], *CDH23* (*sputnik*) [19,20], *PCDH15* (*orbiter*) [21] and *USH1C* [22]. Regarding USH2 genes, Ebermann et al. (2010) [23] used “knock down” models for *USH2A* and *GPR98* in a study focused on the modifier gene *PDZD7*. Additionally, the *GPR98* gene was also characterized in zebrafish by Gibert et al. (2005) [24]. In most of these fish mutants, there is early photoreceptors cell death, which is absent in the majority of murine models, making fish a good model to study the combined eye and ear physiopathology of USH [16].

Small laboratory fish such as zebrafish and medaka, the Japanese killifish, are attractive vertebrate animal models that are easy to handle and are ideally suited for genetic studies because of their large numbers of progeny per generation [25]. Draft genome sequences for both zebrafish and medaka (Medaka Genome Project [http://dolphin.lab.nig.ac.jp/medaka]) are already available. Thus, fish are becoming increasingly important models in biomedical research [26]. A compact genome that lacks the complex repetitive elements observed in zebrafish, and the availability of several inbred strains [27] make the medaka fish model especially suited for genome-based analyses. Because the medaka fish is an attractive and economic model organism for genetic-based studies in biomedical research, we analyzed the gene structure, expression and function of human *USH2A* ortholog in this teleost species.

Materials and Methods

Animal Strains and Maintenance

This study was carried out in strict accordance with the recommendations contained in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Welfare Ethical Committee (CEBA) of the “Hospital Universitario La Fe”. Adult medaka (*Oryzias latipes*) CAB strain animals were kept in recirculating water aquaria at 28°C on at 14-hr light/10-hr dark daily cycle. Embryos were collected by natural spawning in Yamamoto solution [28] and staged as previously described [29]. Embryos were raised at 25°C.

Cloning of Medaka Fish *Ush2a* cDNA and Sequencing

Blast searches, using Zebrafish USH2A protein (CAK04893.2) as bait, and intron-exon borders determination were performed as previously described [30,31]. Alignment of protein sequences were done using ClustalW program from the Network Protein Sequence Analysis [32]. The signal peptide sequence was determined with the program SignalP 4.0 Server [33] with a cut-off of 0.650. The domains LamGL, LamNT, EGF-Lam, LamG and FN3 with E-value lower than 1×10^{-3} and the transmembrane domain were found out using the Conserved Domain Search Service (CD Search) from the National Center for Biotechnology Information and the program TMHMM server v2.0 [34,35]. Finally, the PDZ1-binding domain was located as previously stated [5]. Primers based on cDNA sequence predictions obtained were used to amplify the full-length *Ol-Ush2a* open reading frame from whole larvae and adult eye cDNA (see Table 1 for primer sequences). PCR products were separated and purified using gel electrophoresis and sequenced on an ABI 3500XL analyzer using the fluorescent dideoxy terminator method (Applied Biosystems).

Table 1. Primers used to amplify and sequence the full-length open reading frame (ORF) of *Ol-Ush2a*.

NAME	SEQUENCE 5'-3'
P1D	ATGGAACCATGACCCTCATCAC
P1R	TGCTCTCAGATGTATTCGGAC
P2D	AACAGCCCTGGTTGGACTGG
P2R	TCCATCCTTAGACCAACAAGC
P3D	GTGTGATCAAAACT
P3R	GAGTCTGGATGGCAGGAAC
P4D	TTTGTGTCCAACACGCCATG
P4R	GCAGGTAAAGTCAGTCCTGTG
P5D	TACACAGGTGGAACAATCATC
P5R	AACCAAGTTACAGCGGCACTG
P6D	CAGAAGCCATGGACTACAGTC
P6R	TCAACACAACCTGAGTGTAC
P7D	CTCGGCACATACTCAGACTG
P7int_aD	AATTCCAACGCTCCAGGTCC
P7int_bD	TTGTGCACCTTCTCCGTAC
P7intR	AGACCAGCAGGTGCGATG
P7R	TGGTAACAGGAGTTGGGAGAC
P8D	ACCCAATGGAGAGGTTTCATGG
P8R	GAGACCAGGTAGTTGGTACTG
P9D	GTTTCTTCTCACTGTAGAGC
P9R	TATCCTTACAGCACTGGTGC
P10D	GACATCCTGCTTCGATCCAG
P10R	ACGGTAACGTTCTTGCCATC
P11D	CTACAGCAGTGACAACCAAGT
P11R	GGCAATCTTTCAGGTGTAGTC
P12D	TCAATGCTGGAAGTCGCAAG
P12R	GGCTTGTTGGTCGACACAG
P13D	TCCTTACAGTGACAGATTTGC
P13R	GAAGGCAGTGGAGATATTCTG
P14D	GTGACTGGTCTCTGTACTC
P14R	GTGGGCTCCAAGTAAGTGAC
P15D	GGCTGGTTACAAGAAGCACC
P15intD	CCATTGAGAAAGCTTCACACC
P15R	CAGCAGTATGAACACAGTTC
P16D	CAGGTGACGTGTACAACAGAC
P16R	CTCGTCTAAAGATGTGTGTC

