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斑馬魚內耳中含有 ZP domain 蛋白的基因表現與特性分析

Expression and characterization of ZP domain-containing proteins in zebrafish ear

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本論文係 楊仲翔 君（D96B46002）在國立臺灣大學生化科學研究所完成之碩（博）士學位論文，於民國 100 年 7 月 26 日承下列考試委員審查通過及口試及格，特此證明。

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_____ 黃銓珍 (指導教授)

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中文摘要

Zona pellucida（透明帶)為包覆在卵外面的一個聚合結構，其功能是避免精子重複進入卵中而特化出來的。多年來的研究顯示，哺乳類的卵上的透明帶是由特殊的蛋白所構成，分別是 ZP1，ZP2 以及 ZP3。將這些蛋白進一步的比對分析其胺基酸序列可以發現到一個高度保留的 ZP domain（透明帶結構域)，而且研究也顯示這個區域與蛋白的功能息息相關。透明帶結構域已知是許多細胞外蛋白質所共同擁有的特殊構造之一。

β-tectorin 為 336 個胺基酸所組成，並帶有一個高度保留的 ZP domain(透明帶結構域)， 它是內耳中 tectorial membrane("覆膜")的主要成分之一: 覆膜在內耳中專司將聲音訊號轉換為神經訊號，為一個細胞外構造並與內耳中的聽毛細胞連結在一起。我的研究著重於分析 β-tectorin 這一類帶有透明帶結構域的蛋白質，是否在斑馬魚的內耳發育上扮演著重要的角色。首先，藉由生物資訊學的方法分析斑馬魚的基因資料庫並成功取得了斑馬魚 β-tectorin 的胺基酸序列。進一步比對分析，發現斑馬魚的 β-tectorin 與其他物種有接近相似度以及保有一段在哺乳類以及鳥禽類 β-tectorin 所保留的透明帶結構域。利用斑馬魚原位雜交法以及反轉錄酵素聚合酶連鎖反應，分析 β-tectorin 在時間和空間上的表現，發現 β-tectorin 專一的表現在內耳的感覺器官 macula 中。由 morpholino 來 knockdown β-tectorin 的表現，會影響發育的過程中內耳的耳石位置和數量，並且影響到內耳內部構造的型態。觀察這些耳石異常的斑馬魚可以發現牠們在游泳的行為也有所異常。

除了 β-tectorin 的研究外，我們也研究了另一個帶有透明帶結構域的 zpDL1 蛋白。同樣地藉由生物資訊學的方法分析斑馬魚的基因資料庫取得了 zpDL1 蛋白的序列。由於關於 zpDL1 蛋白的研究尚未有任何文獻報導，首先利用了原位雜交法探測 zpDL1 的表現位置。zpDL1 專一地表現在內耳的感覺器官 crista，呈現出三個點狀。觀察我們所建構以啟動子 (promoter)穩定表現綠色螢光蛋白的斑馬魚亦可以得到與原位雜交法一樣的結論。而這樣的 *Tg(zpDL1:GFP)*有助於我們觀察感覺器官 crista 的發育，或者是進行以雷射進行破壞 crista 進而觀察 hair cell

是否能再生的實驗。

我的研究結果顯示了斑馬魚β-tectorin 蛋白專一的表現在內耳的 macula 並且在斑馬魚內耳發育扮演重要的角色。一旦缺乏 β-tectorin 將會導致內耳發育上的缺失，耳石形成和功能上的異常。此外，從 zpDL1 研究的也顯示了 zpDL1 表現在另一種內耳內的感覺器官 crista 的 hair cell 中，意謂著 ZP domain 蛋白與這些感覺器官的功能息息相關。整體而言，我的研究成果有助於了解含有 ZP domain 蛋白在內耳中所扮演的角色。

Abstract

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 one component of a gel-like structure called tectorial membrane which is involved in transmitting sound wave into neuronal signals and is important for normal auditory function. β-tectorin is specifically expressed in mammalian and avian inner ear. We identified and cloned the gene encoding zebrafish β-tectorin. Through whole-mount *in situ* hybridization, we demonstrated that *β-tectorin* mRNA was expressed in the otic placode and in the specialized sensory patch of the inner ear during zebrafish embryonic stages. Analysis by western blot and immunofluorescence staining suggested that β-tectorin may undergo some posttranslational modifications. Morpholino knockdown of zebrafish β-tectorin affected the position and number of otoliths in the ear of morphants. Finally, the swimming behaviors of β-tectorin morphants were abnormal since the development of inner ear was compromised.

In addition to studies of β-tectorin, we had also studied another ZP domain containing protein, zpDL1. Through bioinformatics method, we predicted and identified zpDL1. Since zpDL1 has not been reported in any literature yet, we first characterized its expression by whole-mount *in situ* hybridization. The expression profile of zpDL1 displayed a specific pattern in the three crista of the inner ear, with an appearance correlated to the onset of crista emerging (about 48 hpf to 72 hpf). A transgenic *Tg(zpDL1:GFP)* line was generated using 2 kilo bps promoter region upstream the start codon to drive GFP (green fluorescent protein) expression. Specific expression of GFP in the crista will be used to label the hair cells in the crista and further applied in hair cell ablations by intense laser beam.

Our results reveal that zebrafish β-*tectorin* is specifically expressed in the zebrafish inner ear, specifically in the macula, and is important for regulating the development of zebrafish inner ear. Lack of zebrafish β-*tectorin* caused severe defects of inner ear, formation of otoliths and function. Furthermore, zpDL1, another ZP domain containg protein, also showed expression pattern in hair cells related sensory organ, crista. Taken together, our works contributed to better knowledge of the role of ZP domain containing proteins in the zebrafish inner ear.

Abbreviations

ac	anterior crista
am	anterior macula
bps	base pairs
cDNA	Complementary deoxyribonucleic acid
dpf	day past fertilization
ER	endoplasmic reticulum
hpf	hour past fertilization
kDa	kilodalton
mc	medial crista
mRNA	Messenger RNA
pc	posterior crista
pm	posterior macula
RT-PCR	Reverse transcription-polymerase chain reaction
TM	Tectorial membrane
ZP	zona pellucida

Introduction

ZP domain containing proteins

The zona pellucida (ZP) was first discovered and recognized as a structural element by Bork & Sander in 1992[1]. This specialized ZP domain was originally identified in glycoproteins that are components of the extracellular matrix, zona pellucida, surrounding the mammalian oocyte. The Zona pellucida contains glycoproteins, mammalian ZP1, ZP2 & ZP3 which are receptors responsible for inducing sperm acrosome reaction, forming interconnected fibrilous structures around the oocyte. The association of these proteins relies on a common motif called ZP-domain[1,2,3]. Since then, various proteins are known containing the ZP domain and involved in different function, i.e., being expressed in differentiated epithelia and neural tissues.

The ZP domain is found in various proteins, such as previously mentioned mammalian ZP1, ZP2, and ZP3. Among others are the non-mammalian egg-coating proteins, Tamm-Horsfall protein (THP)/uromodulin, 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 [2]. The ZP domain containing proteins have diverse functions. For examples, the Tamm-Horsfall/uromodulin protein (THP), a major glycoprotein secreted by the human kidney, is known to prevent urinary tract infection[4]. Owing to their propensity to form a gel, THP filaments may ensure the water impermeability of the thick ascending limb of Henle's loop[5]. Whereas, Glycoprotein (GP)-2, a major zymogen granule membrane protein in pancreatic tissue, is reported to be involved in the formation and secretion of zymogen granules or binding scavenger receptors [6,7].

As for α- and β-tectorins , they are the main components of the avian and mammalian tectorial membrane, an extracellular matrix in the organ of Corti[2,8,9]. Transforming growth factor-*β* , TGF-*β* receptor type III (TGFR3), or betaglycan, are the most abundant TGF-*β* binding proteins at the cell surface. In mammals, TGFR3 seems to be essential for restructuring of blood vessels during angiogenesis [10]. Endoglins, which is structurally related to TGFR3, are involved in angiogenesis, cardiovascular development and vascular remodeling[11].

ZP domain-containing proteins are highly conserved among all species and are often glycosylated [1]. They are generally modified with a variable number of high-mannose type, N-linked oligosaccharides in the ER (endoplasmic reticulum). 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. However, there are still few ZP domain containing proteins lacking both N- and O-linked oligosaccharides[12]. Therefore, even though glycosylation is a modification common to the majority of ZP domain proteins, its extent and relevance in terms of protein structures and functions can differ significantly[2].

Functions of ZP domain containing proteins

It was first proposed in 1995 that the ZP domain might play a role in polymerization of ZP domain–containing proteins into filaments and/or matrices[13,14]. An assay was developed at that time. Epitotpe-tagged recombinant ZP glycoproteins were examined for whether they can be integrated into ZP of the mouse oocytes[15]. By this assay, ZP domain together with the N-terminal signal peptide and C-terminal propeptide are both necessary and sufficient for assembling nascent protein into ZP

ZP domain-containing proteins, often present in filaments and/or matrices, play important roles in protein polymerization [2]. Ultrstructural studies of mouse egg ZP proteins and other ZP domain containing proteins demonstrated that ZP domain containing proteins are fibrillar in nature [2,16]. The ZP domain is a frequently found module in extracellular proteins that polymerize into higher-order structures, such as filaments and matrices.

Structure of ZP domain containing proteins

The ZP domain consists of ~260 amino acids (aa) and 8 conserved cysteine (Cys) residues that participate in intramolecular disulfides forming [17] (Appendix Fig. 1A). In addition, most ZP domain copntaining proteins are glycosylated and possess an N-terminal signal peptide and either a C-terminal putative transmembrane domain (TMD) or glycosyl phosphatidylinositol-(GPI-) anchor(Appendix Fig. 1). ZP domain containing proteins usually are composed of a signal sequence that drives the protein to the endoplasmic reticulum (ER) [1] [17,18].

ZP domain containing proteins often form long interconnected fibrils or filaments that exhibit a repeated structure[16,19]. Furthermore, the ZP domain is actually a module consisting of two domains, called ZP-N (N-terminal) and ZP-C (C-terminal), separated by a short linker region. The ZP-N domain, in some cases, seems sufficient to form polymers *in vitro* in some proteins[2].

Additional motifs or domains between the N-terminal signal peptide and the ZP-domain are present in many ZP-domain proteins. These domains include plasminogen activator N-terminal (PAN), Epidermal growth factor (EGF and EGF-like), von Willebrand factor (vWA), scavenger receptor cysteine rich (SRCR), and entactin domains[2]. Mutations in these additional domains may also affect the activity of these ZP domain proteins[20,21]. Therefore, although the ZP-domain has

an important role in the activity of ZP domain proteins, additional domains and sequences may also contribute to the functional diversification of ZP domain proteins, as well as the modifications of the juxtamembrane extracellular region.

Tectorial membrane

The Tectorin membrane is a specialized extracellular matrix that takes the role in converting sound vibrations into neuronal signals. It is a gel-like matrix that located in the inner ear organ, namely the organ of Corti, in the cochlea of inner ear (Appendix Fig. 2). The tectorial membrane is coupled to the hair cells and attached to the tip of hair cell stereocilium bundles. In higher vertebrates, when the sound waves travel to the middle and inner ear, the acoustic energy is then transferred to mechanical energy by the ossicles (Known to consist of three bones--the hammer, anvil and stirrup). Stirrup converts the vibration of sound to the fluid-filled cochlea of the inner ear (Appendix Fig. 2). The organ of corti is responsible for transferring the vibration of sound into a neuronal transduction signals that are sent to the brain. The organ of corti is composed of three canals, the upper canal (vestibular), the middle canal (cochlear duct) and the lower canal, and filled with endolymph (Appendix Fig. 2). Since the tectorial membrane is attached to the stereocillia bundles of the hair cells, sound vibration can cause a distortion in basilar membrane that bends and deflects the stereocillia in the fluid filled canal. The fluid is comprised of endolymph that rich in potassium ions and the influx of ions will trigger depolarization of hair cells, thereby, excitating them and then transmitting the signals to the brain through VIIIth auditory nerve.

The mammalian tectorial membrane (TM) 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 [2]. The TM is composed of several

genetically distinct types of collagen, collagen Types II, V, IX and XI and three non-collagenous glycoproteins, α-tectorin (Tecta), β-tectorin (Tectb) and otogelin [2-8]. Tecta, Tectb and otogelin are proteins that are only expressed at high levels in the inner ear and they account for ~50% of the protein present in the TM. The cDNA sequences for otogelin and Tecta predict large, modular glycoproteins (313 and 239 kDa respectively), whilst the sequence for Tectb encodes a much smaller protein (36 kDa). As mentioned in previous text, both *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. For example, 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) [20] or a recessive form at locus DFNB21 [22]. On the other hand, the *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 [23]. Taken together, both types of tectorins are important for maintaining normal auditory functions of the inner ear .

