

Zona Pellucida Domain-Containing Protein β -Tectorin is Crucial for Zebrafish Proper Inner Ear Development

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Abstract

Background: The zona pellucida (ZP) domain is part of many extracellular proteins with diverse functions from structural components to receptors. The mammalian β -tectorin is a protein of 336 amino acid residues containing a single ZP domain and a putative signal peptide at the N-terminus of the protein. It is 1 component of a gel-like structure called the tectorial membrane which is involved in transforming sound waves into neuronal signals and is important for normal auditory function. β -Tectorin is specifically expressed in the mammalian and avian inner ear.

Methodology/Principal Findings: We identified and cloned the gene encoding zebrafish β -tectorin. Through whole-mount *in situ* hybridization, we demonstrated that β -tectorin messenger RNA was expressed in the otic placode and specialized sensory patch of the inner ear during zebrafish embryonic stages. Morpholino knockdown of zebrafish β -tectorin affected the position and number of otoliths in the ears of morphants. Finally, swimming behaviors of β -tectorin morphants were abnormal since the development of the inner ear was compromised.

Conclusions/Significance: Our results reveal that zebrafish β -tectorin is specifically expressed in the zebrafish inner ear, and is important for regulating the development of the zebrafish inner ear. Lack of zebrafish β -tectorin caused severe defects in inner ear formation of otoliths and function.

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Introduction

The zona pellucida (ZP) was first discovered as a glycoprotein surrounding the plasma membrane of an oocyte, and it is important in fertilization. The ZP domain is a sequence shared by many extracellular proteins with diverse functions from structural components to receptors. Among these proteins are the mammalian ZP1, ZP2, and ZP3, and non-mammalian egg-coating proteins, Tamm-Horsfall protein (THP), glycoprotein (GP)-2, α - and β -tectorins, transforming growth factor (TGF)- β receptor III, endoglin, deleted in malignant brain tumor (DMBT)-1, no-mechanoreceptor potential-A (Nomp A), Dumpy, and cuticlin-1 [1]. Each of these ZP-containing proteins is composed of a signal sequence driving these proteins to the endoplasmic reticulum (ER), and each possesses a ZP domain of approximately 260 amino acids long that is comprised of 8~10 conserved cysteine residues, a C-terminal, a hydrophobic transmembrane-like region, and a short cytoplasmic tail [2,3]. ZP domain-containing proteins are highly conserved among all species and are often glycosylated [4]. They are generally modified with a variable number of high-mannose type, N-linked oligosaccharides in the ER. These proteins can be further modified by the addition of O-linked oligosaccharides and by processing of high-mannose-type,

N-linked oligosaccharides to the complex type when transferred to Golgi apparatuses. ZP domain-containing proteins are often present in filaments and/or matrices which play important roles in protein polymerization [1].

In the inner ear organ of Corti, the tip of hair cell stereocilium bundles is covered by a gel-like matrix called the tectorial membrane. The mammalian tectorial membrane is formed by 3 different collagens (types II, V, and IX) combined with 3 non-collagenous, glycosylated polypeptides, called α -tectorin, β -tectorin, and otogelin [1]. The tectorial membrane is a particular structure that deflects the stereocilia of hair cells during sound-triggered vibrations of the basilar membrane, and hair cells facilitate the transduction of sounds into neural signals. α - and β -tectorins belong to the ZP domain-containing protein family, and mutations in α -tectorin or β -tectorin were reported to result in human nonsyndromic deafness. Studies of the human α -tectorin gene, *TECTA*, showed that it is related to dominant forms of prelingual, nonprogressive deafness: DFNA8 (MIM601543) and DFNA12 (MIM601842) [5] or a recessive form at locus DFNB21 [6]. On the other hand, *TECTB*, which encodes β -tectorin, also plays an important role in maintaining the normal function of the tectorial membrane. Previous studies on knockout mice reported that the structure of the striated-sheet matrix is disrupted and

cochlear tuning is sharpened in *TECTB*^{-/-} mice [7]. Together, both types of tectorins are important for maintaining normal auditory function of the inner ear.

Currently, only mammalian and chicken tectorins have been identified and cloned [8]. Little is known about zebrafish β-tectorin and its role during embryonic development. Zebrafish have many benefits as a model animal for studying genes involved in inner ear development: first, zebrafish eggs develop *in vitro*; second, the zebrafish ear is transparent for the first few weeks of life; and third, forward genetic screens and antisense technology are well-established [9]. The zebrafish inner ear system consists of semicircular canals, otoliths, and different sensory patches that are formed by special type of cells, called hair cells. The semicircular canals are attached to the sensory epithelium called cristae which are located at the base of each canal. Cristae are important for sensing the position of the head and angular acceleration. Polycrystalline masses called otoliths are connected to 2 or more macular organs, called saccules and utricles [9]. Zebrafish is considered to be a fish with excellent hearing in the teleost family due to the function of Weberian ossicles which connect the swim bladder to the saccule allowing sound amplification [10,11]. Although, the structure of the zebrafish inner ear greatly differs from that of the mammalian inner ear due to the lack of a cochlea, the convenience in handling it and the evolutionarily conserved molecular mechanisms of inner ear development make zebrafish a good animal model for studying development of the inner ear.

In order to investigate the role of ZP domain-containing proteins in zebrafish inner ear development, we predicted and identified a zebrafish ZP domain-containing protein, zebrafish β-tectorin, through a bioinformatics method, and designed a specific morpholino to knock down the expression of zebrafish β-tectorin. We also characterized the temporal and spatial expressions of zebrafish β-tectorin by whole-mount *in situ* hybridization, and demonstrated that it is specifically expressed in the zebrafish inner ear in the early stages of development. Morpholino knockdown of β-tectorin expression in zebrafish resulted in abnormal inner ear development. Otoliths of β-tectorin morphants showed delocalization or a fused pattern. Taken together, we propose that the zebrafish β-tectorin plays an important role in the proper formation of zebrafish inner ear components, and therefore a lack of it will cause serious defects in development of the inner ear.

Results

Cloning of β-tectorin from zebrafish

The overall deduced amino acid sequences of zebrafish β-tectorin respectively showed 49%, 50%, 50%, and 49% identities to those of human, mouse, chicken, and *Xenopus* β-tectorin (Fig. 1). The zebrafish β-tectorin protein contains a conserved ZP domain, which has highly conserved cysteines of C1 to C8, and Cx, Cy, Ca, and Cb. A signal peptide of 16-amino acids, MAAVGLF-FILLPVTWA, in the NH₂-terminal was predicted by the online software, SignalP 3.0 (<http://www.cbs.dtu.dk/services/SignalP/>), which likewise occurs in mammalian β-tectorin proteins. Mammalian β-tectorin proteins have a signal peptide of 17 amino acids. A hydrophobic C-terminus characteristic of proteins that are membrane bound via a putative GPI-anchor was reported in avian and mammalian β-tectorins [12,13]. Moreover, human β-tectorin is a glycoprotein and contains 4 N-linked sugar chains on Asn80, Asn104, Asn116, and Asn145. Similarly, the zebrafish β-tectorin protein contains 4 N-linked glycosylation sites of ⁷⁷NHS, ¹⁰⁴NDS, ¹¹⁶NYT, and ¹⁴⁵NGS (Fig. 1).