doi:10.1371/journal.pone.0074995.t001

RNA Isolation and RT-PCR

Total RNA was isolated from total embryo, larvae and adult tissues using Trizol (Invitrogen, Carlsbad, CA). Reverse transcription was performed according to manufacturer instructions using the Gene Amp Gold RNA Core Kit (Applied Biosystems). Sequence primers used to analyze the spatiotemporal expression pattern of *Ol-Ush2a* were Direct Primer: 5'-AACAGCCC-TGGTTGGACTGG and Reverse Primer: TGCTCTCAGATG-TATTCGGAC- 3'.

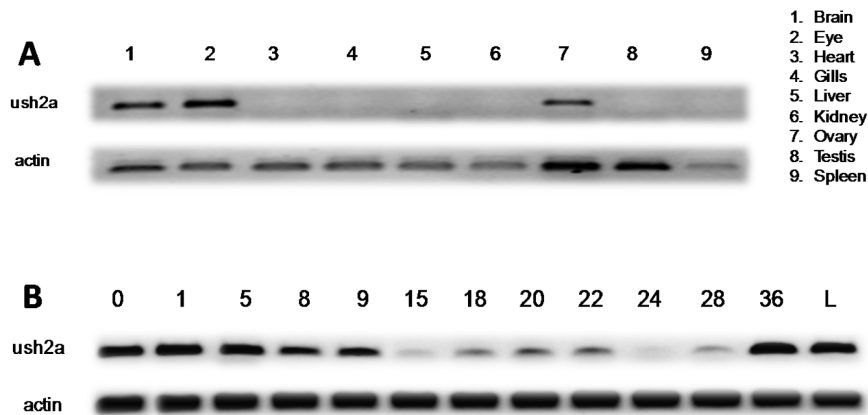


Figure 3. Spatial and temporal *Ol-Ush2a* expression pattern analysis performed by RT-PCR amplification. **A:** RT-PCR amplification from adult organs. **B:** RT-PCR amplification from different embryonic developmental stages.
doi:10.1371/journal.pone.0074995.g003

1.00mM of *Ol_ush2a*MO1 (MO1) targeted to the translation start site (5'-TCATCAGCAGCAGCACTGGAGACAT-3'), 0.05–1.00 mM of *Ol_ush2a*MO2 (MO2) also targeted to the translation start site (5'-TCCAAAGTTTCTCTCTCATGCC-3'), 0.10–1.00 mM of *Ol_ush2a*MO3 (MO3) targeted to the splice donor site of exon 5 (5'-CTGTCATGGATCTGGAAATC-CAAAA-3') and 1.00 mM of *Ol_ush2a*MO4 (MO4) targeted to the splice donor site of exon 29 (5'-CATCTGAATTCCT-TAATGTGAAGTA-3'). The sequence of control MO for *Ol_ush2a*MO1 (MO1C) was 5'-TCATgAcCAcCAcCACTcGA-GACAT-3', this sequence correspond to a 5-mispair oligo and it is a specific control for *Ol_ush2a*MO1. The sequence of control MO (MOC) used for the rest of experiments was 5'-CCTCTTACCTCAGTTACAATTTATA-3'.

Immunohistochemistry

For whole-mount imaging of stereocilia and retina, embryos were fixed in 'BT' fix (4% PFA, 0.15 mM CaCl₂, 4% sucrose in PBS) o/n at 4°C, rinsed four times for 5 minutes each in PBS +0.01% Triton X-100, and then permeabilized with 2% Triton X-100 in PBS o/n at 4°C. They were rinsed four times for 5 minutes each in PBS +0.01% Triton X-100, blocked for 2 hours at room temperature in 10% NGS +0.01% Triton X-100, then incubated with Alexa-Fluor-488-phalloidin (Invitrogen) and *Zpr-1* antibody

(The zebrafish international resource Center, Eugene, OR, USA) diluted in block o/n at 4°C. Following four 5-minute PBS +0.01% TritonX-100 washes, embryos were incubated in secondary antibody and TOPRO solution for one hour at room temperature. After rinsing four times for 5 minutes each in PBS-Triton, embryos were mounted using fluoromount G medium. In some experiments, embryos were raised with 0.003% phenylthiourea (PTU) to inhibit pigment formation.

Results

Ol-Ush2a cDNA and Protein Structure

Using bioinformatics tools, a single ortholog of human *USH2A* was identified in medaka fish genome at chromosome 3. By PCR amplification of embryonic and ocular cDNA based on these predictions followed by sequencing, the *Ol-Ush2a* gene was characterized. It was assembled from 16 overlapping fragments. This transcript is 16.5 kb in length, with an ORF of 5.445 aa codons (GenBank Accession Number: KF278972). Medaka fish and human predicted proteins have the same motif arrangement, with a large extracellular domain consisting of LamGL, LamNT, EGF-Lam, LamG, and FN3 motif repeats, a single membrane-spanning segment and a PDZ-binding C terminus predicted to reside intracellularly (Fig. 1).