Beta tectorin

Beta tectorin was identified as a glycoslyated ZP domain containing protein in the vertebrates[13]. It is exclusively expressed in the inner ear and formed a part of tectorial membrane. Along with alpha tectorin, beta tectorin is believed to form the complex extracellular structure via its ZP domain. Beta tectorin double knockout mice displayed dysfunction of tectorial membrane structure and defects in normal cochlear function[23]. The expression of Beta tectorin is downregulated on a the doubly knockout mice that lacks midkine and pleiotrophin genes; suggesting that these

growth factor or cytokine are important for regulating Beta tectorin expression[24]. In addition, mice deficient in the thyroid hormone supply also displayed reduced beta tectorin expression which leads to malfunctions of the tectorial membrane[25]. Taken together, Beta tectorin is important in maintaining the normal function of tectorial membrane. Even though many studies had been done in mice and chickens, few information regarding Beta tectorins is found in the zebrafish.

Introduction to the zebrafish inner ear anatomy

The zebrafish inner ear is composed of a complex vestibular system possessing many special features. These features are three semicircular canals and two or more macular organs[26]. At the end of each semicircular canal is a round mound of sensory epithelium called the crista. The crista is specialized in providing information about the position of the head and angular acceleration; movements of the head caused the fluid inside canals to impinge and deflect the kinocilia bundles of the hair cells that are within the crista, transmitting the information to the brain by depolarizing the hair cells.

The early zebrafish larva possesses two macular organs called the saccule (posterior macula) and utricle (anterior macula). Besides, a third macular organ, lagena, will form at the juvenile stage. Otoliths are calcium carbonate crystals serving as mechanoreceptors which stimulate the kinocillia of the hair cells lying in the macular organ (Appendix Fig. 3) When the body moves, otoliths are also moved and deflects the underlying hair bundles [26]. The hair bundles in turn transmit the information to the brain, which allows the animal to sense gravity and linear acceleration. Previous reports show that a mutant fish with dysfunction in forming utriclar otoliths and semicircular canals has deficiency in the gravity sensing [27].

In lower vertebrates, such as frogs and fish, macular organs are also important

for hearing [28]. Fish can manipulate their saccular organs (posterior macula) to "hear" frequencies between 10–4000 Hz. Some fish can be classified as experts in hearing due to presence of a series of bones known as Weberian ossicles that connect the swim bladder to the saccule. Sound sets the air-filled bladder into motion and this motion is transmitted to the sensory epithelium through ossicles and thereby amplifying the sound. The zebrafish is considered as hearing specialist.

Basically, the organization and morphology of the inner ear neuroepithelium or hair cells in fish resembles those of higher vertebrates[29]. In addition to hair cells in the inner ear, fish possess other sensory organs called neuromasts, or lateral lines. Therefore, the zebrafish becomes an excellent model in deciphering the mechanisms of genetic hearing loss.

The development of zebrafish inner ear

At the mid-somite stage, an ectodermal thickening, called the otic placode, develops the ear [30] . The otic placode is visible on either side of the hindbrain. The main components of the larval zebrafish ear includes five sensory epithelium, two maculae and three cristae. Sensory patches are thickened regions of epithelium containing two major cell types,i.e., hair cells and supporting cells. It is known that the supporting cells surround and isolate hair cells from one another[29].

Thickenings forof the maculae do not appear until hair cells begin to differentiate. The ventral floor of the ear becomes thicker than the dorsal roof of the vesicle at first . The first hair cells of the utricular (anterior) and saccular (posterior) maculae appears at the rostral and caudal ends, respectively. [31] The zebrafish provides an important model for vertebrate inner ear developments. The otic placode becomes visible at approximately 16 hours and forms a vesicle with a lumen by cavitation at approximately 18 hours. Two otoliths appear in the lumen by 19.5 hours, and at about

24 hours when the first sensory hair cells are seen, they are grouped in two small patches, one beneath each otolith, corresponding to future maculae. Otoliths attached to the kinocilia around 20 hpf [32].Staining the hair cells with fluorescent phalloidin, reveals about 10-20 hair cells in each macula around 42 hours. Between 3 days and 7 days the numbers increase to about 80 per macula[31]. At around 24 hours, visualization of neurons of the statoacoustic ganglion can be done by staining with HNK-1 antibody. It is shown that the neuronal precursors originate by delamination from the ventral face of the otocyst; with the culmination of delamination takes place between 22 hours and 30 hours. Between 42 hours and 72 hours, the system of semicircular canals formed by outgrowth of protrusions from the walls of the otocyst forms pillars of tissue spanning the lumen. 3 further clusters of hair cells also become visible in this period, forming the 3 cristae [31]. As a result, all key components of the ear are appears by the end of the first week. The following growth includes developments of thousands more hair cells and additional neurons derived probably from proliferation of ganglion neuronal precursors. Even though the timetable is different from species to species, the fundamental principles of inner ear developments in the zebrafish seem to be similar to those in other vertebrates[31].

The zebrafish is considered 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 [28,30]. 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.

Genes involved in inner ear development of zebrafish

The otic induction is the first step of inner ear development in zebrafish. Transplantation experiments in very early stage demonstrated that the hindbrain tissue is the source of inductive cues for otic placode development[33]. The candidate genes involved inducting of otic placode are the fibroblast growth factors (Fgfs), evident by the fact that they are expressed in the region next to the otic placode. Several laboratories discovered that *fgf3* and *fgf8* are required for otic induction[34,35,36]. Since then, targets of the *fgf3* and *fgf8* signaling dependent and independent pathway are also examined; The *Sox9a*, regulated by the *fgf3* and *fgf8* signaling pathway, is showed to rescue the absence of otic placodes in the mutant zebrafish line Df^{b380}[37]. Other factors involved in otic induction such as the *dlx3b* and *dlx4b* were also reported, however, in a *fgf3* and *fgf8* independent manner.

The paired boxtranscription factors, Pax2 and Pax8, are also downstream targets of Fgf signaling[38]. Loss of *pax2a* or *pax8* alone results in abnormally small ears. Further studies also concluded that Foxi1, which resides upstream of *pax8* and regulates the expression level of *pax8,* also takes part in otic placode development[38,39]. In addition, Dlx3b was shown to regulate the expression of *pax2a*. The development of otic placode is blocked upon removal of both *foxi1* and *Ddlx3b*.

Morphogenesis of the otic vesicle can be affected by mutations in some genes. Studies of zebrafish mutants revealed such genes, for example, the *jekyll* mutant, with defects in the inner that the protrusion of semicircular canals is affected. The gene mutated in the *jekyll* mutant is the Ugdh [uridine 5'-diphosphate (UDP)-glucose dehydrogenase], an enzyme that is crucial for production of the extracellular matrix component hyaluronic acid (HA) [40]. Disorganized epithelial columns are also a

prominent feature of *dog-eared/eya1* mutants. Disruption of *eya1* affects the survival of sensory neural precursors [41]. Furthermore, Hedgehog pathway mutants, *chameleon* (con(tf18b)) and *slow muscle omitted* (smu(b641)) showed disorganized canals and abnormal ear morphology, exhibiting a partial mirror image duplication of anterior otic structures, concomitant with a loss of posterior otic domains [42].

Hair cell function in zebrafish

The hair cells are presented in two systems in zebrafish, i.e., the lateral line system and the inner ear. The overall morphology of these hair cell types closely resemblse that seen in the vestibular hair cells of other vertebrates. Genes required for normal hair cell functioning in the zebrafish are associated with auditory defects in mice and humans, thus suggesting the function conservations of of these genes. In higher vertebrates, sound is transferred into a force that deflects the hair bundles if hair cell in the cochlear. The deflection of hair bundles by vibrations triggers the opening of mechanically gated ion channels, influx of endolymph that containing high concentration of potassium ions will cause hair cell depolarizations[43].

FM1-43 Dye

FM1-43 is a small styryl dye, a vital fluorescent dye (used to stain living cells), that can be used to label sensory hair cells in the inner ears and the lateral lines (neuromasts) of the vertebrates. FM1-43 enters hair cells through the tips of stereocillia; Blockades of the mechanically gated transduction channels will also block the entry of FM1-43 into hair cells[44]. FM1-43 can be uptaken by hair cells as early as 27 hpf, approximately the time when macular hair cells appear. Therefore, FM1-43 can be used to label vital and functional hair cells.

Morpholino Oligos knock-down

As a common reverse genetic method used in zebrafish, the microinjection of zebrafish embryos with morpholino oligos, is an effective and specific way to knock-down gene expressions. Morpholino oligos are small fragment of DNA homologs binding to target RNAs and forms DNA-RNA hybrid that interrupt the normal function of target RNA. The use of mophorlino oligos provides a non-toxic and efficient technique to study the physiology of a target gene [45].

Purpose of the study

In this study, we aimed to investigate the role of ZP domain-containing proteins in the inner ear development. We used zebrafish as animal models due to easy handling as well as high reproducibility. The inner ear of zebrafish is transparent during the first two weeks of development, which make it a much more advantageous for observing and tracing under a light microscope. This study was intended to set up a model of ZP domain containing proteins involving in the morphogenesis of fish inner ear.

Specific Aims

(1) To predict and identify the two zebrafish ZP domain-containing protein, zebrafish β-tectorin and zpDL1 through bioinformatics method. We also aim to compare identity and similarity of their amino acid sequence with other species via protein alignments.

(2) To characterize the expression profile of these proteins through RT-PCR and whole-mount *in situ* hybridization. These results will provide information about the possible functions of the ZP domain containing proteins.

(3) To perform functional assay by designing specific morpholino oligos to knock-down the expression of the ZP domain containing proteins; zebrafish β-tectorins and analyse its role involved in the development of zebrafish inner ears. The swimming behavior of the morphants will also be tested.

(4) To determine the possible promoter of zpDL1 and establish a *Tg(zpDL1:GFP)* stable line to evaluate hair cell regenerations after ablationds.

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* [46]. 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 (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 μg 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'.)

Cell cultures

Monkey kidney fibroblast COS-1 cells were cultured in high-glucose Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (Hyclone, UT, USA) in a humidified atmosphere of 5% CO_2 at 37℃.

In vivo and *in vitro* expression of zebrafish β-tectorin

In vivo expression of zebrafish β-tectorin was performed by transfection of expression plasmids into COS-1 cells. Transfection is conducted by using the PolyJet *In Vitro* DNA Transfecton Reagent (SignaGen Laboratories, Ijamsville, MD, USA) and followed procedures according to the manufacturer's protocol. COS-1 cells transfected either with pcDNA3.1-β-tectorin-GFP or pcDNA3.1-β-tectorin-myc and transfected cells were harvested at 24 hr or 48 hr and fixed with 4% paraformaldehyde and permeabilized in PBS with 0.1% Triton X-100. Western blot analyses were performed with anti-GFP polyclonal antibody (rabbit, 1:3000 dilution) or anti-myc monoclonal antibody (Sigma, m4439, clone 9E10, mouse, 1:3000 dilution). Immunostaining was performed using anti-GFP polyclonal antibody (rabbit, 1:500 dilution) or anti-myc monoclonal antibody (mouse, 1:500 dilution) at 4 °C overnight, followed by incubation with a Cy3-conjugated goat-anti-rabbit antibody or a Cy3-conjugated rabbit-anti-mouse antibody for 30 min and DAPI for 5 min at room temperature. Photo images were prepared using an Olympus IX70-FLA inverted fluorescence microscope (Olympus, Tokyo, Japan) equipped with the SPOT system (Diagnostic Instruments, Sterling Heights, MI, USA).

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') [47].

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 [48]. 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-(dibutylamino)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 [49]. The formula of extracellular solution is described as follow, 134 mM NaCl, 2.9 mM KCl, 1.2 mM $MgCl_2$, 2.1 mM $CaCl_2$, 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.

Cloning of zpDL1 promoter region and establish zebrafish *TG(zpDL1:GFP)* stable line

The 2 kilo bps promoter region of zpDL1 was cloned by PCR using following primers; *zpDL1-Promoter* (zpDL1-P1, 5'- CCT GAA TAC TTA GGA CCA TGT GAT AGT TCA -3' and zpDL1-P2, 5'-TGT ACA GAT TAT TTC AAT TTC AAC ATT ACT -3'), the BAC clone CH211-103F14 was used as template for amplification of the promoter region. The approximate 2 kilo bps PCR product is first cloned to pGEM-T-easy and then subcloned to pITR-GFP vector. Linearization of the vector was done by cleavage using ClaI. To obtain stable transgenic fish, embryos at the one-cell stage were co-injected with 10 ng μl^{-1} linearized pITR-zpDL1-GFP. To generate germline transgenic zebrafish, the injected embryos were raised to adulthood and 3-month-old fish (F0) was crossed to wild-type fish. F1 embryos were examined by fluorescence microscopy to identify germline-transmitted F0 founders. Positive F1 embryos were raised to adulthood and were then screened in the same way to estimate copy numbers of the transgene and to establish stable transgenic lines. Three independent lines (F2 and more advanced generations) were obtained. This line recapitulates the endogenous expression pattern of zpDL1.