Genomic structure of the zebrafish β-tectorin gene

We then used the 1542 bp of zebrafish β-tectorin cDNA (with accession no. FJ374270) to perform an online BLAST search of the GenBank database. The zebrafish β-tectorin cDNA matched 10 non-contiguous regions of a 92,355-bp zebrafish BAC clone, CH73-92E20 (GenBank accession no. CU462848). Subsequently, a BLAST 2-sequence comparison of BAC CH73-92E20 with the zebrafish β-tectorin cDNA indicated that β-tectorin cDNA contained 10 putative exons and 9 introns spanning at least 8.6 kb (Fig. 2). Using these putative exons as a model, a sequence alignment was produced such that each intron concurred with the GT/AG intron donor/acceptor site rule [14]. Exon 1 contained the 5'-UTR, while exon 2 contained the putative translation initiation site. Exon 2 contained 9 bp of the 5'-UTR and 66 bp of the first coding sequences of β-tectorin cDNA. Exon 10 contained the last 69 bp of the coding sequences and 362 bp of 3'-UTR. The size of the introns considerably varied, ranging 81 (intron 8) to 2419 bp (intron 9).

Comparison of the exon-intron organization of zebrafish and mouse β-tectorin genes indicated that their genomic structures were similar with 10 exons and 9 introns. The mouse β-tectorin gene spanned approximately 15.4 kb. In addition, the average intron size of the mouse β-tectorin gene (1411 bp) was larger than that of the zebrafish β-tectorin gene (799 bp) (Fig. 2).

Expression profiles of zebrafish β-tectorin messenger (m)RNA in adult tissues and embryos at different developmental stages

Expression levels of zebrafish β-tectorin transcripts in adult tissues and embryos from different developmental stages were examined by an RT-PCR analysis. A pair of primers was used to amplify a DNA fragment that spanned exons 2 to 4 to avoid genomic DNA interference in the PCRs. The amplified product of this pair of primers was about 1208 bp long. As shown in Fig. 3A, a high level of β-tectorin expression was detected in the brain, with moderate expression in the kidneys and less in the intestines.

During embryogenesis, β-tectorin transcripts were not detected at 1 day post-fertilization (dpf), and their expression levels constantly increased thereafter; however, a significant decrease in the expression level was observed at 5 dpf and thereafter (Fig. 3A).

Spatial and temporal expression patterns of zebrafish β-tectorin were further analyzed by whole-mount *in situ* hybridization. During different stages of development, expression of the β-tectorin transcript was specifically detected in the anterior and posterior maculae on both sides of the zebrafish from 48~120 h post-fertilization (hpf) (Fig. 3B, panels a~l). It was interesting to note that the expression of β-tectorin mRNA in the anterior macula was much weaker than that of the posterior macula in 48-hpf embryos. The signals of β-tectorin *in situ* hybridization are restricted to the macula of the inner ear, no signals in other parts of the embryos can be detected in various stages (Fig. 3B, panels c, f, i and l). Through longitudinal section of the zebrafish inner ear at 72 hpf, β-tectorin is both expressed in the hair cells and supporting cells of macula, and the expression pattern resembles the early expression of *pax5* in the macula [15]. The overall expression pattern of zebrafish β-tectorin in the inner ear was quite similar to that of the *Starmaker* gene, which is also expressed in the anterior and posterior maculae on both sides of the inner ear [16].

Abnormal otolith formation in β-tectorin morphants

Morpholino (MO)-mediated knockdown of genes in zebrafish embryos has become a routine and efficient method to provide information about gene function *in vivo* [17]. To examine the

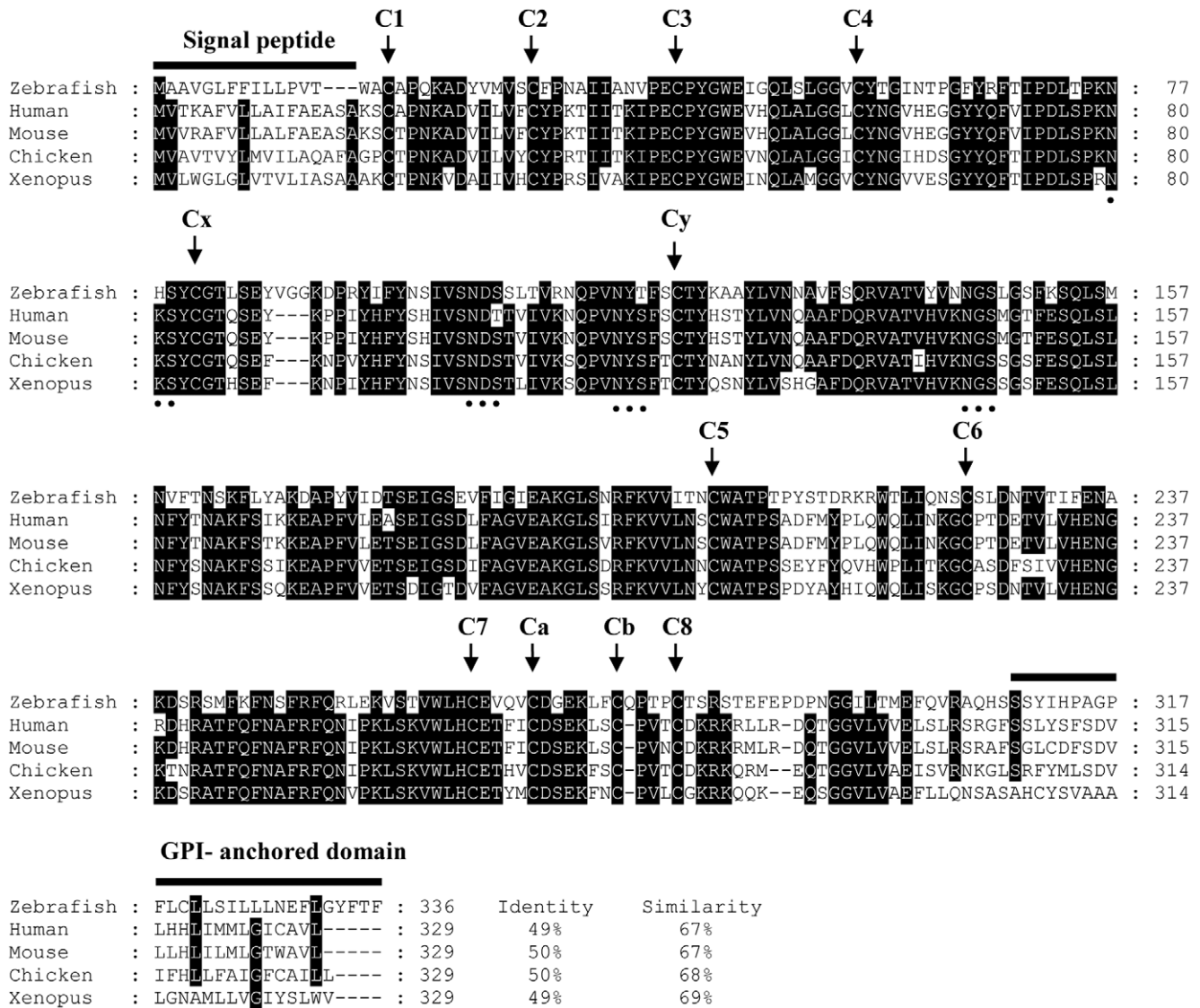


Figure 1. Zebrafish β-tectorin amino acid sequence alignment with those of other species. Deduced amino acid sequences of zebrafish β-tectorin were aligned with those of human, mouse, chicken, and *Xenopus*. All β-tectorin proteins contained a conserved zona pellucida (ZP) domain of approximately 260 amino acids, with 12 highly conserved cysteine residues (indicated by arrows). Identical residues in 4 or 5 proteins are highlighted. Signal peptide and putative GPI-anchored domains are heavily overlined. The putative N-linked glycosylation sites are indicated by dots (...). The accession numbers of each β-tectorin from different species are listed below: human (XM_521604), mouse (X99806), chicken (AAA92461), and *Xenopus* (CAJ82963). doi:10.1371/journal.pone.0023078.g001

function of β-tectorin *in vivo*, we designed zebrafish β-tectorin MOs, which targeted the sequence located at the 5'-UTR of β-tectorin mRNA, to specifically knock down the translation of endogenous β-tectorin mRNA.