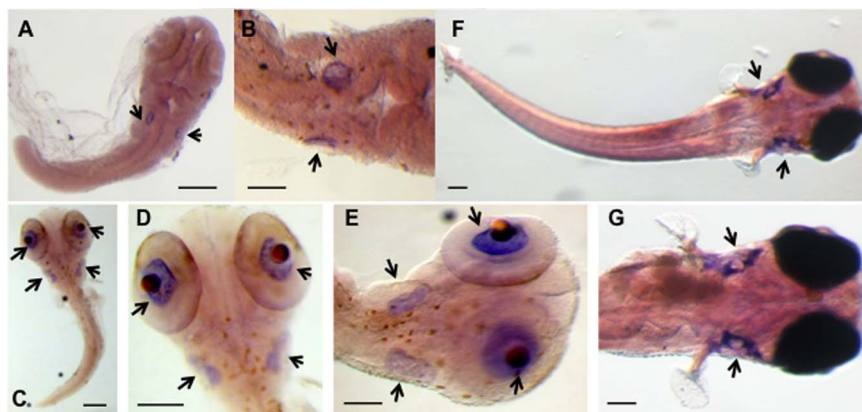


Figure 4. *Ol-Ush2a* expression pattern obtained by ISH experiments. **A, B:** Stages 22–23. **C, D, E:** Stage 30, treated with PTU. **F, G:** Stages 36–38. Scale bars: A, C, F and G: 100 μ m. B, D and E: 50 μ m.
doi:10.1371/journal.pone.0074995.g004

Table 2. Phenotypic analysis of *Ol-Ush2a* loss of function.

	normal phenotype %	<i>Ol-Ush2a</i> -specific phenotype %	Mortality %	n
MO C	92	0	3	356
MO1 C	95	0	2	215
MO1	3	78	14	324
MO2	4	82	11	196
MO3	0	0	100	153
MO4	94	0	4	124

Nonspecific phenotypes (i.e., injection phenotypes) are not represented in the table, but represent the difference between the reported values and 100%.
doi:10.1371/journal.pone.0074995.t002

Human *USH2A* has several alternative splice variants. We have investigated the presence of the most frequent of these isoforms (isoform_a) in medaka fish embryos. An evolutionary analysis of *Ush2a_iso_a* points out that the short isoform would be specific for eutherian species (Fig. 2).

Ol-Ush2a Expression Pattern

To determine the spatial and temporal *Ol-Ush2a* expression patterns we performed RT-PCR and whole mount *in situ* hybridization analysis of gene expression. *Ol-Ush2a* RT-PCR amplification from adult organs showed *Ol-Ush2a* expression restricted to the brain, eye and ovary (Fig. 3A). When *Ol-Ush2a* expression was analyzed at different embryonic stages, strong *Ol-Ush2a* expression was detected at early developmental stages (st. 0–9). The high *Ol-Ush2a* expression level decreases during gastrulation (st. 15), increases at neurula stage (st. 18) and is maintained during somite formation stages (st. 20–28). During late embryonic stages (st. >30) and after hatching (36) *Ol-Ush2a* expression is upregulated (Fig. 3B). WMISH experiments showed that *Ol-Ush2a* mRNA expression was first detected in the embryonic ear at embryonic stages 22–23, when otic vesicles appear, persisting through all later stages examined (Fig. 4A–G). We also observed *Ol-Ush2a* expression in the retina of embryos treated with PTU at 30–32 developmental stages (Fig. 4C–E).

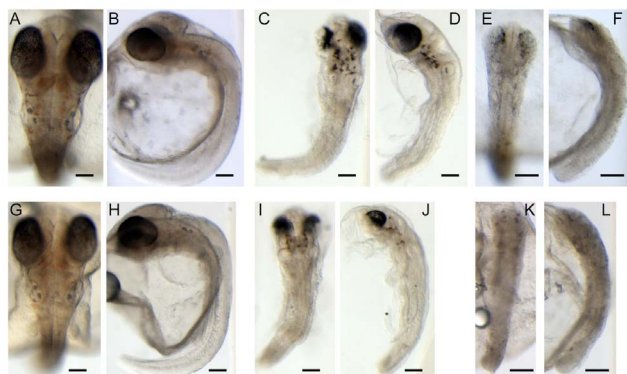


Figure 5. Medaka embryos obtained after MO1 and MO2 injections. A, B, G, H: MO1 C (A, B) and MO2 C (G, H) embryos at 72 hpf. C, D, I, J: MO1 (C, D) and MO2 (I, J) injected embryos at 72 hpf, showing a **mild phenotype**. E, F, K, L: MO1 (E, F) and MO2 (K, L) injected embryos at 72 hpf, showing a **severe phenotype**. Scale bars: 100 μ m. Embryos in frontal (A, C, E, G, I, K) and lateral position (B, D, F, H, J, L).
doi:10.1371/journal.pone.0074995.g005