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, MAAVGLFFILLPVTWA, in the NH_2-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 [13,14]. 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 ^{77}NHS, ^{104}NDS, ^{116}NYT, and ^{145}NGS (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 [50]. 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).

Molecular charcterization of zebrafish Beta tectorin protein

To confirm whether zebrafish β-tectorin protein undergoes posttranslational modification to become a GPI-anchored protein, zebrafish β-tectorin protein was analysed by *in vitro* transcription and translation and western blot assay. We generated full-length zebrafish *β-tectorin* through PCR amplification and subcloned it into expression vector pcDNA3.1 that contains a cytomegavirus (CMV) promoter for both *in vitro* and *in vivo* expression. We designed two different forms of recombinant zebrafish β-tectorin. One contains a green fluorescent protein (GFP), while the other contains a myc-tag at the C-terminal end of zebrafish β-tectorin. Both forms were able to be transcribed and translated *in vitro* with correct sizes (Fig. 3A). However, when both forms were expressed in COS-1 cells, only zebrafish β-tectorin fused with GFP could be detected by western blot (Fig. 3B). The myc-tagged zebrafish β-tectorin could not be detected (Fig. 3C).

Previous reports have suggested that a putative tetrabasic (KRKR) cleavage site for endopeptidase recognitions is located upstream of the hydrophobic carboxyl tail of the avian and the mammalian β-tectorin [13,14,51]. However, these basic residues are

not present in the zebrafish β-tectorin (Fig. 1). Thus, some questions are raised upon whether zebrafish β-tectorin can be proteolytically processed at its C-terminal tail. .The undetectability of myc-tagged zebrafish β-tectorins in western blot may be the consequences of proteolytic cleavages. Further study using the immunofluorescence staining assay supports this hypothesis. Our immunofluorescence staining data demonstrated that myc-tagged zebrafish β-tectorin was barely detectable as compared to either GFP alone or GFP-tagged zebrafish β-tectorin (Fig. 3D). Based on these results, we hypothesized that a putative cleavage site may be present at the C-terminal of zebrafish β-tectorin and the fusion of GFP proteins hinders the proteolytic cleavage due to their large size. Such a hypothesis requires further experiments to investigate the specific cleavage site.

Expression profiles of zebrafish *β-tectorin* messenger (m)RNAs 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. 4A, 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. 4A).

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. 4B, 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. 4B, panels c, f, i and l). Through longitudal 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 [52]. 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 [53].

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* [54]. To examine the 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. 8) 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. 5A, panel a). *β-Tectorin* morphants displayed 2 different phenotypes including the fusion of 2 otoliths (Fig. 5A, panel b, *n*= 50/168, 29%; Fig. 5B) as well as a single otolith (Fig.5A, panel c, *n*=5/168, 2.9%; Fig. 5B). 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 observed with abnormal semicircular canal outgrowth (Fig. 5A, panels e, h, f, and i, arrows), whereas the control showed 2 normal otoliths and outgrowth of the semicircular canals (Fig. 5A, panels d and g).

We also generated splice MOs to block splicings of the *β-tectorin* sequence within exon 2 and exon 3 (Fig. 9A). The splice MO 1 which blocks a splicing donor site locates at the boundry of Exon 2 and intron 2 while the splice MO 2 targets the acceptor site at the boundry 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 (Fig. 9B). After injecting a mixture of splice MOs 2ng 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. 5B) in the resulting morphants. There were no other severe morphological defects in the ATG MO (Fig. 10). 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 5B).

To rule out the possibilities that the phenotypes of these morphants are the results of off-target effect of the morpholino used [55], 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. 5B), 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. 5B). 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 [53]. In control MO-injected zebrafish, the *stm* transcript was expressed in anterior and posterior maculae on both sides at 96 hpf (Fig. 6A, 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. 6A, 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 [56]. 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. 6B). On the other hand, another gene marker, *zona pellucida-like domain-containing protein-like 1 (zpDL1)* (Genbank accession no. XM_001921951.2), was used to label anterior, lateral, and posterior cristae of otoliths of control MO-injected zebrafish at 96 hpf (Fig 6C, 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. 6C, arrow).

FM1-43, a fluorescent dye, is known for labeling hair cells of the inner ear by entering the mechanotransduction channels [44]. This function of FM1-43 allows us to monitor the formation of active hair cells in the morphants. For this purpose, zebrafish at diffenerent stages were injected with FM1-43 dyes specifically in 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 [44]. 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 (Fig. 6D). 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.

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 [57].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. 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. 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). 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 7, B1 to B4). *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 7, A1 to A4).

Alignment of zebrafish *zpDL1*

Through bioinformatics and data, we obtain zebrafish *zpDL1* mRNA and amino acid sequence. Zebrafish zpDL1 is a protein with 419 amino acid residues. The deduced amino acid sequence of zebrafish zpDL1 (zpDL1 z) showed overall respectively about 63% identities to those of human, mouse and cattle (Fig. 12). The alignment results also showed that zebrafish zpDL1 has an average similarity as high as 75%, compared to zpDL1 of other species. A conserved region of approximately 260 a.a. and conserved cysteine residues were also present.

Expression profiles of *zpDL1*

In order to characterize the expression profile of zpDL1, we conducted whole mount *in situ* hybridization. Probe targeting the specific 3'UTR region of *zpDL1* mRNA was designed. No signals were detected before 72 hpf on any part of the zebrafish embryos. However, strong signals had appeared at the 3 cristae, the sensory organ that are coupled to the semicircular canals, at 72 hpf and 120 hpf (Fig. 13A , panel a to d). The expression of *zpDL1* is unique to the crista in the ear suggest that the ZP domain containing proteins are important in maintaining normal inner ear function, especially related to the sensory hair cells. However, we could not clearly distinguish whether zpDL1 is expressed in the hair cells only or may be also

expressed in the surrounding supporting cells.

Establish a stable line zebrafish; *Tg(zpDL1:EGFP)*

We had cloned the upstream region of zpDL1 ATG codon start site with a size of approximately 2000 bps. Then, we generate a ITR (inverted terminal repeats) -mediated transgene construct pITR-zpDL1-GFP via subcloning [58]. pITR-zpDL1-GFP vector was linearized by restriction enzyme and purified. The purified DNA was microinjected into embryos of one to two cell stage. Embryos expressed the zpDL1 driven green fluorescent protein approximately at 72 hpf, which is consistent with the results of whole mount *in situ* hybridization.(Fig.13B). We had established a stable line harboring this transgene by breeding the offspring that displayed the green flourescence labeled crista (Fig. 13B). This stable line is useful in observation of hair cell regeneration after photosensitizer or high energy laser beam induced ablation. Future perspectives of this stable line remain to be evaluated.

Conclusion and perspective

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 [9,14]. 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. 11).

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 [2]. 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 [59]. 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 [60]. In transgenic mice with a specific mutation in α-tectorin, the structure of the tectorial membrane is disrupted leading to hearing loss [61]. Similarly, mice lacking β-tectorin have sharpened cochlear tuning leading to low-frequency hearing loss [23]. 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. 4B), which is similar to the expression of *Starmaker* mRNA [53]. 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. 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. 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 [56], 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 [53]. 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 *zpDL1* mRNA in zebrafish *β-tectorin* morphants. *zpDL1* can be used as a marker to label the 3 sensory cristae of zebrafish embryos at 4 dpf. As shown in Fig. 5C, *zpDL1* 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.

zpDL1 protein is conserved among many species and yet, information about its function and expression profile is limited. Our results provided a new insight into deciphering the possible function of *zpDL1*. Since, zpDL1 is specifically expressed in the zebrafish inner ear, especially crista, the sensory organ, suggesting a conserved function of ZP domain containing proteins associated with the hair cells. Mutations in this gene may therefoe involved in human hearing loss or other defects related with hair cells. Since, it is already known mutations in human tectorin proteins (*α*-tectorin, *β*-tectorin) are associated with hearing loss[20] [22] [23]. One may think there is a

possible role of zpDL1 involving in the human hearing disorder as well.

We have established *Tg(zpDL1:GFP)* transgenic zebrafish, that specifically labels the 3 cristae during the time while the cristae are developed. This stable line will be useful in observation and tracing the development of cristae. Cristae are the sensory organ that couples to the semicircular canals, loss of cristae may cause serious faults to the perception and swimming behavior of zebrafish. *Tg(zpDL1:GFP)* transgenic line allows us to ablate the GFP positive cells through femtosecond laser pulses under a laser device [62]. Ablation of cristae at early stage of inner ear development by femtosecond laser pulses, can be used to analyse whether or not the development of the zebrafish inner ear is affected. Moreover, regeneration of hair cells after ablation can be studied using this technique, too.

References

1. Bork P, Sander C (1992) A large domain common to sperm receptors (Zp2 and Zp3) and TGF-beta type III receptor. FEBS Lett 300: 237-240.
2. Jovine L, Darie CC, Litscher ES, Wassarman PM (2005) Zona pellucida domain proteins. Annu Rev Biochem. 2005/06/15 ed. pp. 83-114.
3. Wassarman PM, Litscher ES (2008) Mammalian fertilization: the egg's multifunctional zona pellucida. Int J Dev Biol 52: 665-676.
4. Saemann MD, Weichhart T, Horl WH, Zlabinger GJ (2005) Tamm-Horsfall protein: a multilayered defence molecule against urinary tract infection. Eur J Clin Invest 35: 227-235.
5. Serafini-Cessi F, Malagolini N, Cavallone D (2003) Tamm-Horsfall glycoprotein: biology and clinical relevance. Am J Kidney Dis 42: 658-676.
6. Schmidt K, Schrader M, Kern HF, Kleene R (2001) Regulated apical secretion of zymogens in rat pancreas. Involvement of the glycosylphosphatidylinositol-anchored glycoprotein GP-2, the lectin ZG16p, and cholesterol-glycosphingolipid-enriched microdomains. J Biol Chem 276: 14315-14323.
7. Holzl MA, Hofer J, Kovarik JJ, Roggenbuck D, Reinhold D, et al. (2011) The zymogen granule protein 2 (GP2) binds to scavenger receptor expressed on endothelial cells I (SREC-I). Cell Immunol 267: 88-93.
8. Steel KP, Kros CJ (2001) A genetic approach to understanding auditory function. Nat Genet 27: 143-149.
9. Goodyear RJ, Richardson GP (2002) Extracellular matrices associated with the apical surfaces of sensory epithelia in the inner ear: molecular and structural diversity. J Neurobiol 53: 212-227.
10. Bandyopadhyay A, Zhu Y, Malik SN, Kreisberg J, Brattain MG, et al. (2002) Extracellular domain of TGFbeta type III receptor inhibits angiogenesis and tumor growth in human cancer cells. Oncogene 21: 3541-3551.
11. Fonsatti E, Altomonte M, Nicotra MR, Natali PG, Maio M (2003) Endoglin (CD105): a powerful therapeutic target on tumor-associated angiogenetic blood vessels. Oncogene 22: 6557-6563.
12. Brivio MF, Bassi R, Cotelli F (1991) Identification and characterization of the major components of the Oncorhynchus mykiss egg chorion. Mol Reprod Dev 28: 85-93.
13. Killick R, Legan PK, Malenczak C, Richardson GP (1995) Molecular cloning of chick beta-tectorin, an extracellular matrix molecule of the inner ear. J Cell Biol 129: 535-547.
14. Legan PK, Rau A, Keen JN, Richardson GP (1997) The mouse tectorins. Modular matrix proteins of the inner ear homologous to components of the sperm-egg