To determine the specificity of the MO used, a control approach was used. The 25-bp target sequence of the β-tectorin MO was cloned upstream of the green fluorescent protein (GFP) ORF into a pCMV backbone expression vector (bTec-GFP). As for the control, a target sequence containing 5 mismatches was used (MM b-Tec-GFP). The MM b-Tec-GFP or bTec-GFP plasmid was injected into zebrafish embryos in the absence or presence of a 9-ng β-tectorin MO. Co-injection of bTec-GFP RNA and the β-tectorin MO completely blocked GFP expression ($n = 22/24$; Fig. S1A). Conversely, GFP expression was not affected when 5 bp of the target sequence was exchanged (MM b-

Tec-GFP) ($n = 36/36$; Fig. S1B) indicating the specificity of the β-tectorin MO.

After injecting 4 ng/embryo of β-tectorin MO, we observed abnormal otolith morphology in embryos at 120 hpf. There are 2 otoliths on each side in wild-type (WT) zebrafish embryos; the one in the anterior has a flattened-oval shape, whereas the other one in the posterior, is larger, and has a round shape (Fig. 4A, panel a). β-Tectorin morphants displayed 2 different phenotypes including the fusion of 2 otoliths (Fig. 4A, panel b, $n = 50/168$, 29%; Fig. 4B) as well as a single otolith (Fig. 4A, panel c, $n = 5/168$, 2.9%; Fig. 4B). Changes in morphology of the otoliths were correlated with the irregular formation of the vestibular system in the inner ear; development of semicircular canals seemed to be affected in β-tectorin morphants when observed at 72~120 hpf. β-Tectorin morphants with either fused or single otoliths were

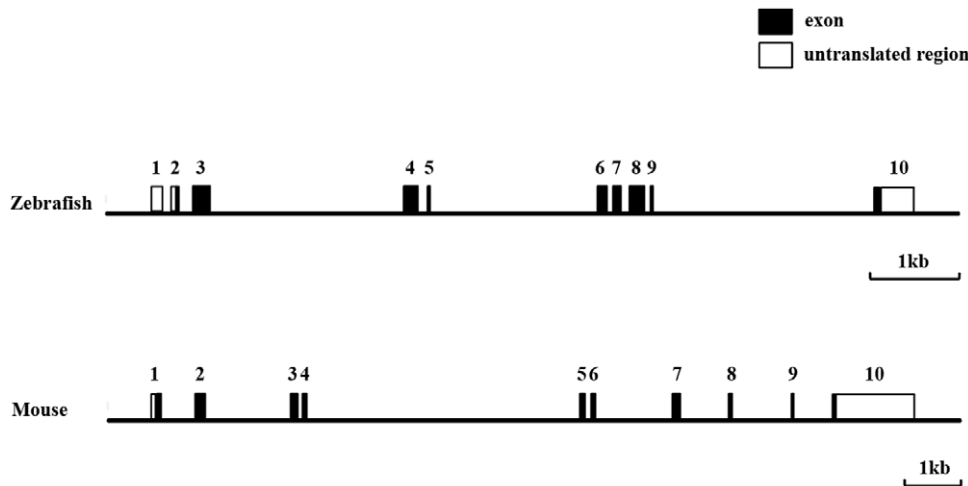


Figure 2. Genomic organization of zebrafish and mouse β -tectorin genes. Coding regions are shown as filled boxes numbered from 1 to 10 in both zebrafish and mouse β -tectorin genes. The 5'- and 3'-untranslated regions are shown as open boxes, while introns and 5'- and 3'-flanking regions are indicated by solid lines. doi:10.1371/journal.pone.0023078.g002

observed with abnormal semicircular canal outgrowth (Fig. 4A, panels e, h, f, and i, arrows), whereas the control showed 2 normal otoliths and outgrowth of the semicircular canals (Fig. 4A, panels d and g).

We also generated splice MOs to block splicings of the β -tectorin sequence within exon 2 and exon 3 (supplementary Fig. 2A). The splice MO 1 which blocks a splicing donor site locates at the boundary of Exon 2 and intron 2 while the splice MO 2 targets the acceptor site at the boundary of intron 2 and Exon 3. Each of the splice MOs was used individually, producing abnormal phenotypes similar to those of the ATG MOs, differing only in efficiency (data not shown). The combination of the two splice MOs is more efficient in generating abnormal phenotypes that include both the fused and single otolith phenotype like the ATG MO. The efficiency of the splice MO mixture was analyzed by RT-PCR (supplementary Fig. 2B). After injecting a mixture of splice MOs 2 ng each, we observed the appearance of the fusion of 2 otoliths (n = 113/287, 39.37%; Fig. 4B) and single otolith (n = 7/287, 2.43%; Fig. 4B) in the resulting morphants. There were no other severe morphological defects in the ATG MO (supplementary Fig. 3). Gradient increases in the amount of ATG MO injected into the embryos also showed increases in the abnormal phenotypes in a dosage-dependent manner (Fig. 4B).

To rule out the possibilities that the phenotypes of these morphants are the results of off-target effect of the morpholino used [18], the p53 MO was used in co-injecting with ATG MO into embryos and the phenotypes of these embryos were observed. The percentage of abnormalities in the β -Tectorin morphants coinjected with p53 MO was approximately equal to that of β -Tectorin morphants (Fig. 4B), suggesting that the phenotypes of these morphants were not related to off-target effect of the morpholino.

To further confirm the specificity of gene knockdowns by the β -tectorin MO, mRNA rescue was performed. Full length β -Tectorin mRNAs, which were synthesized in vitro and injected into embryos in one- or two- cell stage, were used to investigate the rescue of β -Tectorin morphants. Misexpressions of the β -Tectorin mRNA have no effect on morphologies of the control embryos without MO injection. Coinjection of 8 ng ATG MO with ~100 pg β -Tectorin mRNA into each embryo resulted in a

reduction in the percentage of the β -Tectorin morphant with abnormal ear phenotypes, fused otoliths (n = 12/35, 34%) and single otolith (n = 3/35, 8.5%), whereas injection of 8 ng ATG MO alone showed a percentage of as high as 84% abnormalities in the inner ear, fused otoliths (n = 35/45, 78%), single otolith (n = 3/45, 6%) (Fig. 4B). These results demonstrated that the β -Tectorin mRNA can rescue defects in β -Tectorin morphants, validating the specificity of β -Tectorin MO.

Development of the inner ear was affected in the β -tectorin MO morphants as shown by whole-mount *in situ* hybridization

To further analyze inner ear defects observed in morphants, whole-mount *in situ* hybridization was first performed with *Starmaker (stm)*, which was reported to regulate the growth, shape, and crystal lattice of otoliths [15]. In control MO-injected zebrafish, the *stm* transcript was expressed in anterior and posterior maculae on both sides at 96 hpf (Fig. 5B, lateral, panel a, dorsal panel a'). However, the *stm* transcript was less expressed in the anterior macula of β -tectorin morphants either with fused otoliths or a single otolith compared to control MO-injected zebrafish (Fig. 5B, panels b, b' c, and c'; arrows). Otolith matrix protein 1 (*omp-1*) is important for otolith growth and correct anchoring of otoliths to the maculae [19]. The expression pattern of *omp-1* in β -tectorin morphants was also reduced in the anterior macula compared to the control, and its distribution seemed to differ from that of control MO-injected zebrafish (Fig. 5C). On the other hand, another gene marker, *zona pellucida-like domain-containing protein-like 1 (zpDL1)* (Genbank accession no. XM_00192195), was used to label anterior, lateral, and posterior cristae of otoliths of control MO-injected zebrafish at 96 hpf (Fig. 5D, panels a and b). In β -tectorin morphants with fused otoliths, the *zpDL1* signal was detected only in the anterior and posterior cristae of the inner ear, and the signal in the lateral crista was lost (Fig. 5D, arrow).