Morpholino Knockdown of *Ol-Ush2a* in Medakafish Embryos

In order to analyze the role of *Ol-Ush2a* during early development, we used morpholino oligonucleotides to knock down the function of *Ol-Ush2a*: MO1 and MO2 were designed to block the translation initiation site, MO3 blocked splicing at the intron-4-exon-5 boundary and MO4 blocked splicing at intron-28-exon-29 boundary.

Depletion of *Ol-Ush2a* after injection of MO1 or MO2 caused the same phenotype with a delay in embryonic development and evident embryonic malformations (Fig. 5, Table 2). At 72 hpf, MO1 and MO2 injected embryos with less severe phenotypes presented small eyes and heads and otolith malformations (Mild phenotype. Fig. 5C, D, I, J). These embryos were shorter and had curved tails and also presented pigmentation defects. Morphant embryos with more severe phenotypes presented serious generalized malformations (Severe phenotype. Fig. 5E, F, K, L). Not surprisingly MO1 and MO2 injections prevented hatching. Early embryonic lethal phenotypes (at 24 hpf) were obtained with MO3 at concentrations 1–0.5 mM. No effect in embryo development and larvae behavior was observed after injection of MO4 at 1 mM concentration. Using RT-PCR analysis of MO4 injected embryos, we identified an aberrant splice form skipping exon 29, producing an in-frame splicing of exons 28–30 which may explain the lack of phenotype of this morpholino (Fig. 6).

We next investigated the structural architecture of inner ear and retinas of 0.1 mM MO1 morphant embryos using immunofluorescence. Medaka embryos have three different sensory areas in their inner ear containing hair cells necessary for hearing, movement and equilibrium (Fig. 7A and Fig. S1A). In mild morphant embryos, the sensory patches of hair cells observed in control embryos were reduced to only one area with less stereocilia with apparent normal morphology (Fig. 7 and Fig. S1A and S1B). In severe morphant embryos, recognizable stereocilia could not be

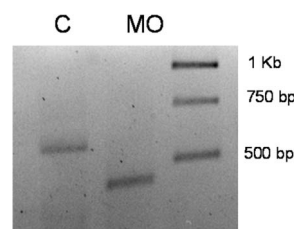


Figure 6. RT-PCR results from total RNA extracted from control and morphant MO3 embryos at 96 hpf. C. Control embryos. Expected size: 573 bp. MO. Morphant embryos. Expected size: 444 bp.
doi:10.1371/journal.pone.0074995.g006

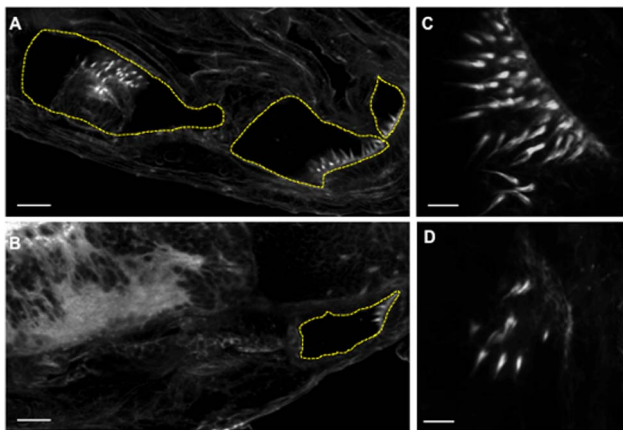


Figure 7. Ultra structural architecture analysis of inner ear and stereocilia performed on MO1 morphant embryos using fluorescent staining. A. Phalloidin staining of WT larvae inner ear highlighting the three sensory areas containing stereocilia. B. Phalloidin staining of 0.1 mM 8 dpf morphant embryos highlighting the unique sensory area containing stereocilia. C. Stereocilia morphology of WT inner ear. D. Stereocilia morphology of morphant inner ear. Scale bars: A and B: 20 μm . C and D: 5 μm .

doi:10.1371/journal.pone.0074995.g007

detected after phalloidin staining. These results suggest that *Ol-Ush2a* participates in the embryonic development of the inner ear.

The other area of *Ol-Ush2a* expression was the developing retina (Fig. 8). *Ol-Ush2a* severe morphant embryos presented disorganized small eyes; however, this effect could be due to the severity of the embryonic malformations affecting general organ structure. In order to detect more specific retinal effects, we analyzed the retinal structure of mild *Ol-Ush2a* morphant embryos that presented inner

ear defects. No apparent differences were observed in retinal lamination between control and mild morphant embryos after phalloidin+anti-zpr1+TOPRO immunostaining (Fig. 8). This result indicates that inner ear development is more sensitive to depletion of *Ol-Ush2a* protein levels than retina development.