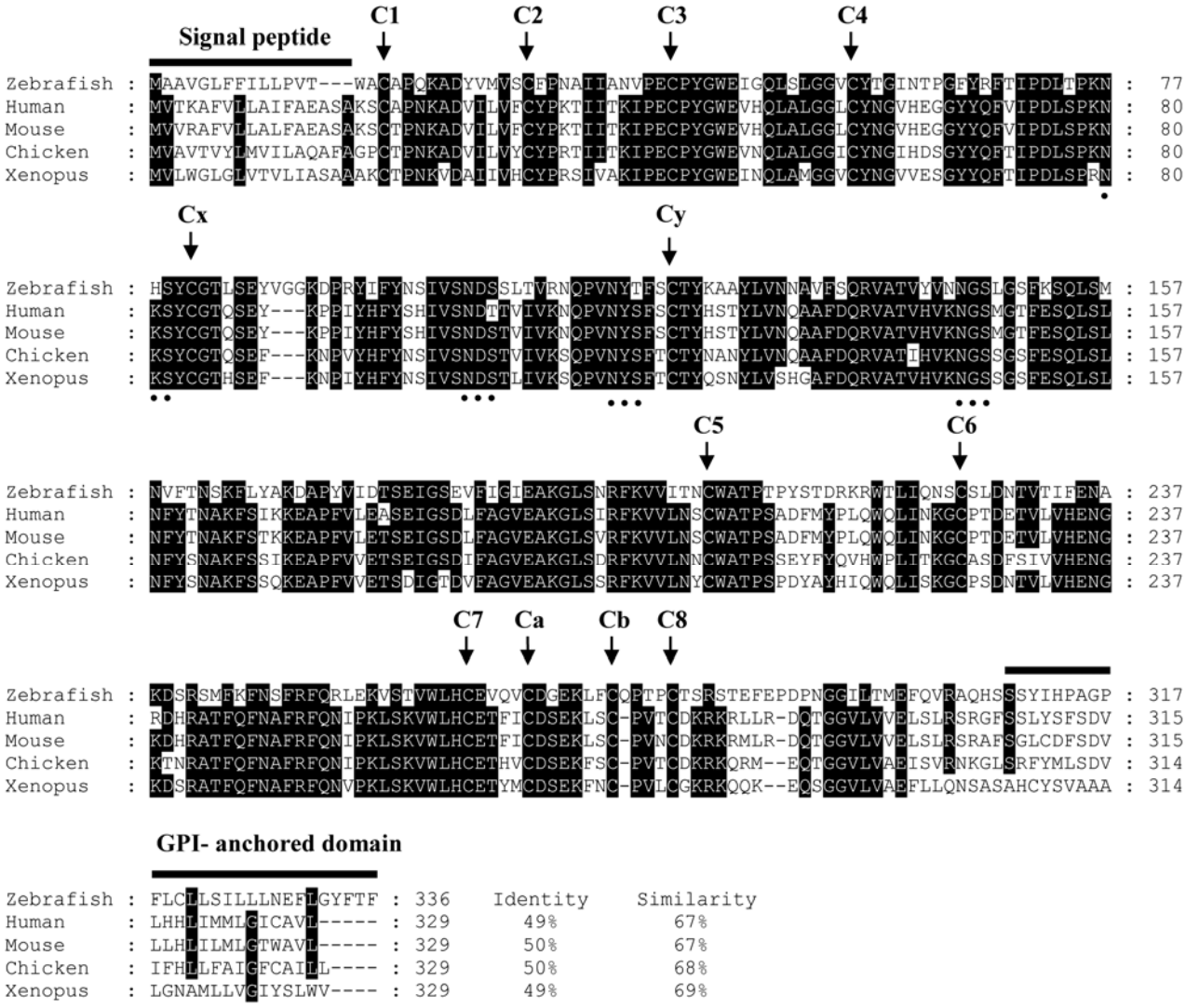
adhesion system. J Biol Chem 272: 8791-8801.
15. Qi H, Williams Z, Wassarman PM (2002) Secretion and assembly of zona pellucida glycoproteins by growing mouse oocytes microinjected with epitope-tagged cDNAs for mZP2 and mZP3. Mol Biol Cell 13: 530-541.
16. Greve JM, Wassarman PM (1985) Mouse egg extracellular coat is a matrix of interconnected filaments possessing a structural repeat. J Mol Biol 181: 253-264.
17. Jovine L, Qi H, Williams Z, Litscher E, Wassarman PM (2002) The ZP domain is a conserved module for polymerization of extracellular proteins. Nat Cell Biol 4: 457-461.
18. Wassarman PM, Jovine L, Litscher ES (2001) A profile of fertilization in mammals. Nat Cell Biol 3: E59-64.
19. Green DP (1997) Three-dimensional structure of the zona pellucida. Rev Reprod 2: 147-156.
20. Verhoeven K, Van Laer L, Kirschhofer K, Legan PK, Hughes DC, et al. (1998) Mutations in the human alpha-tectorin gene cause autosomal dominant non-syndromic hearing impairment. Nat Genet 19: 60-62.
21. Fernandes I, Chanut-Delalande H, Ferrer P, Latapie Y, Waltzer L, et al. (2010) Zona pellucida domain proteins remodel the apical compartment for localized cell shape changes. Dev Cell 18: 64-76.
22. Meyer NC, Alasti F, Nishimura CJ, Imanirad P, Kahrizi K, et al. (2007) Identification of three novel TECTA mutations in Iranian families with autosomal recessive nonsyndromic hearing impairment at the DFNB21 locus. Am J Med Genet A 143A: 1623-1629.
23. Russell IJ, Legan PK, Lukashkina VA, Lukashkin AN, Goodyear RJ, et al. (2007) Sharpened cochlear tuning in a mouse with a genetically modified tectorial membrane. Nat Neurosci 10: 215-223.
24. Zou P, Muramatsu H, Sone M, Hayashi H, Nakashima T, et al. (2006) Mice doubly deficient in the midkine and pleiotrophin genes exhibit deficits in the expression of beta-tectorin gene and in auditory response. Lab Invest 86: 645-653.
25. Knipper M, Richardson G, Mack A, Muller M, Goodyear R, et al. (2001) Thyroid hormone-deficient period prior to the onset of hearing is associated with reduced levels of beta-tectorin protein in the tectorial membrane: implication for hearing loss. J Biol Chem 276: 39046-39052.
26. Nicolson T (2005) The genetics of hearing and balance in zebrafish. Annu Rev Genet 39: 9-22.
27. Mizuno R, Ijiri K (2003) Otolith formation in a mutant Medaka with a deficiency in gravity-sensing. Adv Space Res 32: 1513-1520.

28. Popper AN, Fay RR (1993) Sound detection and processing by fish: critical review and major research questions. Brain Behav Evol 41: 14-38.
29. Hudspeth AJ (1992) Hair-bundle mechanics and a model for mechanoelectrical transduction by hair cells. Soc Gen Physiol Ser 47: 357-370.
30. Whitfield TT, Riley BB, Chiang MY, Phillips B (2002) Development of the zebrafish inner ear. Dev Dyn 223: 427-458.
31. Haddon C, Lewis J (1996) Early ear development in the embryo of the zebrafish, Danio rerio. J Comp Neurol 365: 113-128.
32. Riley BB, Phillips BT (2003) Ringing in the new ear: resolution of cell interactions in otic development. Dev Biol 261: 289-312.
33. Van de Water TR, Represa J (1991) Tissue interactions and growth factors that control development of the inner ear. Neural tube-otic anlage interaction. Ann N Y Acad Sci 630: 116-128.
34. Phillips BT, Bolding K, Riley BB (2001) Zebrafish fgf3 and fgf8 encode redundant functions required for otic placode induction. Dev Biol 235: 351-365.
35. Leger S, Brand M (2002) Fgf8 and Fgf3 are required for zebrafish ear placode induction, maintenance and inner ear patterning. Mech Dev 119: 91-108.
36. Maroon H, Walshe J, Mahmood R, Kiefer P, Dickson C, et al. (2002) Fgf3 and Fgf8 are required together for formation of the otic placode and vesicle. Development 129: 2099-2108.
37. Liu D, Chu H, Maves L, Yan YL, Morcos PA, et al. (2003) Fgf3 and Fgf8 dependent and independent transcription factors are required for otic placode specification. Development 130: 2213-2224.
38. Hans S, Liu D, Westerfield M (2004) Pax8 and Pax2a function synergistically in otic specification, downstream of the Foxi1 and Dlx3b transcription factors. Development 131: 5091-5102.
39. Solomon KS, Kudoh T, Dawid IB, Fritz A (2003) Zebrafish foxi1 mediates otic placode formation and jaw development. Development 130: 929-940.
40. Busch-Nentwich E, Sollner C, Roehl H, Nicolson T (2004) The deafness gene dfna5 is crucial for ugdh expression and HA production in the developing ear in zebrafish. Development 131: 943-951.
41. Kozlowski DJ, Whitfield TT, Hukriede NA, Lam WK, Weinberg ES (2005) The zebrafish dog-eared mutation disrupts eya1, a gene required for cell survival and differentiation in the inner ear and lateral line. Dev Biol 277: 27-41.
42. Hammond KL, Loynes HE, Folarin AA, Smith J, Whitfield TT (2003) Hedgehog signalling is required for correct anteroposterior patterning of the zebrafish otic vesicle. Development 130: 1403-1417.
43. Schwander M, Kachar B, Muller U (2010) Review series: The cell biology of hearing. J Cell Biol 190: 9-20.

44. Meyers JR, MacDonald RB, Duggan A, Lenzi D, Standaert DG, et al. (2003) Lighting up the senses: FM1-43 loading of sensory cells through nonselective ion channels. J Neurosci 23: 4054-4065.
45. Heasman J (2002) Morpholino oligos: making sense of antisense? Dev Biol 243: 209-214.
46. Westerfield M, editor ((2007)) THE ZEBRAFISH BOOK,5th Edition; A guide for the laboratory use of zebrafish (Danio rerio): Eugene, University of Oregon Press. Paperback.
47. Chen J, Ruan H, Ng SM, Gao C, Soo HM, et al. (2005) Loss of function of def selectively up-regulates Delta113p53 expression to arrest expansion growth of digestive organs in zebrafish. Genes Dev 19: 2900-2911.
48. Chen YC, Cheng CH, Chen GD, Hung CC, Yang CH, et al. (2009) Recapitulation of zebrafish sncga expression pattern and labeling the habenular complex in transgenic zebrafish using green fluorescent protein reporter gene. Dev Dyn 238: 746-754.
49. Tanimoto M, Ota Y, Inoue M, Oda Y (2011) Origin of inner ear hair cells: morphological and functional differentiation from ciliary cells into hair cells in zebrafish inner ear. J Neurosci 31: 3784-3794.
50. Breathnach R, Benoist C, O'Hare K, Gannon F, Chambon P (1978) Ovalbumin gene: evidence for a leader sequence in mRNA and DNA sequences at the exon-intron boundaries. Proc Natl Acad Sci U S A 75: 4853-4857.
51. Coutinho P, Goodyear R, Legan PK, Richardson GP (1999) Chick alpha-tectorin: molecular cloning and expression during embryogenesis. Hear Res 130: 62-74.
52. Kwak SJ, Vemaraju S, Moorman SJ, Zeddies D, Popper AN, et al. (2006) Zebrafish pax5 regulates development of the utricular macula and vestibular function. Dev Dyn 235: 3026-3038.
53. Sollner C, Burghammer M, Busch-Nentwich E, Berger J, Schwarz H, et al. (2003) Control of crystal size and lattice formation by starmaker in otolith biomineralization. Science 302: 282-286.
54. Nasevicius A, Ekker SC (2000) Effective targeted gene 'knockdown' in zebrafish. Nat Genet 26: 216-220.
55. Robu ME, Larson JD, Nasevicius A, Beiraghi S, Brenner C, et al. (2007) p53 activation by knockdown technologies. PLoS Genet 3: e78.
56. Murayama E, Herbomel P, Kawakami A, Takeda H, Nagasawa H (2005) Otolith matrix proteins OMP-1 and Otolin-1 are necessary for normal otolith growth and their correct anchoring onto the sensory maculae. Mech Dev 122: 791-803.
57. Granato M, van Eeden FJ, Schach U, Trowe T, Brand M, et al. (1996) Genes

controlling and mediating locomotion behavior of the zebrafish embryo and larva. Development 123: 399-413.
58. Hsiao CD, Hsieh FJ, Tsai HJ (2001) Enhanced expression and stable transmission of transgenes flanked by inverted terminal repeats from adeno-associated virus in zebrafish. Dev Dyn 220: 323-336.
59. Wassarman PM (1999) Mammalian fertilization: molecular aspects of gamete adhesion, exocytosis, and fusion. Cell 96: 175-183.
60. Rau A, Legan PK, Richardson GP (1999) Tectorin mRNA expression is spatially and temporally restricted during mouse inner ear development. J Comp Neurol 405: 271-280.
61. Legan PK, Lukashkina VA, Goodyear RJ, Lukashkin AN, Verhoeven K, et al. (2005) A deafness mutation isolates a second role for the tectorial membrane in hearing. Nat Neurosci 8: 1035-1042.
62. Kohli V, Elezzabi AY (2008) Laser surgery of zebrafish (Danio rerio) embryos using femtosecond laser pulses: optimal parameters for exogenous material delivery, and the laser's effect on short- and long-term development. BMC Biotechnol 8: 7.