FM1-43, a fluorescent dye, is known for labeling hair cells of the inner ear by entering the mechanotransduction channels [20]. This function of FM1-43 allows us to monitor the formation of active hair cells in the morphants. For this purpose, zebrafish at different stages were injected with FM1-43 dyes specifically in

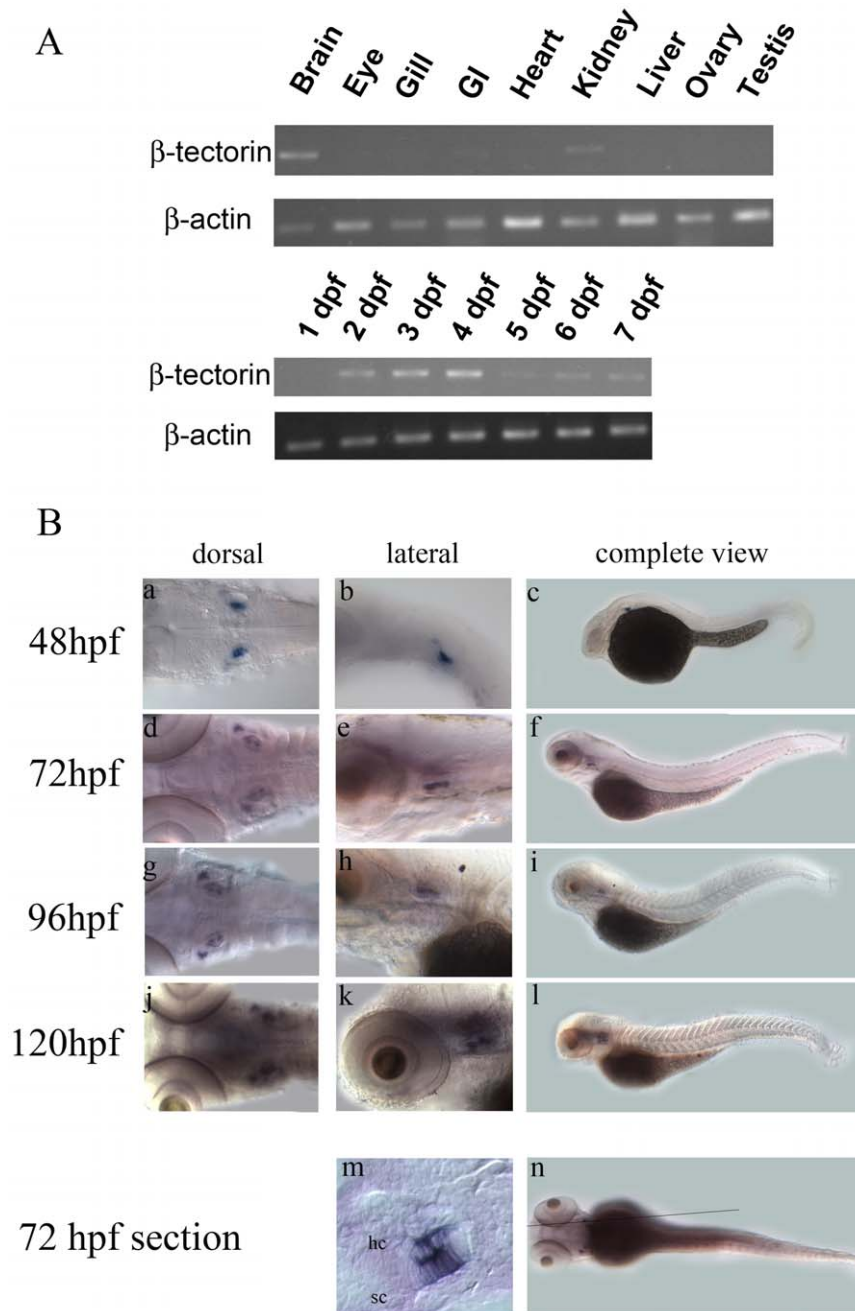


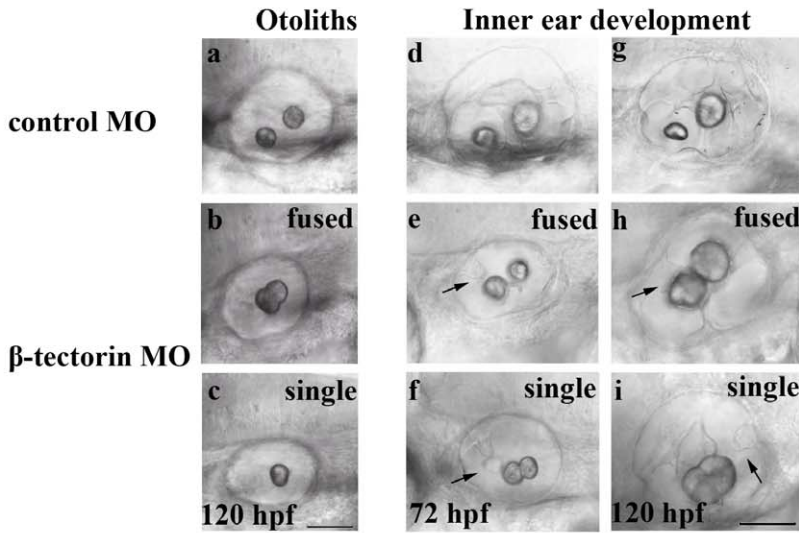
Figure 3. Expression profiles of zebrafish β -tectorin mRNAs by RT-PCR and whole-mount *in situ* hybridization. (A) RT-PCR of the β -tectorin transcript was performed using a pair of primers to produce a DNA fragment of 1208 bp. β -Actin bands were also used to normalize the amount of cDNA prepared from different tissues and embryos at different developmental stages. (B) Whole-mount *in situ* hybridization with antisense β -tectorin at different developmental stages was performed. The images were taken from the dorsal (a, d, g, j) and the lateral view (b, e, h, k), and complete lateral view (c, f, i, l) with the anterior to the left and dorsal to the top. Longitudinal sections of the embryo were at 72 hpf with anterior to the left and dorsal to the top (panel m). The straight line in panel n represents the region of sections in panel m. hc, hair cell; sc, supporting cell.
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lumen of the otic vesicle through an injection tube. FM1-43 dyes, which were taken up by macula and cristae, could be easily observed under a confocal fluorescence microscopy [20]. ATG MO-injected zebrafish embryos were further injected with FM1-43 dyes at 72 hpf to investigate whether the functions of these hair cells are affected. β -tectorin morphants with fused otoliths of 72 hpf had lost its lateral crista as compared to control zebrafish.

These results are consistent with the data gained from whole-mount *in situ* hybridization, as signals of *zpDL1* lost in the lateral crista in the β -tectorin morphants.

Taken together, the altered expression patterns observed in ear-marker genes indicated that β -tectorin may play an important role in both otolith and inner ear formation during zebrafish development.