Discussion

We have characterized the medaka fish *Ol-Ush2a* gene. *Ol-Ush2a* is 5,445 amino acids in size and is encoded by a 16.5 Kb mRNA sharing the same motif arrangement with its human ortholog. Searches of genomic databases revealed that *USH2A* is remarkably conserved across phyla. It is conserved in the deuterostome lineage as well as in some (annelids and mollusks), but not all (arthropods and nematodes) protostomes [37]. Thus, *USH2A* could be an example of a gene lost in Ecdysozoa [38].

Two variants of *USH2A* are present in human and mice [5,14]. The long variant (*USH2A_iso_b*, full length), participates in the formation of the ankle-link complex that anchors adjacent stereocilia to each other in the inner ear [13,39]. In the retina, it is part of the complex participating in cargo delivery to the outer segment of vertebrate photoreceptor cells [15]. The short variant (*USH2A_isoform_a*), comprising only the first 5 Kb, is predicted to be a secreted, extracellular protein and was found in a subset of basement membranes [40], although functional characterization is lacking. The evolutionary analysis performed with several putative *USH2A* short isoform sequences from vertebrates, suggest that *USH2A_isoform_a* arose in eutherians and is not present in reptiles, birds, amphibians and fishes.

RT-PCR expression analysis at different medaka embryonic stages showed a strong *Ol-Ush2a* expression at early stages (0–9). These transcripts are of maternal origin, which would be in agreement with *Ol-Ush2a* expression being detected in the adult ovary. Furthermore, this early expression could explain the severe

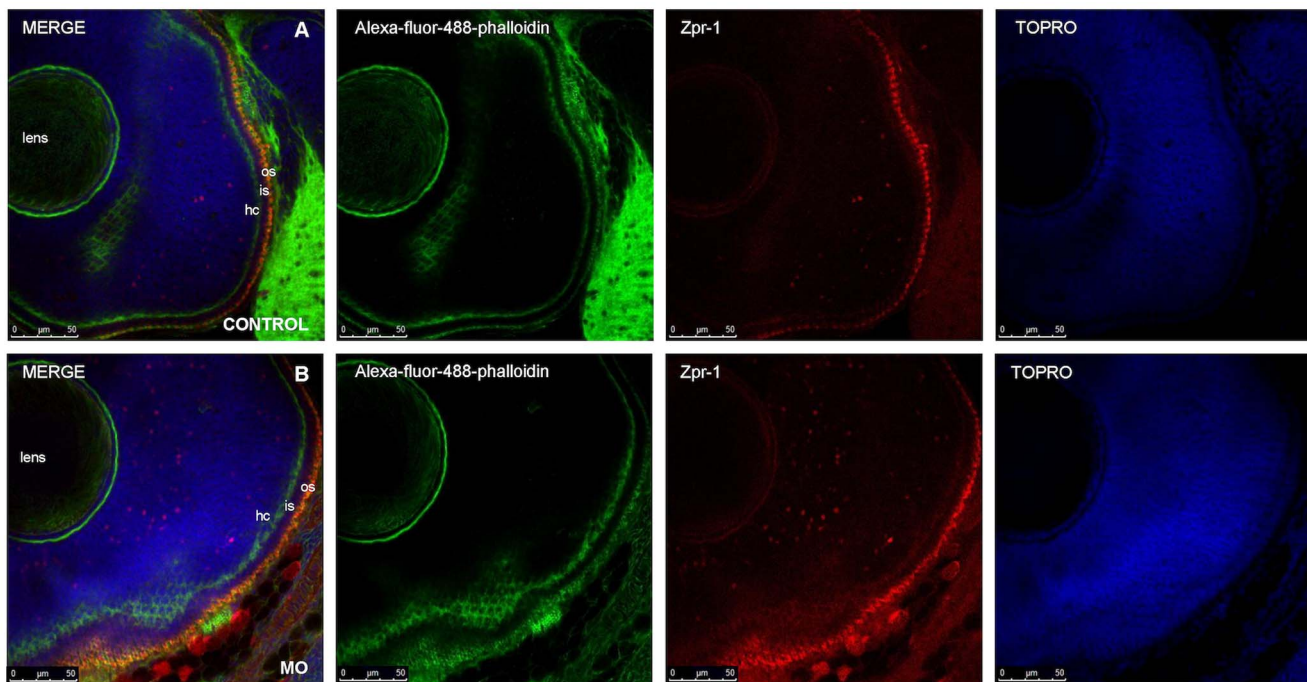


Figure 8. Retinal lamination observed in WT and 0.1 mM 5 dpf morphant embryos treated with PTU. Green: Alexa-fluor-488-phalloidin: actin. Blue: TOPRO: nuclei. Red: Zpr-1: double cone photoreceptors. hc: horizontal cells. os: outer segments. is: inner segments si.

doi:10.1371/journal.pone.0074995.g008

defects in complete embryonic development observed after injection of specific MOs against *Ol-Ush2a*.