Figures


Signal peptide
C1
C2
C3
C4
Cx
Cy
C5
C6
C7
Ca
Cb
C8
GPI- anchored domain
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Human : MVTKAFVLLAIFAEASAKSCAPNKADVILVFCYPKTIITKIPECPYGWEVHQLALGGLCYNGVHEGGYYQFVIPDLSPKN : 80
Mouse : MVVRAFVLLALFAEASAKSCTPNKADVILVFCYPKTIITKIPECPYGWEVHQLALGGLCYNGVHEGGYYQFVIPDLSPKN : 80
Chicken : MVAVTVYLMVILAQAFAGPCTPNKADVILVYCYPRTIITKIPECPYGWEVNQLALGGICYNGIHDSGYYQFTIPDLSPKN : 80
Xenopus : MVLWGLGLVTVLIASAAAKCTPNKVDAIIVHCYPRSIVAKIPECPYGWEINQLAMGGVCYNGVVESGYYQFTIPDLSPRN : 80
Zebrafish : HSYCGTLSEYVGGKDPRYLFYNSIVSNDSSLTVRNQPVNYTFSCTYKAAYLVNNAVFSQRVATVYVNNGSLGSFKSQLSM : 157
Human : KSYCGTQSEY---KPPIYHFYSHIVSNDTTVIVKNQPVNYSFSCTYHSTYLVNQAAFDQRVATVHVKNGSMGTFESQLSL : 157
Mouse : KSYCGTQSEY---KPPIYHFYSHIVSNDSTVIVKNQPVNYSFSCTYHSTYLVNQAAFDQRVATVHVKNGSMGTFESQLSL : 157
Chicken : KSYCGTQSEF---KNPVYHFYNSIVSNDSTVIVKSQPVNYSFTCTYNANYLVNQAAFDQRVATIHVKNGSSGSFESQLSL : 157
Xenopus : KSYCGTHSEF---KNPIYHFYNSIVSNDSTLIVKSQPVNYSFTCTYQSNYLVSHGAFDQRVATVHVKNGSSGSFESQLSL : 157
Zebrafish : NVFTNSKFLYAKDAPYVIDTSEIGSEVFIGIEAKGLSNRFKVVITNCWATPTPYSTDRKRWTLIQNSCSLDNTVTIFENA : 237
Human : NFYTNAKFSIKKEAPFVLEASEIGSDLFAGVEAKGLSIRFKVVLNSCWATPSADFMYPLQWQLINKGCPTDETVLVHENG : 237
Mouse : NFYTNAKFSTKKEAPFVLETSEIGSDLFAGVEAKGLSVRFKVVLNSCWATPSADFMYPLQWQLINKGCPTDETVLVHENG : 237
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Human : RDHRATFQFNAFRFQNIPKLSKVWLHCETFICDSEKLSC-PVTCDKRKRLLR-DQTGGVLVVELSLRSRGFSSLYSFSDV : 315
Mouse : KDHRATFQFNAFRFQNIPKLSKVWLHCETFICDSEKLSC-PVNCDKRKRMLR-DQTGGVLVVELSLRSRAFSGLCDFSDV : 315
Chicken : KTNRATFQFNAFRFQNIPKLSKVWLHCETHVCDSEKFSC-PVTCDKRKQRM--EQTGGVLVAEISVRNKGLSRFYMLSDV : 314
Xenopus : KDSRATFQFNAFRFQNVPKLSKVWLHCETYMCDSEKFNC-PVLCGKRKQQK--EQSGGVLVAEFLLQNSASAHCYSVAAA : 314
Zebrafish : FLCLLSILLLNEFLGYFTF : 336 Identity Similarity
Human : LHHLIMMLGICAVL----- : 329 49% 67%
Mouse : LLHLILMLGTWAVL----- : 329 50% 67%
Chicken : IFHLLFAIGFCAILL---- : 329 50% 68%
Xenopus : LGNAMLLVGIYSLWV---- : 329 49% 69%

Fig. 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).

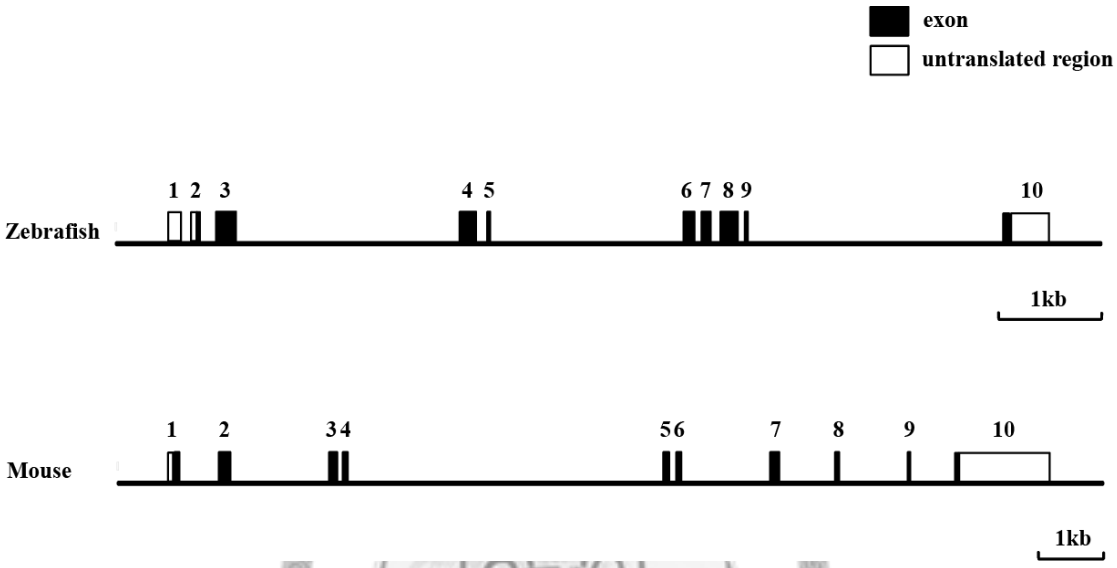
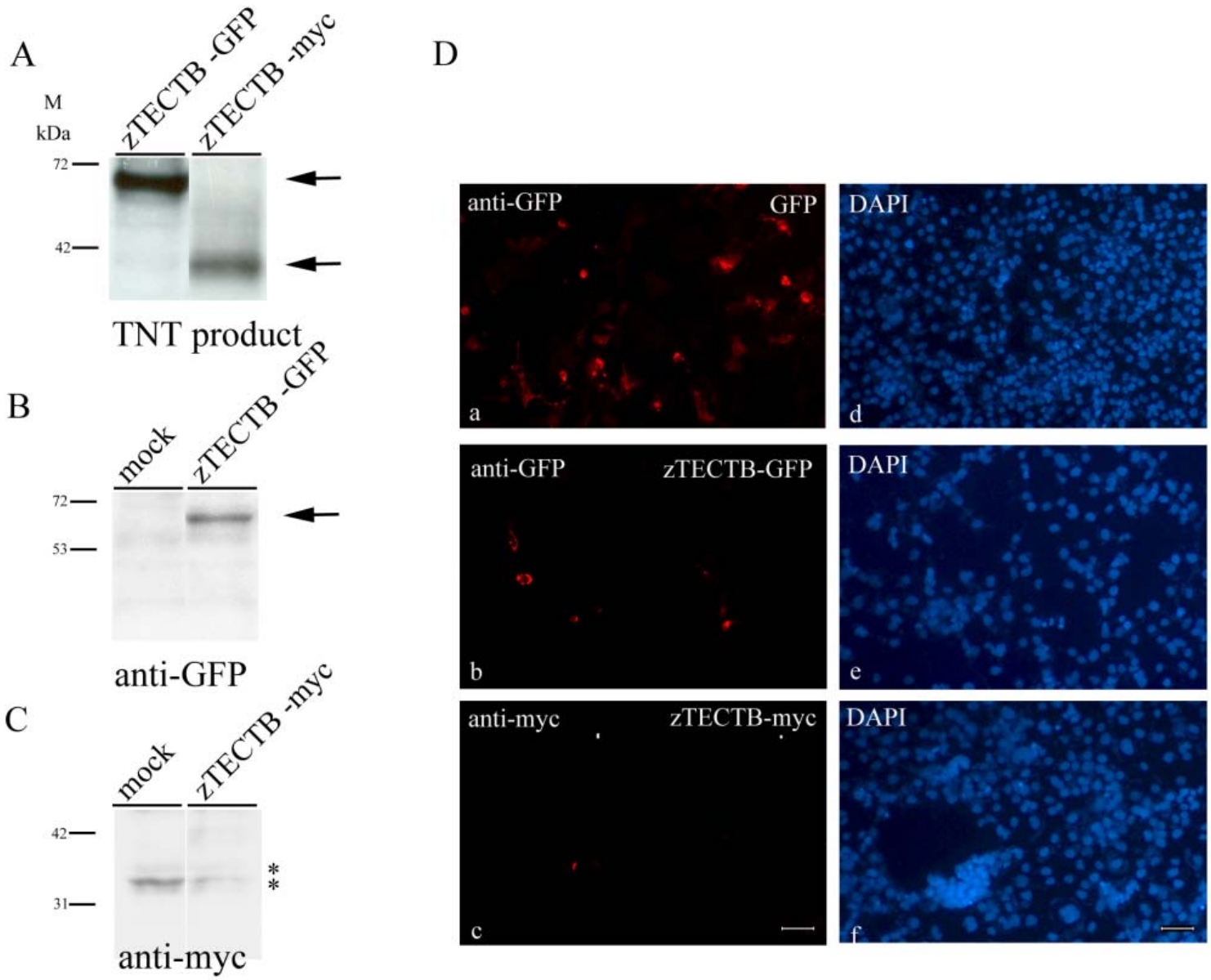

exon
untranslated region
Zebrafish
1 2 3
4 5
6 7 8 9
10
1kb
Mouse
1
2
3 4
5 6
7
8
9
10
1kb

Fig. 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.


A
M
kDa
zTECTB -GFP
zTECTB -myc
72
42
TNT product
B
mock
zTECTB -GFP
72
53
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C
mock
zTECTB -myc
42
31
anti-myc
D
anti-GFP
GFP
a
DAPI
d
anti-GFP
zTECTB-GFP
b
DAPI
e
anti-myc
zTECTB-myc
c
DAPI
f

Fig. 3. Molecular charcterization of zebrafish β-tectorin protein. (A) *In vitro* transcription and translation of β-tectorin-GFP or β-tectorin-myc protein, respectively. Two products of approximate 65 kDa and 39 kDa were indicated by arrows. (B) Western blot analysis of total cell lysates from cells transfected with pcDNA3.1-β-tectorin-GFP by using polyclonal antibody against GFP. Cells were harvested at 48 h after transfection. A product of approximate 65 kDa was detected and shown by an arrow. (C) Western blot analysis of total cell lysates from cells transfected with pcDNA3.1-β-tectorin-myc by using anti-myc monoclonal antibody.. (D) For immunofluorescence staining, transfected COS-1 cells were immunostained with anti-GFP antibody or anti-myc antibody at 24 h after transfection. The cell nuclei were stained with DAPI (blue). Bars, 100 μm.