A



B

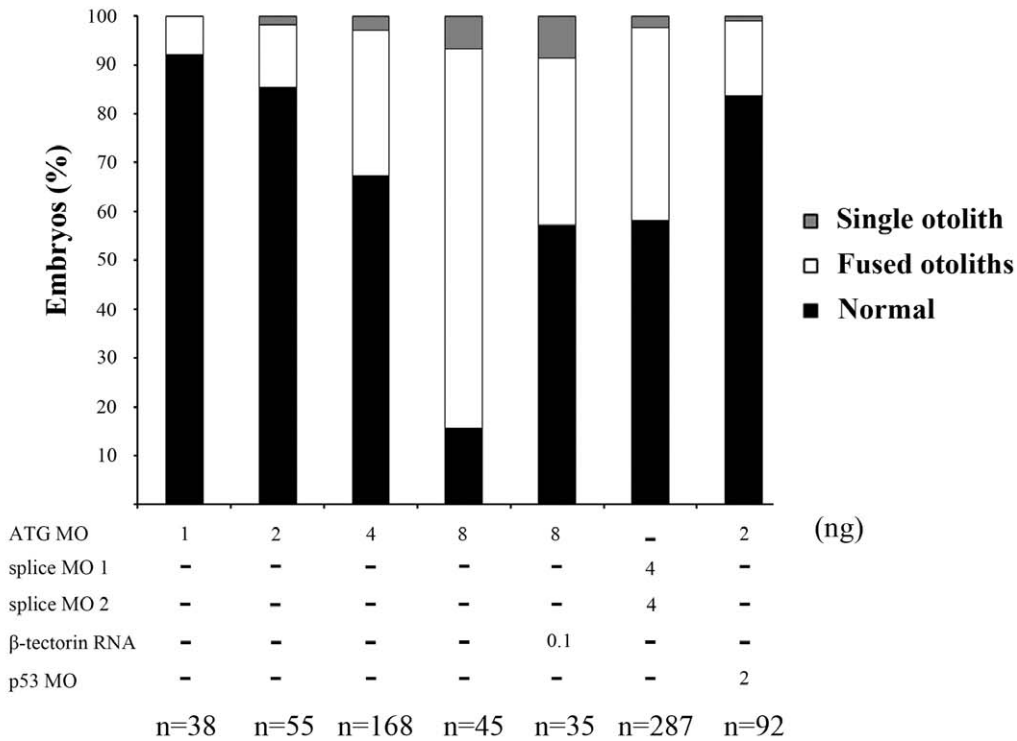


Figure 4. Abnormal otolith phenotypes in β -tectorin morphants. (A) The otolith phenotypes of β -tectorin morphants are classified into normal (normal, panel a), fused (fused, panel b) and single otoliths (single, panel c). Abnormal development of the vestibular system is shown by arrows in β -tectorin morphants from 72 to 120 hpf (panels d to i). (B) The percentage of abnormal otolith phenotypes in zebrafish embryos injected with different β -tectorin MOs or combined with β -tectorin mRNAs or p53 MOs. All samples are observed at 72 hpf. Bars, 50 μ m. doi:10.1371/journal.pone.0023078.g004

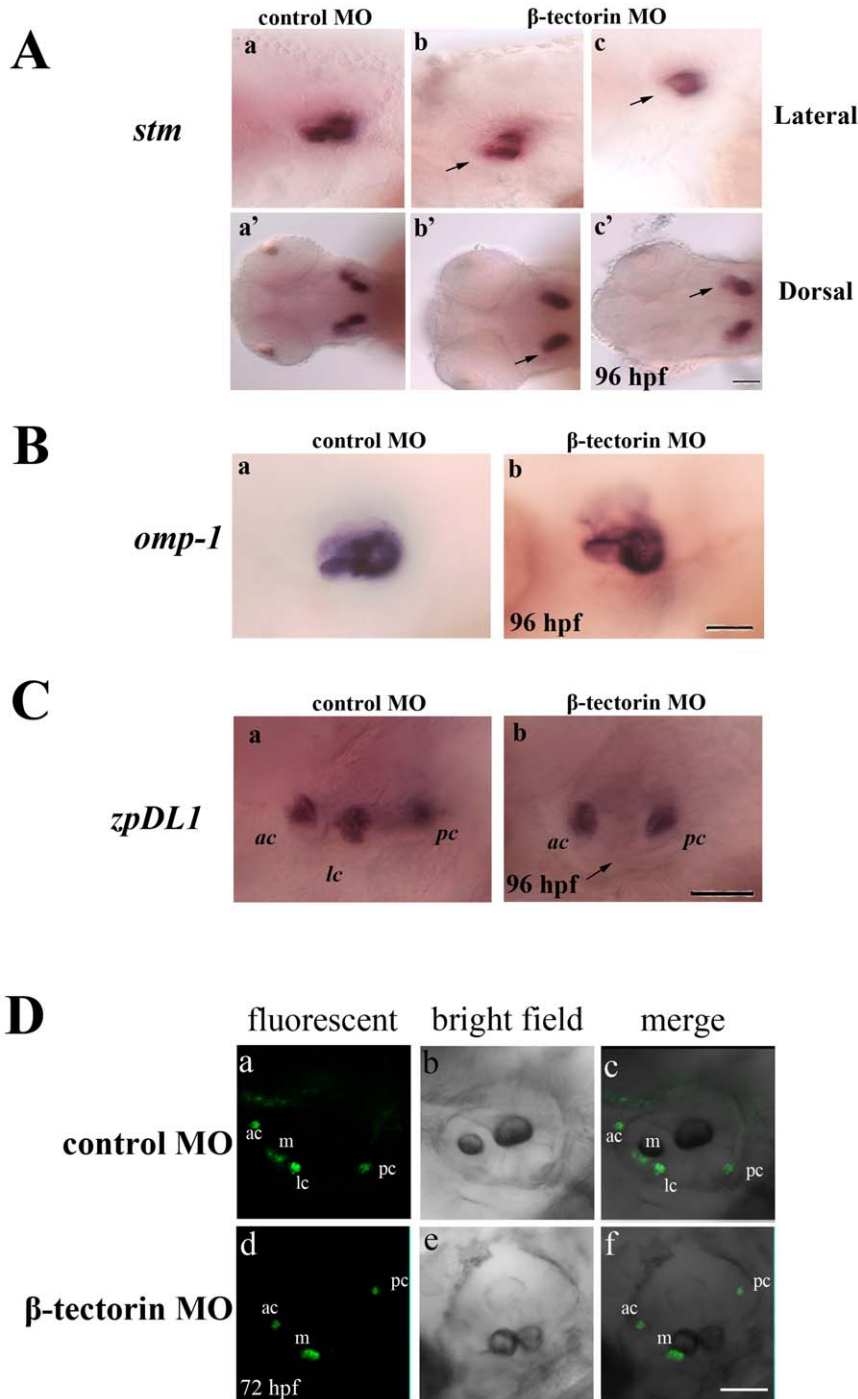


Figure 5. Characterization of ear defects in β -tectorin morphants. The expression levels of the following inner ear marker genes, such as starmaker (*stm*) (A), otolith matrix protein 1 (*omp-1*) (B), and zona pellucida-like domain-containing protein-like 1 (*zpDL1*) (C), were examined by whole-mount *in situ* hybridization in β -tectorin morphants. *Stm* signals in the anterior macula (am) of β -tectorin morphants decreased or even disappeared in fishes with either fused (panels b and b') or single otoliths (panels c and c'), as indicated by arrows. *zpDL1* signals in the lateral crista (lc) of β -tectorin morphants vanished. Black bars, 100 μ m (D) Confocal microscopy image analysis of β -tectorin morphants injected with FM1-43 dyes into the otic vesicle at 72 hpf. After injection, hair cells in anterior crista (ac), lateral crista (lc), macula (m) and posterior crista (pc) of control MO-injected embryos can take up FM1-43 dyes. White bar, 50 μ m.
doi:10.1371/journal.pone.0023078.g005

Behavioral defects in β -tectorin morphants

Altered swimming behaviors and a lack of balance are indices of abnormal ear function, for example, swimming in a corkscrew or circular path [21]. Those β -tectorin morphants with either fused otoliths or a single otolith were further tested for their ability to

maintain balance and swim after stimulation. At 5 dpf, about 40% of noninjected embryos (WT) and control MO-injected embryos (N = 70) displayed floating in an upright position and sometimes swam spontaneously in random directions. Another 45% of control MO-injected embryos remained lying down on the bottom

of the Petri dish. In order to test whether these 5-dpf zebrafish could respond to vibration, several short vibrations were created by deploying an ultrasonic processor in the water, and all zebrafish swam away immediately (Video S1). The remaining 15% of control MO-injected embryos were further stimulated using a glass tube to touch the head of a zebrafish, and they swam away in a straight manner (Video S3). On the other hand, β -tectorin morphants with single or fused otoliths showed a failure to maintain balance, and all stayed at the bottom of the Petri dish. About 10% of those β -tectorin morphants responded to short vibrations created by the ultrasonic processor in the water with short irregular movements ($n = 70$) (Video S2). The remaining β -tectorin morphants with fused otoliths were stimulated on the head with a glass tube about 5 times, and they either did not respond to the stimuli or swam a very short range in a circular path (Fig. 6, B1 to B4) (Videos S4 and S5). β -Tectorin morphants with a single otolith had similar behavioral defects as those with fused otoliths described above. Some swam in a corkscrew path which implied profound defects (Fig. 6, A1 to A4) (Videos S6 and S7).