At the beginning of gastrulation there is a sudden *Ol-Ush2a* expression decrease to almost undetectable levels. A low expression level is detected again from neurula stages until the somite period. After stage 30, *Ol-Ush2a* expression becomes stronger and persists in the larvae after hatching. In adult tissues, RT-PCR analysis showed that *Ol-Ush2a* was expressed in eye, but also in brain and ovary. WMISH analysis showed that the *Ol-Ush2a* expression pattern in the embryo was restricted to otoliths and retina, suggesting that USH2A might play a conserved role in the development and/or maintenance of retinal photoreceptors and cochlear hair cells. Van Wijck *et al.*, (2004) [5] also found a restricted expression pattern for the long isoform_b of USH2A in humans. It was only detected in adult retina (predominantly), heart, and kidney.

Suppression of *Ol-Ush2a* using two specific MOs against the ATG starting codon site resulted in abnormal development phenotypes (small eyes and heads and shorter curved tails), similar to those phenotypes obtained in zebrafish for other ciliary proteins, encoded by genes like *RPGRIP1L*, *ZFRPGR2* or *ZFRP2* [41,42,43] and for the novel discovered USH gene *CIB2* [44]. Surprisingly, these malformations were not observed in zebrafish after *Ush2a* suppression using a specific MO against the splice donor site of exon 6 [23]. *Ush2a* knockdown experiments in zebrafish produced moderate levels of photoreceptor cell death in larvae. This cell death was restricted to photoreceptors and the retinas were morphologically normal. They also reported swimming and balance defects in *Ush2a* morphant larvae due to abnormal hair bundle morphology. Thus, both teleost fish present morphologically normal retinas and defects in the inner ear. Phenotype differences may be due to different penetrance levels of the morpholino effects or other technical challenges. In this sense, the use of conditional techniques to modulate the time and level of *Ol-Ush2a* knock down effect may be helpful to further develop the model and analyze the molecular mechanisms of Ush2a action. Nevertheless, one must not forget that zebrafish and medaka

belong to different fish evolutive branches that are separated by an evolutive distance of about 110–200 million years ago (Mya). And so, important differences between both organisms exist that could explain the discordances observed between phenotypes [26,45].

Additionally, *USH2A* function during mammals' embryonic development must be redundant or practically restricted to hearing and vision since *USH2A* knockout mice and the human mutations described do not exhibit embryonic lethality.

Our data suggest that *Ol-Ush2a* is essential for medaka embryo development since loss of *Ol-Ush2a* during early development results in embryo lethality. The type of embryo defects observed in our study and in other ciliary disorders are associated with defective cell movement specifically implicated in left-right (LR) axis determination and planar cell polarity (PCP), in part due to abnormal Wnt signaling [46,47], and can result in shortened body axis, broad notochord, thin extended somites, failure of tail extension and small head and eyes [42,48].

Supporting Information

Figure S1 Extended analysis of ultra structural architecture of otoliths in WT (A) and morphant (B) specimens. Arrows indicate stereocilia areas.

(TIF)

Acknowledgments

The authors thank all members of “Laboratorio de Investigación en Enfermedades Neurosensoriales del IIS-La Fe” for their help and cooperation. We are also grateful to animal facilities staff from “Centro de Investigación del Hospital La Fe”.

Author Contributions

Conceived and designed the experiments: EA AVS AGE JLM. Performed the experiments: EA AVS JUC GGG MPG AGE. Analyzed the data: EA AVS JUC AGE JLM. Contributed reagents/materials/analysis tools: PU LC MDL JMM. Wrote the paper: EA AVS AGE JMM JLM.