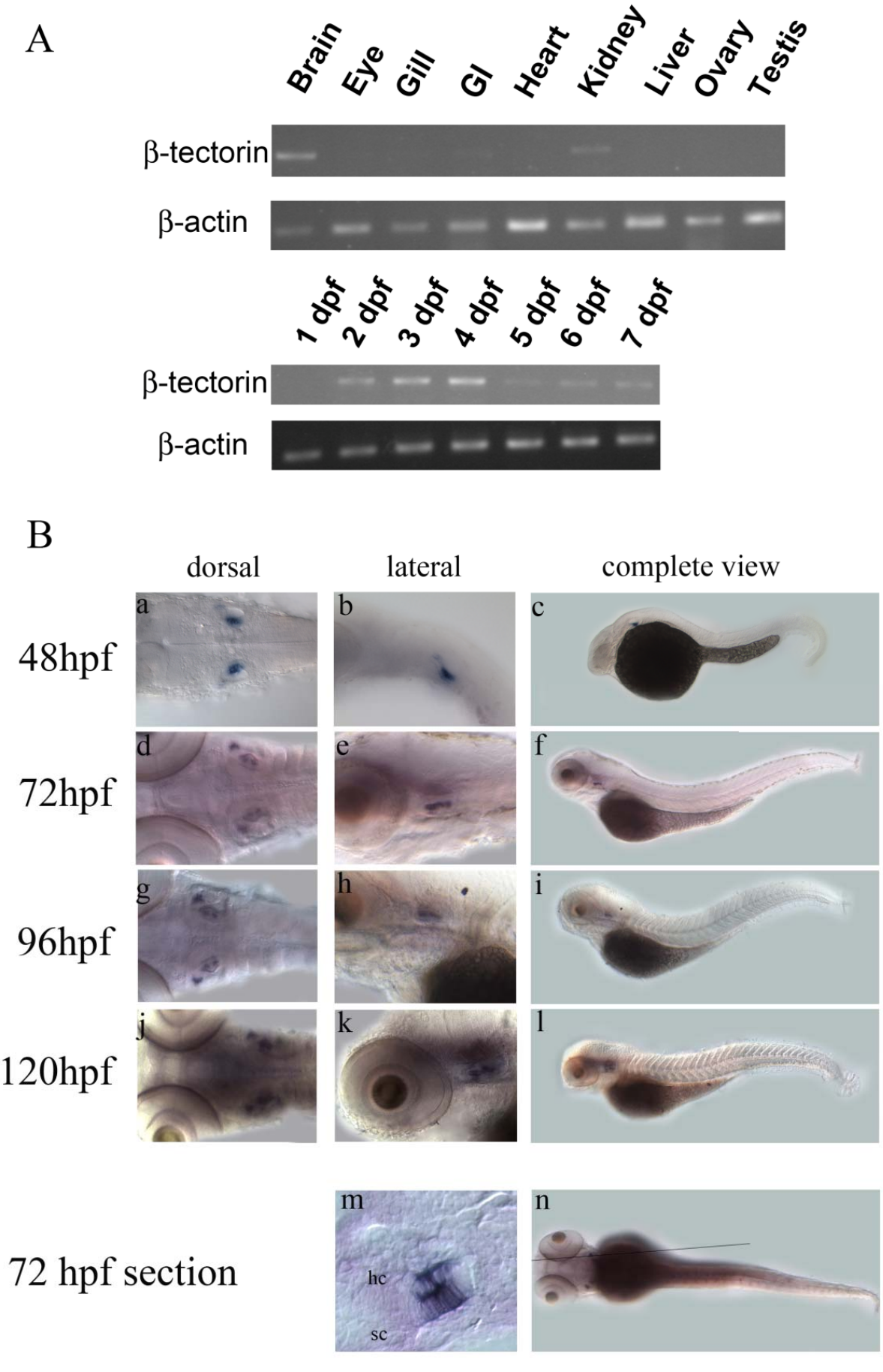
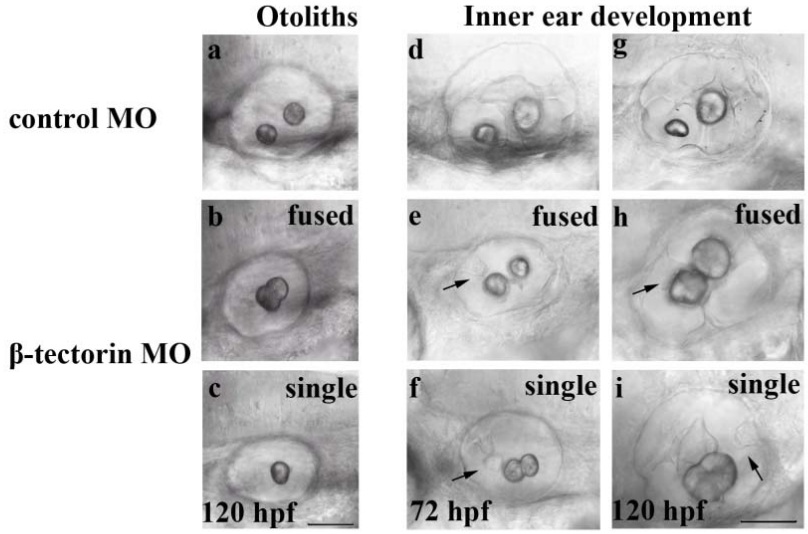

A
Brain
Eye
Gill
GI
Heart
Kidney
Liver
Ovary
Testis
β-tectorin
β-actin
1 dpf
2 dpf
3 dpf
4 dpf
5 dpf
6 dpf
7 dpf
β-tectorin
β-actin
B
dorsal
lateral
complete view
48hpf
72hpf
96hpf
120hpf
72 hpf section
a
b
c
d
e
f
g
h
i
j
k
l
m
n
hc
sc

Fig. 4. 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.

A


Otoliths
Inner ear development
control MO
β-tectorin MO
a
b fused
c single
120 hpf
d
e fused
f single
72 hpf
g
h fused
i single
120 hpf

B

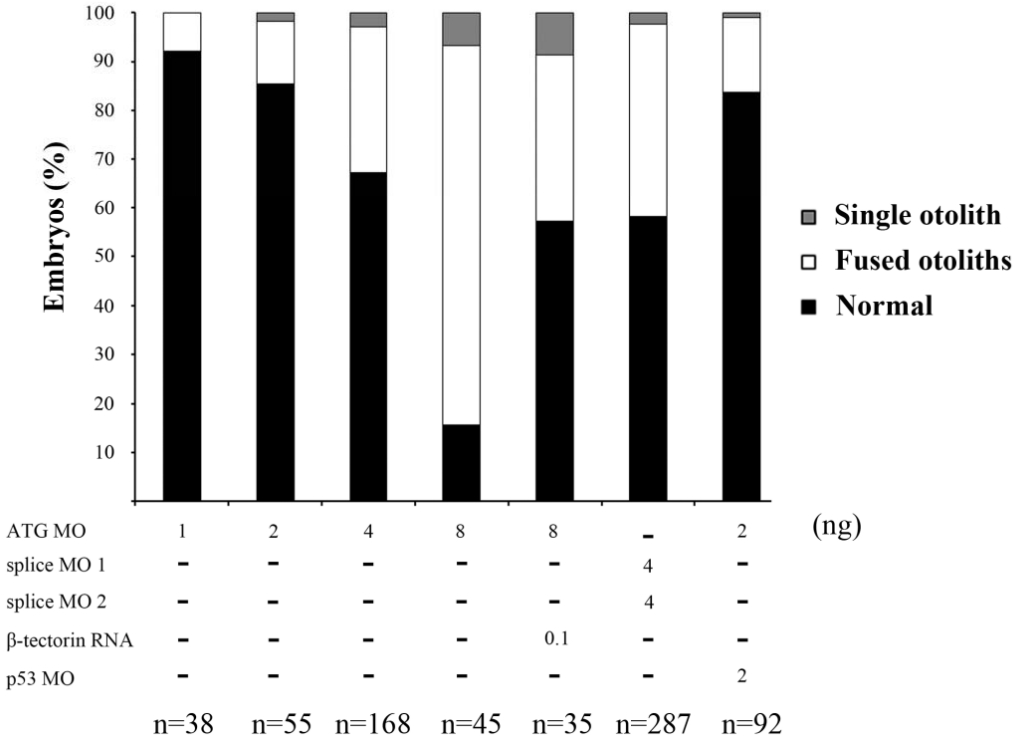

Embryos (%)
100
90
80
70
60
50
40
30
20
10
Single otolith
Fused otoliths
Normal
ATG MO 1 2 4 8 8 - 2 (ng)
splice MO 1 - - - - - 4 -
splice MO 2 - - - - - 4 -
β-tectorin RNA - - - - 0.1 - -
p53 MO - - - - - - 2
n=38 n=55 n=168 n=45 n=35 n=287 n=92

Fig 5. 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, 50μm

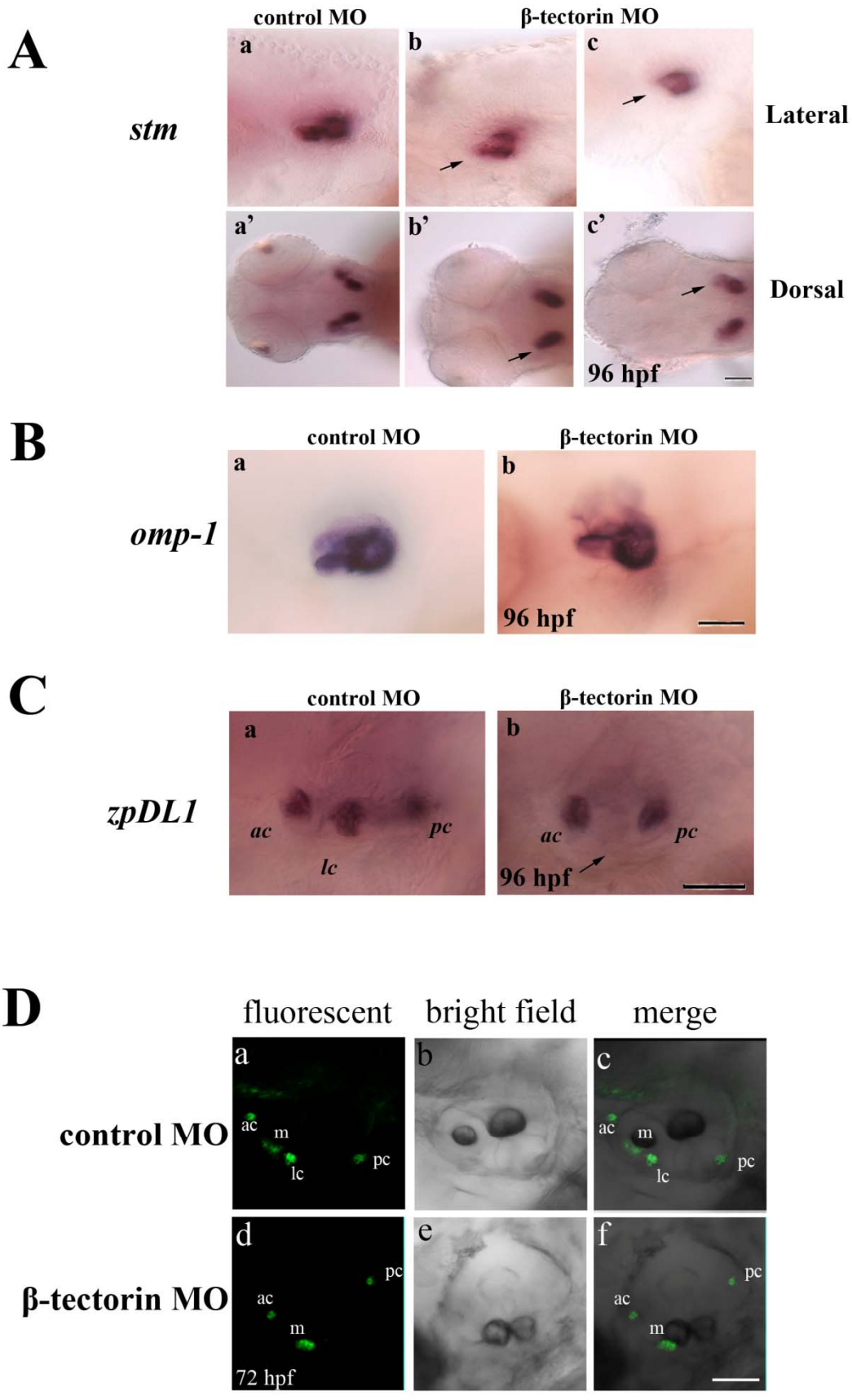

A
control MO
β-tectorin MO
stm
a
b
c
Lateral
a'
b'
c'
Dorsal
96 hpf
B
control MO
β-tectorin MO
omp-1
a
b
96 hpf
C
control MO
β-tectorin MO
zpDL1
a
ac
pc
lc
b
ac
pc
96 hpf
D
fluorescent
bright field
merge
control MO
β-tectorin MO
a
ac
m
lc
pc
b
c
ac
m
lc
pc
d
pc
ac
m
72 hpf
e
f
pc
ac
m

Fig. 6. 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 μm (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 μm