Discussion

In this study, the zebrafish β -tectorin gene and its cDNA were cloned and characterized. The cDNA encodes a protein of 336 amino acids, which displays 49% and 50% identities to human and chick β -tectorins. RT-PCR analyses showed that zebrafish β -tectorin mRNA was primarily expressed in the brain with moderate expression in the kidneys. Whole-mount *in situ* hybridization showed that expression of the β -tectorin transcript was specifically found in the anterior and posterior maculae of the ear. Similar to human β -tectorin, zebrafish β -tectorin contains 4 N-linked glycosylation sites (Fig. 1). Knockdown of zebrafish β -tectorin expression caused the fusion of 2 otoliths or there was only a single otolith, both of which led to severe malfunction of the inner ear.

The predicted amino acid sequence of zebrafish β -tectorin exhibited overall identities of 49%, 50%, 50%, and 50% to β -tectorins from the human, mouse, chick, and *Xenopus* (Fig. 1). However, chick and mouse β -tectorins were homologous with 75% identity at the amino acid level [13,22]. The higher similarity in identity of chick β -tectorin to the human and mouse compared to zebrafish may be due to differences in habitat, terrestrial and aquatic, respectively. This suggests that the environment may influence the evolution of this molecule. If fish β -tectorins from different species were compared, these fish β -tectorins might display higher identities to each other. Indeed, zebrafish β -tectorin showed higher identities of 74%, 76%, and 74%, with 85% similarity, to β -tectorin from Tetraodon, fugu, and medaka (Fig. S4).

In this study, zebrafish β -tectorin contained a highly conserved ZP domain, which was a sequence of approximately 260 amino acid residues with 8 or 10 cysteine residues and was located at the C-terminus (Fig. 1). Many ZP domain-containing proteins with various functions were found in vertebrates [1]. Some of those proteins constitute the extracellular coat of animal eggs, such as ZP1, ZP2 and ZP3. They are responsible for egg/sperm recognition as well as for blocking polyspermy [23]. Other proteins like α - and β -tectorins are 2 major components of the tectorial membrane, which is an extracellular matrix covering the sensory epithelia of the cochlea of the inner ear [24]. In transgenic mice with a specific mutation in α -tectorin, the structure of the tectorial membrane is disrupted leading to hearing loss [25]. Similarly, mice lacking β -tectorin have sharpened cochlear tuning leading to low-frequency hearing loss [7]. Interestingly, zebrafish β -tectorin is not expressed as a tectorial membrane in the cochlea; instead, it is expressed in anterior and posterior maculae of the

zebrafish ear (Fig. 3B), which is similar to the expression of *Starmaker* mRNA [16]. MO knockdown of β -tectorin expression affected otolith formation in zebrafish larvae (Fig. 4A). These β -tectorin morphants showed a failure to maintain balance and float. Only a few β -tectorin morphants (10%) were able to respond to a vibration created by an ultrasonic processor in the water, while most of them continued to lie on the bottom of the Petri dish (Video S2). In addition to their responses to short vibrations, β -tectorin morphants with either fused or single otoliths also showed abnormal swimming patterns after tactile stimulation (Videos S5 and S7). These phenotypes suggest that zebrafish β -tectorin has crucial roles in the development and function of the zebrafish inner ear.

Some extracellular matrix proteins are reported to play important roles in the development of the zebrafish inner ear. For instance, MO knockdown of specific genes like *omp-1* and *otolin-1*, which respectively encode otolith matrix protein 1 and a collagen-like protein [19], also showed abnormal otolith formation and impaired swimming behaviors. The *omp-1* MO resulted in a reduced otolith size, while *otolin-1* MO caused fusion of the 2 otoliths. Therefore, *omp-1* was proposed to play important roles in normal otolith growth, while *otolin-1* is involved in stabilizing the otolith matrix. In this study, β -tectorin morphants also showed similar fused otoliths, but the zebrafish β -tectorin is not a collagen-like protein. Therefore, both *otolin-1* and β -tectorin may interact with each other and polymerize into the otolith matrix.

In addition, the expression patterns of *starmaker* and β -tectorin mRNAs were similar in the inner ear and in anterior and posterior maculae of the ear as shown in Figs. 3B and 5A. The zebrafish *Starmaker* protein is a 66-kD protein that is enriched in strongly acidic amino acid residues and 35% proteins, and is also extremely hydrophilic [16]. During zebrafish development, the *Starmaker* protein is required for otolith biomineralization, and *starmaker* morphants showed starry or chunky otoliths with improper balance in freely swimming zebrafish larvae. Expression of the *starmaker* transcript was slightly reduced in the anterior macula in zebrafish β -tectorin morphants with fused otoliths. However, in zebrafish β -tectorin morphants with a single otolith, a large portion of the *starmaker* signal was lost in the anterior macula. Interactions among *omp-1*, β -tectorin, and *Starmaker* proteins might be the foundation of proper otolith formation.

We also studied the expression of *zpdLLI* mRNA in zebrafish β -tectorin morphants. *ZpdLLI* can be used as a marker to label the 3 sensory cristae of zebrafish embryos at 4 dpf. As shown in Fig. 5C, *zpdLLI* signals were lost in lateral cristae of zebrafish β -tectorin morphants. These data suggest that zebrafish β -tectorin not only regulates anterior macula formation but is also involved in the morphogenesis of cristae. However, the underlying mechanisms require further investigation.

Materials and Methods

Zebrafish care

Zebrafish embryos were raised at 28.5°C, and different developmental stages were determined based on criteria described in the *Zebrafish Book* [26]. All animal procedures were approved by the Animal Use and Care Committee of Academia Sinica (protocol #10-12-114).

Total RNA isolation and reverse-transcription polymerase chain reaction (RT-PCR) analysis of zebrafish β -tectorin mRNA

Total RNA was isolated from different developmental stages and various tissues of adult zebrafish, using the RNazol reagent