References

- Badano JL, Mitsuma N, Beales PL, Katsanis N (2006) The ciliopathies: an emerging class of human genetic disorders. *Annu Rev Genomics Hum Genet* 7: 125–48.
- van Recesswijk J, Arts HH, Roepman R (2011) Scrutinizing ciliopathies by unraveling ciliary interaction networks. *Hum Mol Genet* 15;20(R2): R149–57.
- Millán JM, Aller E, Jaijo T, Blanco-Kelly F, Gimenez-Pardo A *et al.* (2011) An update on the genetics of usher syndrome. *J Ophthalmol* 2011: 417217.
- Eudy JD, Weston MD, Yao S, Hoover DM, Rehm HL *et al.* (1998) Mutation of a gene encoding a protein with extracellular matrix motifs in Usher syndrome type IIa. *Science*. 12;280(5370): 1753–7.
- vanWijk E, Pennings RJ, te Brinke H, Claassen A, Yntema HG *et al.* (2004) Identification of 51 novel exons of the Ushersyndrome type 2A (USH2A) gene that encode multiple conserved functional domains and that are mutated in patients with Usher syndrome type II. *Am J Hum Genet* 74(4): 738–44.
- Weston MD, Luijendijk MW, Humphrey KD, Möller C, Kimberling WJ (2004) Mutations in the VLGR1 gene implicate G-protein signaling in the pathogenesis of Usher syndrome type II. *Am J Hum Genet* 74(2): 357–66. Erratum in: *Am J Hum Genet*. 2004;74(5): 1080.
- Ebermann I, Scholl HP, CharbelIssa P, Becirovic E, Lamprecht J *et al.* (2007) A novel gene for Usher syndrome type 2: mutations in the long isoform of whirlin are associated with retinitis pigmentosa and sensorineural hearing loss. *Hum Genet* 121(2): 203–11.
- Baux D, Larrieu L, Blanchet C, Hamel C, Ben Salah S *et al.* (2007) Molecular and in silico analyses of the full-length isoform of usherin identify new pathogenic alleles in Usher type II patients. *Hum Mutat* 28(8): 781–9.
- Dreyer B, Brox V, Tranebjærg L, Rosenberg T, Sadeghi AM *et al.* (2008) Spectrum of USH2A mutations in Scandinavian patients with Usher syndrome type II. *Hum Mutat* 29(3): 451.
- García-García G, Aparisi MJ, Jaijo T, Rodrigo R, Leon AM *et al.* (2011) Mutational screening of the USH2A gene in Spanish USH patients reveals 23 novel pathogenic mutations. *Orphanet J Rare Dis* 17;6: 65.
- Rivolta C, Sweklo EA, Berson EL, Dryja TP (2000) Missense mutation in the USH2A gene: association with recessive retinitis pigmentosa without hearing loss. *Am J Hum Genet* 66: 1975–1978.
- Aller E, Najera C, Millán JM, Oltra JS, Perez-Garrigues H *et al.* (2004) Genetic analysis of 2299delG and C759F mutations (USH2A) in patients with visual and/or auditory impairments. *Eur J Hum Genet* 12: 407–10.
- Petit C, Richardson GP (2009) Linking genes underlying deafness to hair-bundle development and function. *Nat Neurosci* 12(6): 703–10.
- Liu X, Bulgakov OV, Darrow KN, Pawlyk B, Adamian M *et al.* (2007) Usherin is required for maintenance of retinal photoreceptors and normal development of cochlear hair cells. *Proc Natl Acad Sci* 13;104(11): 4413–8.
- Maerker T, van Wijk E, Overlack N, Kersten FF, McGee J *et al.* (2008) A novel Usher protein network at the periciliary reloading point between molecular transport machineries in vertebrate photoreceptor cells. *Hum Mol Genet* 1;17(1): 71–86.
- Williams DS (2008) Usher syndrome: animal models, retinal function of Usher proteins, and prospects for gene therapy. *Vision Res* 48(3): 433–41.
- Lu B, Wang S, Francis PJ, Li T, Gamm DM *et al.* (2010) Cell transplantation to arrest early changes in an ush2a animal model. *Invest Ophthalmol Vis Sci* 51(4): 2269–76.
- Ernest S, Rauch GJ, Haffter P, Geisler R, Petit C *et al.* (2000) Mariner is defective in myosin VIIA: a zebrafish model for human hereditary deafness. *Hum Mol Genet* 1;9(14): 2189–96.
- Söllner C, Rauch GJ, Siemens J, Geisler R, Schuster SC *et al.* (2004) Mutations in cadherin 23 affect tip links in zebrafish sensory hair cells. *Nature* 29;428(6986): 955–9.
- Glover G, Mueller KP, Söllner C, Neuhaus SC, Nicolson T (2012) The Usher gene cadherin 23 is expressed in the zebrafish brain and a subset of retinal amacrine cells. *Mol Vis* 18: 2309–2322.
- Seiler C, Finger-Baier KC, Rinner O, Makhankov YV, Schwarz H *et al.* (2005) Duplicated genes with split functions: independent roles of protocadherin15 orthologues in zebrafish hearing and vision. *Development* 132(3): 615–23.