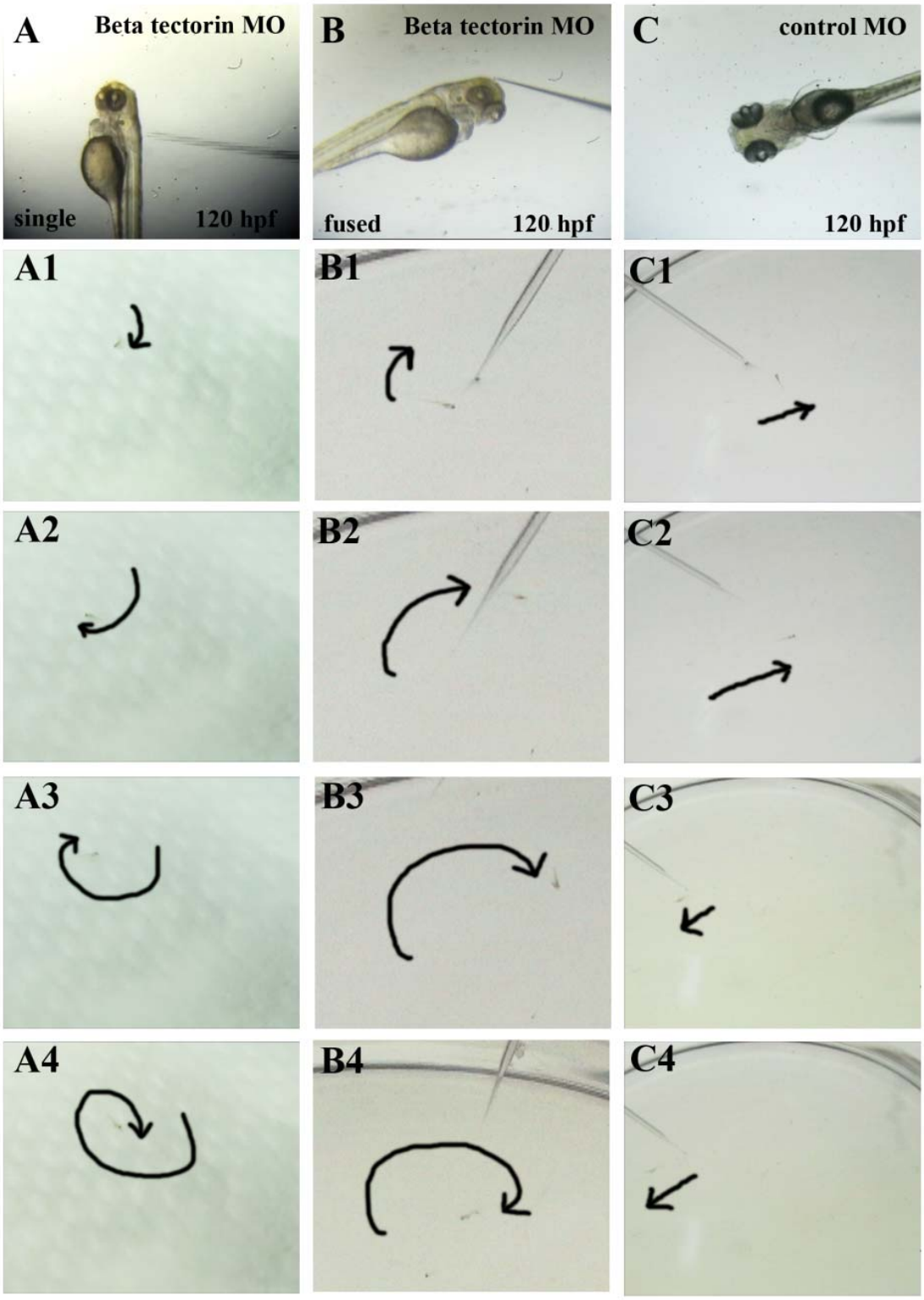

A
Beta tectorin MO
single
120 hpf
B
Beta tectorin MO
fused
120 hpf
C
control MO
120 hpf
A1
B1
C1
A2
B2
C2
A3
B3
C3
A4
B4
C4

Fig. 7. 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.

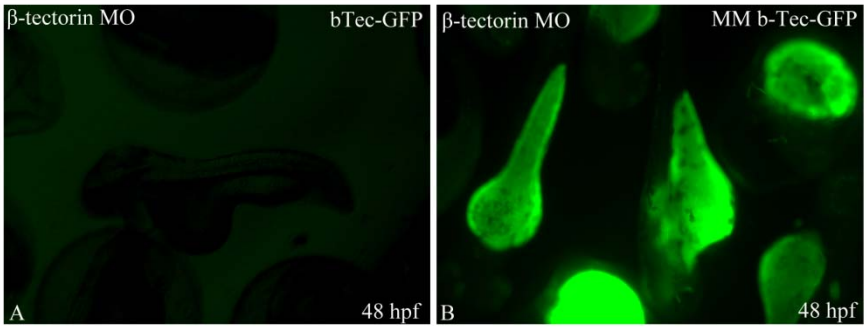
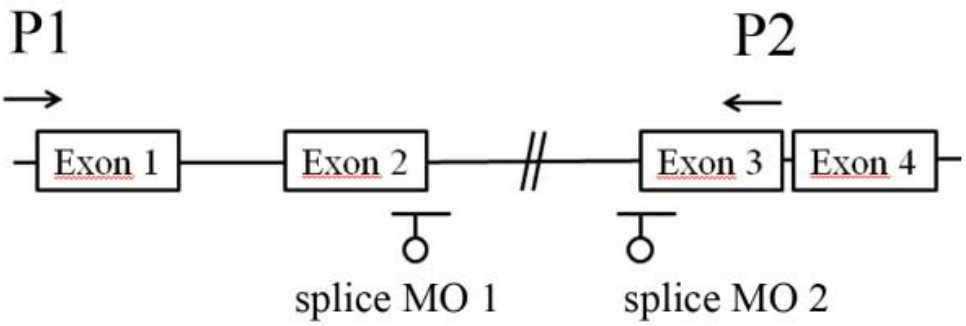

β-tectorin MO
bTec-GFP
A
48 hpf
β-tectorin MO
MM b-Tec-GFP
B
48 hpf

Fig. 8. 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.

A


P1
P2
Exon 1
Exon 2
Exon 3
Exon 4
splice MO 1
splice MO 2

B

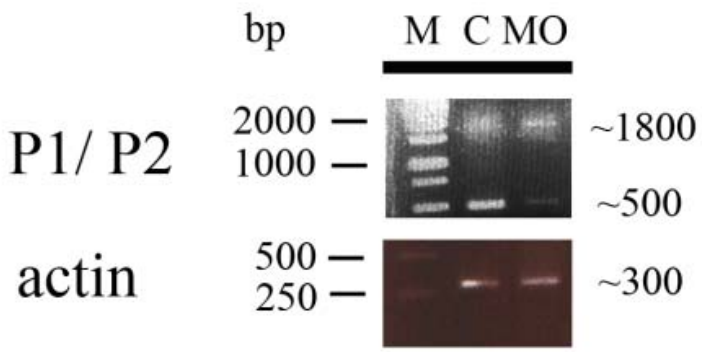

bp
M C MO
P1/ P2
2000
1000
~1800
~500
actin
500
250
~300

Fig. 9. 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 embyos.

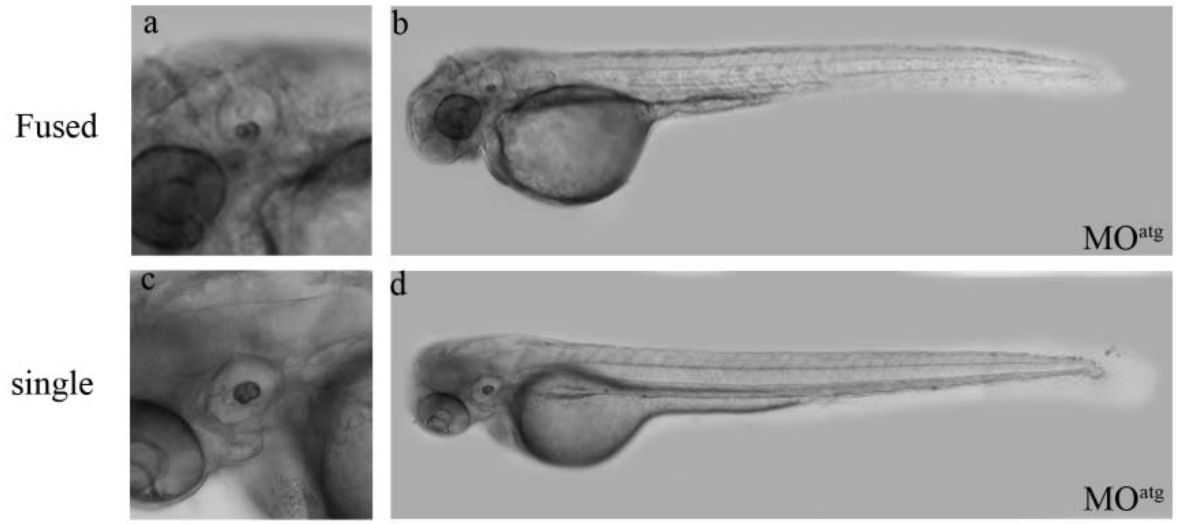

Fused
a
b
MO^atg
single
c
d
MO^atg

Fig. 10. 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.