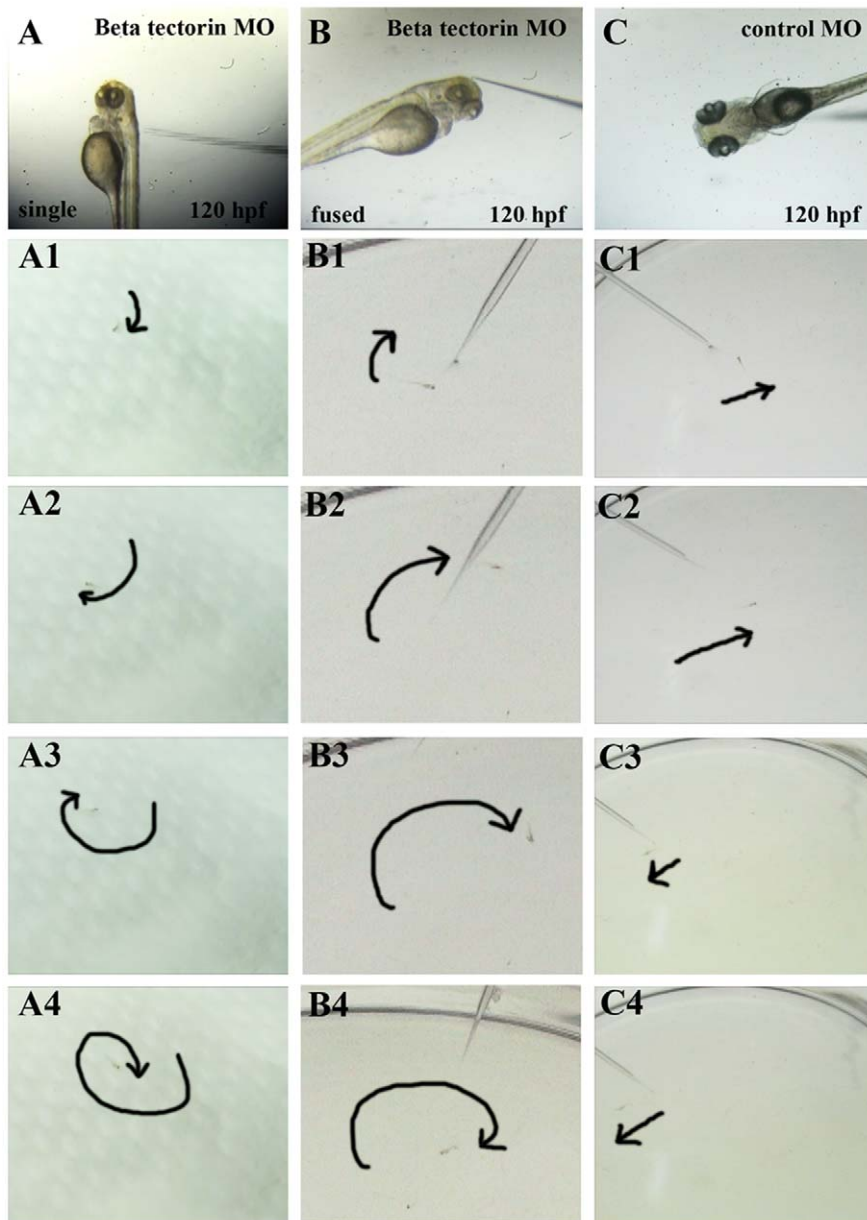


Figure 6. Abnormal swimming behaviors of β -tectorin morphants. β -Tectorin morphants were examined for their abilities to remain balance and react to a stimulus. Tactile stimulation was created by poking a zebrafish on the head with a glass tube: β -tectorin morphants with a single (panel A) and a fused otoliths (panel B), and a control with normal otoliths (panel C). Swimming behaviors of β -tectorin morphants at 5 days post-fertilization under stimulation were recorded with a digital video camera. β -Tectorin morphants with either single or fused otoliths failed to maintain their balance, tended to remain leaning on one side, remained on the bottom (panel A, B), and tended to swim in a corkscrew (panel A, A1 to A4) or circular manner (panel B, B1 to B4). Control zebrafish maintained their balance, had immediate responses to stimulation, and swam in a straight line (panel C, C1 to C4). The trails of the zebrafish movement were illustrated by dark arrows. doi:10.1371/journal.pone.0023078.g006

(Tel-Test, Friendswood, TX, USA) according to the instructions of the manufacturer. After treatment with RQ1 RNase-Free DNaseI (Promega, Madison, WI, USA), 50~100 μ g of total RNA was subjected to the first-strand cDNA synthesis. PCR amplifications were performed with the following zebrafish β -tectorin RT-PCR primers (β -tectorin-RT-F, 5'-GCT GCT GAA GAC CTA CAC AGG AAC-3' and β -tectorin-RT-R, 5'-TGG ATG TAT GCA TGC ATG CGT GTC-3'). Zebrafish β -actin primers (zACT-F, 5'-GTG CTA GAC TCT GGT GAT GGT GTG-3' and zACT-R, 5'-GGT GAT GAC CTG ACC GTC AGG AAG-3') were used for the internal control to amplify a DNA fragment using

cDNA as a template. Primers for examining the efficiency of the splice MOs are as follow (P1, 5'-GCT GCT GAA GAC CTA CAC AGG AAC-3'; P2, 5'-GGC TAA ACA CGG CGT TGT TGA CCA-3').

Cloning of the full-length cDNA encoding zebrafish β -tectorin

To identify zebrafish complementary (c)DNA related to the human β -tectorin gene, we used the coding region of human β -tectorin (accession no. XM_521604) to search GenBank for related expression sequence tags (ESTs) using the tBLAST program and

found some zebrafish EST clones (CN315850 and EG585664) related to human β-tectorin. Using 5'- and 3'-RACE to obtain the 5'- and 3'-untranslated regions (UTRs), we assembled all sequences to obtain 1542-bp cDNA with an open reading frame (ORF) of 1011 bp encoding a protein of 336 amino acid residues. The complete sequence was deposited in GenBank with the accession number of FJ374270.

The full-length cDNA encoding zebrafish β-tectorin was isolated by PCR amplification using gene-specific primers with linkers (β-tectorin-BamH1-F, 5'-CC GGA TCC ATG GCA GCT GTT GGC CTT-3', and β-tectorin-EcoR1-R, 5'-GG GAA TTC AAA AGT AAA GTA TCC TAA-3') according to the sequence submitted with GenBank accession no. FJ374270. Full-length zebrafish β-tectorin was subcloned into the BamH1 and EcoR1 sites of pcDNA3.1-myc to generate pcDNA3.1-β-tectorin-myc. Full length β-tectorin was then further subcloned to T7TS plasmid using BamH1 and Xba1 sites.

Rescue of defects in β-Tectorin morphant by injecting β-Tectorin RNA

Full length β-Tectorin was cloned into T7TS plasmid and synthesized *in vitro*. T7TS-β-Tectorin was linearized to synthesize capping mRNA by using mMESSAGE mMACHINE T7 Kit (Ambion, Foster City, CA, USA). ~100 pg of β-Tectorin RNA was injected into embryos at the one- to two-cell stage.

Morpholino oligonucleotide (MO) injection

Antisense MOs were obtained from Gene Tools (Philomath, OR, USA), and the sequence of zebrafish β-tectorin MO was as follows: 5'-GTG GCA GAA TCC AGA AGA AAT GTT G-3'. The sequence of the two splice MOs used were as follow: splice MO 1 : 5'- AAC CCA TCA AAC ATC TTA CCT CAG A-3' and splice MO 2 : 5'-CCT CCT ACA TAC TGA AAA GAA GGT A-3'. The morpholinos were resolved to 24 μg/μl injection stock, and stored in a -20°C refrigerator. The diluted morpholino was injected into wild-type (WT) zebrafish embryos at the 1~2-cell stage using a microinjection system consisting of an SZX9 stereomicroscope (Olympus, Tokoyo, Japan) and an IM300 Microinjector (Narishige, Tokoyo, Japan). The sequence of p53 MO was as follow (p53 MO: 5'-AAA ATG TCT GTA CTA TCT CCA TCC G-3') [27].

To confirm the specificities of the β-tectorin morpholino, several pCMV-GFP reporter plasmids were created. The morpholino targeted a 25-bp sequence of the PCR by the following primer pairs for the perfect match, bTec-GFP (bTec-GFP-F, 5'- GAT CCC AAC ATT TCT TCT GGA TTC TGC CAC G-3' and bTec-GFP-R, 5'-AAT TCG TGG CAG AAT CCA GAA GAA ATG TTG G-3'). For a 5-base exchanged mismatch, MM-b-Tec-GFP was used (MM-b-Tec-GFP-F, 5'-GAT CCG AAG ATT ACT TCT GCA TTC TGG CAC G-3' and MM-b-Tec-GFP-R,5'-AAT TCG TGC CAG AAT GCA GAA GTA ATC TTC G-3'). The 5' region of the zebrafish β-tectorin mRNA was fused to the N-terminal of the GFP protein. Either construct bTec-GFP or MM-b-Tec-GFP was co-injected with zebrafish β-tectorin morpholino, and the fluorescence was analyzed by a fluorescent microscope at 48 hpf.