22. Phillips JB, Blanco-Sanchez B, Lentz JJ, Tallafuss A, Khanobdee K et al. (2011) Harmonin (Ush1c) is required in zebrafish Müller glial cells for photoreceptor synaptic development and function. *Dis Model Mech* 4(6): 786–800.
23. Ebermann I, Phillips JB, Liebau MC, Koeneke RK, Schermer B et al. (2010) PDZD7 is a modifier of retinal disease and a contributor to digenic Usher syndrome. *J Clin Invest* 120(6): 1812–23.
24. Gibert Y, McMillan DR, Kayes-Wandover K, Meyer A, Begemann G et al. (2005) Analysis of the very large G-protein coupled receptor gene (*Vlgr1/Mass1/USH2C*) in zebrafish. *Gene* 4:353(2): 200–6.
25. Wittbrodt J, Shima A, Schartl M (2002) Medaka - a model organism from the Far East. *Nat Rev Genet* 3: 53–64.
26. Furutani-Seiki M, Wittbrodt J (2004) Medaka and zebrafish, an evolutionary twin study. *Mech Dev* 121: 629–637.
27. Hyodo-Taguchi Y (1996) Inbred strains of the medaka (*Oryzias latipes*). *Fish Biol J Medaka* 8: 29–30.30.
28. Yamamoto T (1975) Medaka (killifish): biology and strains. Tokyo: Heigaku Pub. Co. 1–365p.
29. Iwamatsu T (2004) Stages of normal development in the medaka *Oryzias latipes*. *Mech Dev* 121: 605–618.
30. Camp E, Sánchez-Sánchez AV, García-España A, Desalle R, Odqvist L et al. (2009) Nanog regulates proliferation during early fish development. *Stem Cells* 27: 2081–2091.
31. García-España A, Mares R, Sun TT, Desalle R (2009) Intron evolution: testing hypotheses of intron evolution using the phylogenomics of tetraspanins. *PLoS One* 4: e4680.
32. Combet C, Blanchet C, Geourjon C, Deléage G (2000) NPS@: Network Protein Sequence Analysis. *TIBS* 25, 3(291): 147–150.
33. Petersen TN, Brunak S, von Heijne G, Nielsen H (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nature Methods* 8: 785–786.
34. Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK et al. (2011) CDD: a Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Res* 39: D225–9.
35. Krogh A, Larsson B, von Heijne G, Sonnhammer ELL (2001) Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. *Journal of Molecular Biology* 305(3): 567–580.
36. Sánchez-Sánchez AV, Camp E, Leal-Tassias A, Mullor JL (2010) Wnt signaling has different temporal roles during retinal development. *239(1)*: 297–310.
37. Tucker RP (2010) Expression of usherin in the anthozoan *Nematostella vectensis*. *Biol Bull.* 218(2): 105–12.
38. Putnam NH, Srivastava M, Hellsten U, Dirks B, Chapman J et al. (2007) Sea anemone genome reveals ancestral eumetazoan gene repertoire and genomic organization. *Science* 317: 86–94.
39. Adato A, Lefèvre G, Delprat B, Michel V, Michalski N et al. (2005) Usherin, the defective protein in Usher syndrome type IIA, is likely to be a component of interstereocilia ankle links in the inner ear sensory cells. *Hum Mol Genet* 15;14(24): 3921–32.
40. Pearsall N, Bhattacharya G, Wisecarver J, Adams J, Cosgrove D et al. (2002) Usherin expression is highly conserved in mouse and human tissues. *Hear Res* 174(1–2): 55–63.
41. Khanna H, Davis EE, Murga-Zamalloa CA, Estrada-Cuzcano A, Lopez I et al. (2009) A common allele in RRGRIPI1 is a modifier of retinal degeneration in ciliopathies. *Nat Genet* 41(6): 739–45.
42. Shu X, Zeng Z, Gautier P, Lennon A, Gakovic M et al. (2010) Zebrafish Rprg is required for normal retinal development and plays a role in dynein-based retrograde transport processes. *Hum Mol Genet* 15;19(4): 657–70.
43. Shu X, Zeng Z, Gautier P, Lennon A, Gakovic M et al. (2011) Knockdown of the zebrafish ortholog of the retinitis pigmentosa 2 (RP2) gene results in retinal degeneration. *Invest Ophthalmol Vis Sci* 52(6): 2960–6.
44. Riazuddin S, Belyantseva IA, Giese AP, Lee K, Indzhykulian AA et al. (2012) Alterations of the CIB2 calcium- and integrin-binding protein cause Usher syndrome type IJ and nonsyndromic deafness DFNB48. *Nat Genet* 44(11): 1265–71.
45. Yokoi H, Shimada A, Carl M, Takashima S, Kobayashi D et al. (2007) Mutant analyses reveal different functions of fgfr1 in medaka and zebrafish despite conserved ligand-receptor relationships. *Dev Biol* 1;304(1): 326–37.
46. Ross AJ, May-Simera H, Eichers ER, Kai M, Hill J et al. (2005) Disruption of Bardet-Biedl syndrome ciliary proteins perturbs planar cell polarity in vertebrates. *Nat Genet* 37(10): 1135–40.
47. Caron A, Xu X, Lin X (2012) Wnt/ β -catenin signaling directly regulates Foxj1 expression and ciliogenesis in zebrafish Kupffer's vesicle. *Development* 139(3): 514–24.
48. Khanna H, Hurd TW, Lillo C, Shu X, Parapuram SK et al. (2005) RPGR-ORF15, which is mutated in retinitis pigmentosa, associates with SMC1, SMC3, and microtubule transport proteins. *J Biol Chem* 30;280(39): 33580–7.