Whole-mount *in situ* hybridization

Digoxigenin-labeled RNA probes (Roche, Penzberg, Germany) were generated by *in vitro* transcription using the linearized pGEM-T-easy plasmids (Promega, Madison, WI, USA) carrying the 3'-UTR of the following zebrafish genes. Whole-mount *in situ* hybridization was performed following a previously described protocol [28]. Specific primers for *stm* (stm-F, 5'- GAA TCA ACT

GAG ACA GTC AAG ATA ACC -3' and stm-R, 5'- TGA GAG TGG AGA GCG GGA ATT ATC TGC - 3'), *zpDL1* (zpDL1-F, 5'-GCG GGA CAT CAG TGT GTA TTG TGG AGT TCA -3' and zpDL1-R, 5'- GCA AGC TGT GTG TTG TTG ACC AGG TAT TCC -3'), and omp-1 (zomp-1-F, 5'- CAC ACT ACA GTC TTT GAC AAC ATG - 3' and zomp-1-R, 5'- CAT CAG ATC AAC ACA AAC CTT CAC - 3') were used to amplify the 3'-UTR of each gene. Primers used in the RT-PCR of zebrafish β-tectorin were also used to make the zebrafish β-tectorin probe.

FM1-43 labeling of hair cells

Labeling the hair cells in the inner ear with 40 μM of FM1-43 (N-(3-triethylammoniumpropyl)-4(4-(diethylamino)styryl) pyridinium dibromide), (Invitrogen, Carlsbad, CA, USA) dissolved in the extracellular solution. An injection tube was used to inject FM1-43 into the otic vesicle following the protocols described previously [29]. The formula of extracellular solution is described as follow, 134 mM NaCl, 2.9 mM KCl, 1.2 mM MgCl₂, 2.1 mM CaCl₂, 10 mM HEPES, and 10 mM glucose, and was adjusted to pH 7.8.

Video recording of the swimming behavior of zebrafish β-tectorin morphants

Zebrafish β-tectorin morphants were recorded with a digital video camera (Sony DCR-PC120 digital camera, Tokyo, Japan) at 5 dpf to examine their reactions to short vibrations and tactile stimulation. Short vibrations were created by a Hielscher Up50H ultrasonic processor (Hielscher, Teltow, Germany), at 30 kHz and 50 W with an amplitude of 30% and 0.5 s per cycle. Tactile stimulation was created using a glass tube to touch the head of a zebrafish, and instantaneous responses were recorded with a digital camera. Zebrafish β-tectorin morphants and control MO-injected zebrafish were touched on the head at least 5 times for each test. The swimming behavior of the zebrafish was observed and defined by whether the fish swam in a straight or circular manner.

Supporting Information

Figure S1 Control experiments for morpholino specificity. To determine the specificities of the morpholinos used, pCMV-GFP reporter plasmids containing a perfect (bTec-GFP) or mismatched (MM-b-Tec-GFP) MO target sequence were employed. Both bTec-GFP (A) and MM-b-Tec-GFP were co-injected with the β-tectorin MO. All images were taken from zebrafish embryos at 48 h post-fertilization. (TIF)

Figure S2 The splice MO targeting and RT-PCR analysis of β-tectorin mRNAs of embryos injected with splice MOs. (A) The exon-intron genomic structure from exons 1–4 was shown. Splice MO 1 and MO 2 target the donor and acceptor sites, respectively. (B) Total RNAs were extracted from control MO (C) and splice MO1/MO2-injected (MO) embryos at 72 hpf, then RT-PCR was performed. Primers (P1/P2) flanking the region resulted in a single 500 bp band in the case of control embryos. On the other hand, in the case of morphants, the level of this band was strongly reduced and a second 1800 bp band was visible. The second band resulted from the use of an alternative splice donor. β-Actin bands were used to normalize the amount of cDNA prepared from both embryos. (TIF)

Figure S3 The morphology of β-tectorin morphants. The ATG MO injected zebrafish embryos with fused (a, b), and single

otoliths (c, d) appeared to be normal without obvious defects. All photographs were taken at 72 hpf. (TIF)

Figure S4 Zebrafish β-tectorin amino acid sequence alignment with other fish species. The deduced amino acid sequences of zebrafish β-tectorin were aligned with those from Tetraodon, fugu, and medaka. Identical residues in 3 or 4 proteins are highlighted. The accession numbers of each β-tectorin from different fish species are listed below: Tetraodon (GenBank, accession no: CAG06543), fugu (ensembl no: ENSTRUP00000021095), and medaka (ensembl no: ENSORLP00000014650). (TIF)

Video S1 Control MO-injected zebrafish responded to vibrations made with an ultrasonic processor. Control MO-injected zebrafish at 5 days post-fertilization (dpf) with normal otoliths were placed in a Petri dish, and a transient vibration was generated with a Hielscher Up50H ultrasonic processor, at 30 kHz and 50 W with an amplitude of 30% and 0.5 s per cycle. (WMV)

Video S2 The β-tectorin morphant responded to the vibration made with an ultrasonic processor. The β-tectorin morphant at 5 days post-fertilization with either single or fused otoliths were placed in a Petri dish, and a transient vibration was generated with a Hielscher Up50H ultrasonic processor. (WMV)

Video S3 Response of control MO-injected zebrafish to touch with a glass tube. Control MO-injected zebrafish at 5 days post-fertilization with normal otoliths was touched on the head with a glass tube at least 5 times, and its swimming behavior was observed. The bright-field videomicrograph was taken with an Olympus IX70-FLA inverted fluorescence microscope equipped with a Sony DCR-PC120 digital video camera. (WMV)

Video S4 Response of a β-tectorin morphant with fused otoliths to touch with a glass tube. The β-tectorin morphant at 5 days post-fertilization with fused otoliths was touched on the head with a glass tube at least 5 times. The bright-field videomicrograph was taken with an Olympus IX70-FLA inverted fluorescence microscope equipped with a Sony DCR-PC120 digital video camera. (WMV)

Video S5 Swimming behavior of a β-tectorin morphant with fused otoliths. The β-tectorin morphant at 5 days post-fertilization (dpf) with fused otoliths was touched on the head with a glass tube at least 5 times, and its swimming behavior was observed. The video was taken with a Sony DCR-PC120 digital video camera. (WMV)

Video S6 Response of a β-tectorin morphant with a single otolith to touch with a glass tube. The β-tectorin morphant at 5 days post-fertilization with a single otolith was touched on the head with a glass tube at least 5 times. The bright-field videomicrograph was taken with an Olympus IX70-FLA inverted fluorescence microscope equipped with a Sony DCR-PC120 digital video camera. (WMV)

Video S7 Swimming behavior of a β-tectorin morphant with a single otolith. The β-tectorin morphant at 5 days post-fertilization with a single otolith was touched on the head with a glass tube at least 5 times, and its swimming behavior was observed. The video was taken with a Sony DCR-PC120 digital video camera. (WMV)

Author Contributions

Conceived and designed the experiments: C-HY C-JH. Performed the experiments: C-HY C-HC G-DC W-HL Y-CC K-YH. Analyzed the data: C-HY C-HC G-DC Y-CC C-JH. Contributed reagents/materials/analysis tools: C-HC G-DC P-PH S-PLH. Wrote the paper: C-HY C-JH.

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