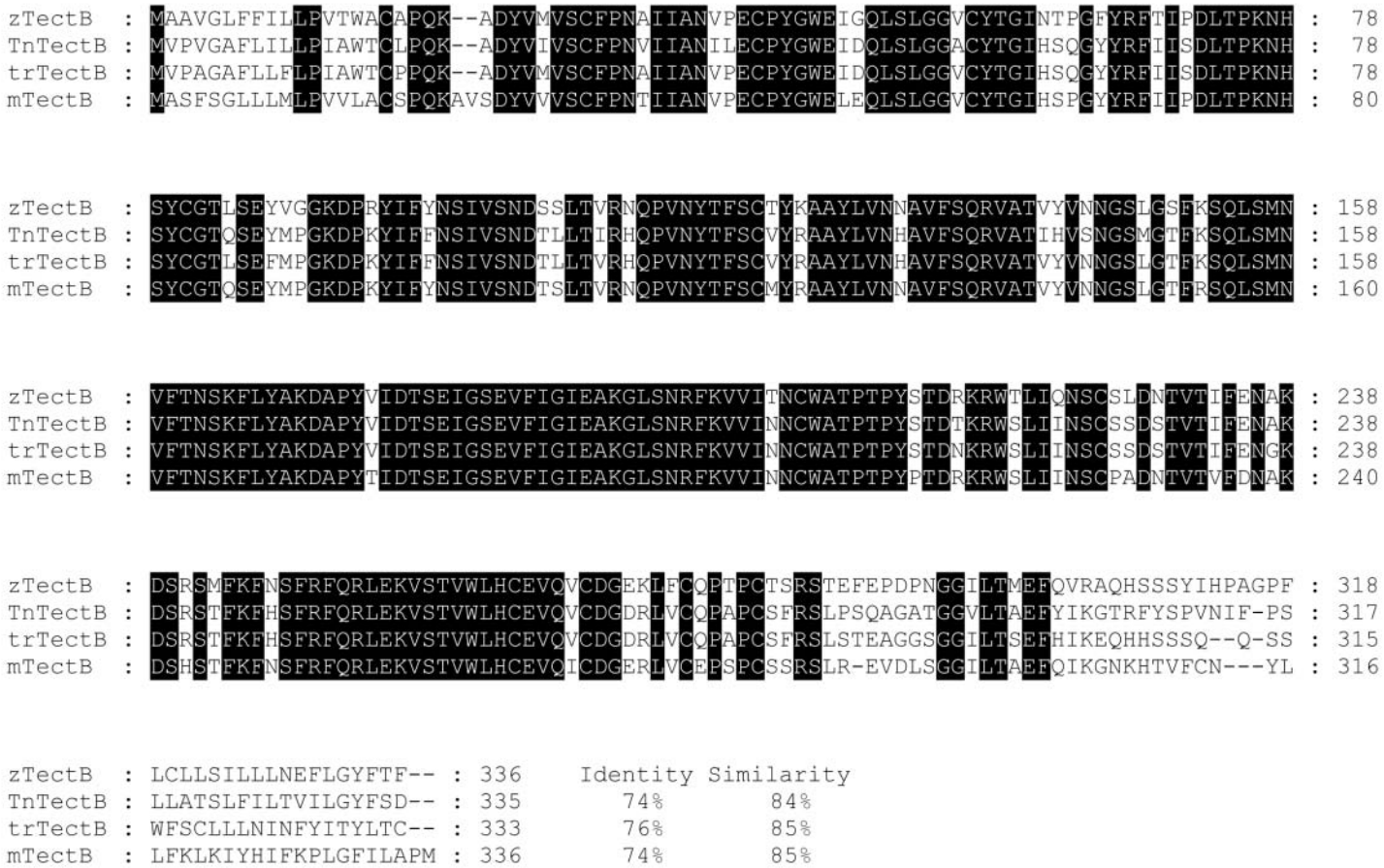

zTectB : MAAVGLFFILLPVTWACAPQK--ADYVMVSCFPNAIIANVPECPYGWEIGQLSLGGVCYTGINTPGFYRFTIPDLTPKNH : 78
TnTectB : MVPVGAFLILLPIAWTCLPQK--ADYVIVSCFPNVIIANILECPYGWEIDQLSLGGACYTGIHSQGYYRFIISDLTPKNH : 78
trTectB : MVPAGAFLLFLPIAWTCPPQK--ADYVMVSCFPNAIIANVPECPYGWEIDQLSLGGVCYTGIHSQGYYRFIISDLTPKNH : 78
mTectB : MASFSGLLLMLPVVLACSPQKAVSDYVVVSCFPNTIIANVPECPYGWELEQLSLGGVCYTGIHSPGYYRFIIPDLTPKNH : 80
zTectB : SYCGTLSEYVGGKDPRYIFYNSIVSNDSSLTVRNQPVNYTFSCTYKAAYLVNNAVFSQRVATVYVNNGSLGSFKSQLSMN : 158
TnTectB : SYCGTQSEYMPGKDPKYIFFNSIVSNDTLLTIRHQPVNYTFSCVYRAAYLVNHAVFSQRVATIHVSNGSMGTFKSQLSMN : 158
trTectB : SYCGTLSEFMPGKDPKYIFFNSIVSNDTLLTVRHQPVNYTFSCVYRAAYLVNHAVFSQRVATVYVNNGSLGTFKSQLSMN : 158
mTectB : SYCGTQSEYMPGKDPKYIFYNSIVSNDTSLTVRNQPVNYTFSCMYRAAYLVNNAVFSQRVATVYVNNGSLGTFRSQLSMN : 160
zTectB : VFTNSKFLYAKDAPYVIDTSEIGSEVFIGIEAKGLSNRFKVVITNCWATPTPYSTDRKRWTLIQNSCSLDNTVTIFENAK : 238
TnTectB : VFTNSKFLYAKDAPYVIDTSEIGSEVFIGIEAKGLSNRFKVVINNCWATPTPYSTDTKRWSLIINSCSSDSTVTIFENAK : 238
trTectB : VFTNSKFLYAKDAPYVIDTSEIGSEVFIGIEAKGLSNRFKVVINNCWATPTPYSTDNKRWSLIINSCSSDSTVTIFENGK : 238
mTectB : VFTNSKFLYAKDAPYTIDTSEIGSEVFIGIEAKGLSNRFKVVINNCWATPTPYPTDRKRWSLIINSCPADNTVTVFDNAK : 240
zTectB : DSRSMFKFNSFRFQRLEKVSTVWLHCEVQVCDGEKLFCQPTPCTSRSTEFEPDPNGGILTMEFQVRAQHSSSYIHPAGPF : 318
TnTectB : DSRSTFKFHSFRFQRLEKVSTVWLHCEVQVCDGDRLVCQPAPCSFRSLPSQAGATGGVLTAEFYIKGTRFYSPVNIF-PS : 317
trTectB : DSRSTFKFHSFRFQRLEKVSTVWLHCEVQVCDGDRLVCQPAPCSFRSLSTEAGGSGGILTSEFHIKEQHHSSSQ--Q-SS : 315
mTectB : DSHSTFKFNSFRFQRLEKVSTVWLHCEVQICDGERLVCEPSPCSSRSLR-EVDLSGGILTAEFQIKGNKHTVFCN---YL : 316
zTectB : LCLLSILLLNEFLGYFTF-- : 336
TnTectB : LLATSLFILTVILGYFSD-- : 335
trTectB : WFSCLLLNINFYITYLTC-- : 333
mTectB : LFKLKIYHIFKPLGFILAPM : 336
Identity Similarity
74% 84%
76% 85%
74% 85%

Fig. 11. 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).

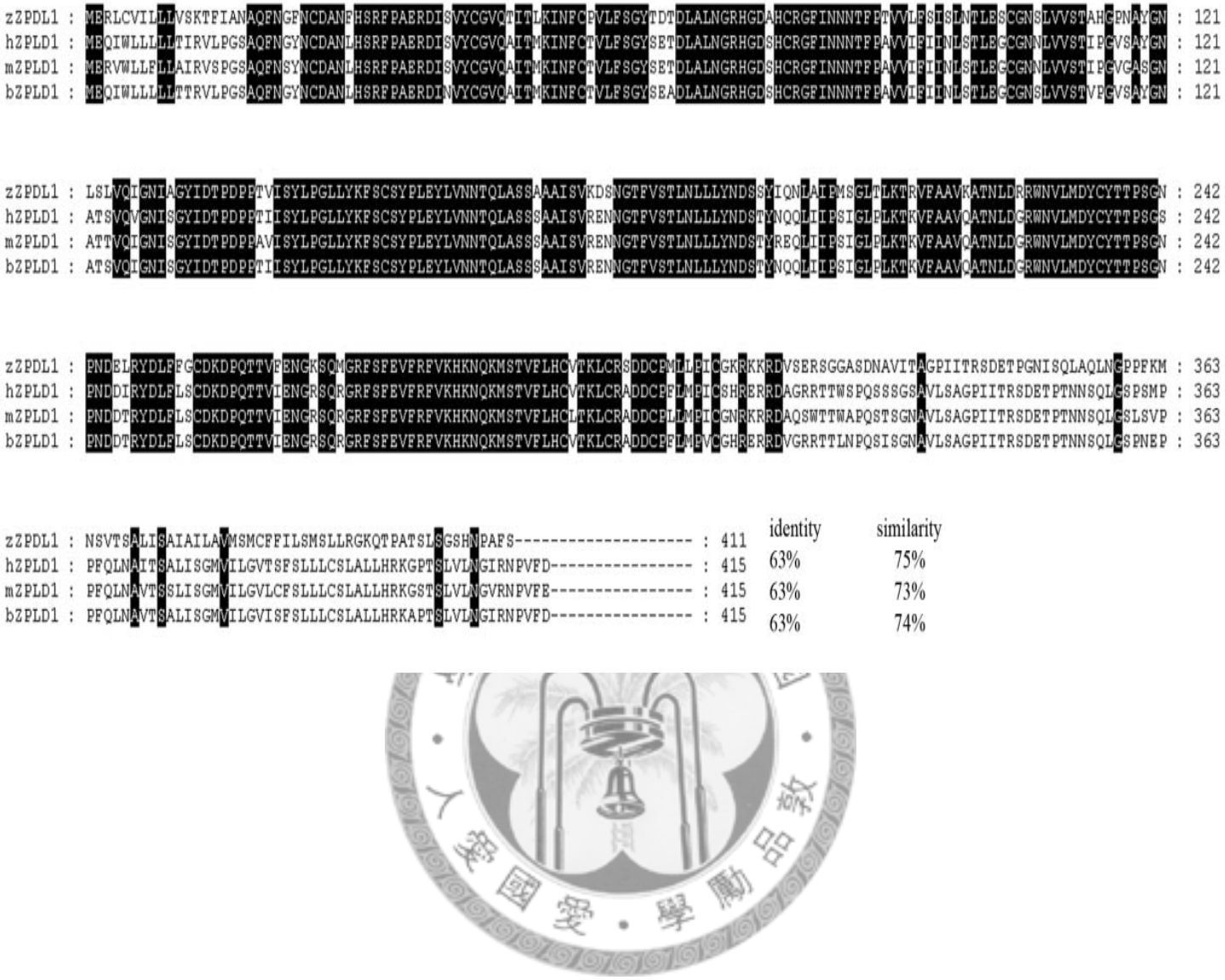
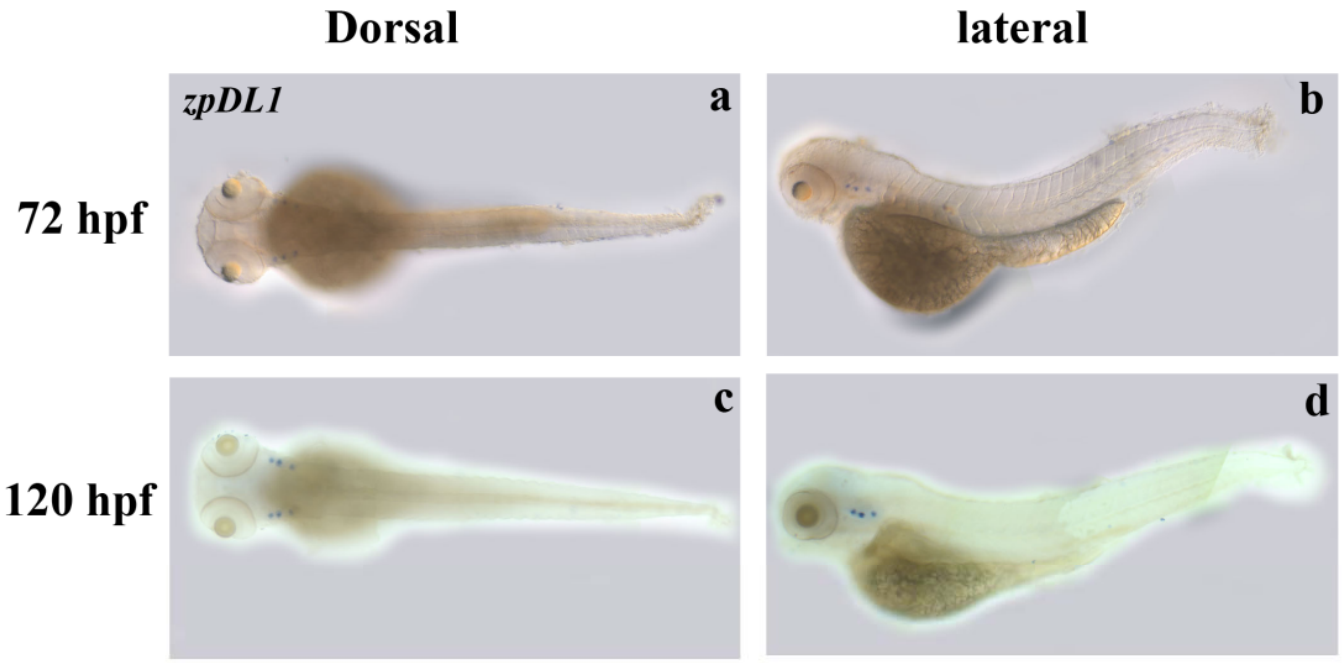

zZPDL1 : MERLCVILLLVSKTFIANAQFNGFNCDANHSRFPAERDISVYCGVQTITLKINFCPVLFSGYTDTDLALNGRHGDAHCRGFINNNTFPAVVIPIINLSTLEGCGNSLVVSTAHGPNAYGN : 121
hZPLD1 : MEQIWLLLLLTIRVLPGSAQFNGYNCDANLHSRFPAERDISVYCGVQAITMKINFCTVLFSGYSETDLALNGRHGDSHCRGFINNNTFPAVVIPIINLSTLEGCGNNLVVSTIPGVSAYGN : 121
mZPLD1 : MERVWLLFLLAIRVSPGSAQFNSYNCDANLHSRFPAERDISVYCGVQAITMKINFCTVLFSGYSETDLALNGRHGDSHCRGFINNNTFPAVVIPIINLSTLEGCGNNLVVSTIPGVGASGN : 121
bZPLD1 : MEQIWLLLLLTTRVLPGSAQFNGYNCDANLHSRFPAERDINVYCGVQAITMKINFCTVLFSGYSEADLALNGRHGDSHCRGFINNNTFPAVVIPIINLSTLEGCGNSLVVSTVPGVSAYGN : 121
zZPDL1 : LSLVQIGNIAGYIDTPDPPTVISYLPGLLYKFSCSYPLEYLVNNTQLASSAAAISVKDSNGTFVSTLNLLLYNDSSKIQNLAIPMSGLTLKTRVFAAVKATNLDRRWNVLMDYCYTTPSGN : 242
hZPLD1 : ATSVQVGNISGYIDTPDPPTIISYLPGLLYKFSCSYPLEYLVNNTQLASSSAAISVRENNGTFVSTLNLLLYNDSTYNQQLIIPSIGLPLKTKVFAAVQATNLDGRWNVLMDYCYTTPSGS : 242
mZPLD1 : ATTVQIGNISGYIDTPDPPAVISYLPGLLYKFSCSYPLEYLVNNTQLASSSAAISVRENNGTFVSTLNLLLYNDSTYREQLIIPSIGLPLKTKVFAAVQATNLDGRWNVLMDYCYTTPSGN : 242
bZPLD1 : ATSVQIGNISGYIDTPDPPTIISYLPGLLYKFSCSYPLEYLVNNTQLASSSAAISVRENNGTFVSTLNLLLYNDSTYNQQLIIPSIGLPLKTKVFAAVQATNLDGRWNVLMDYCYTTPSGN : 242
zZPDL1 : PNDELRYDLEFGCDKDPQTTVRENGKSQMGRFSFEVFRFVKHKNQKMSTVFLHCVTKLCRSDDCPMLLPICGKRKKRDVSERSGGASDNAVITAGPIITRSDETPGNISQLAQLNGPPFKM : 363
hZPLD1 : PNDDIRYDLELSCDKDPQTTVIENGRSQRGRFSFEVFRFVKHKNQKMSTVFLHCVTKLCRADDCPFLMPICSHRERRDAGRRTTWSPQSSSGSAVLSAGPIITRSDETPTNNSQLGSPSMP : 363
mZPLD1 : PNDDTRYDLELSCDKDPQTTVIENGRSQRGRFSFEVFRFVKHKNQKMSTVFLHCLTKLCRADDCPLLMPICGNRKRRDAQSWTTWAPQSTSGNAVLSAGPIITRSDETPTNNSQLGSLSVP : 363
bZPLD1 : PNDDTRYDLELSCDKDPQTTVIENGRSQRGRFSFEVFRFVKHKNQKMSTVFLHCVTKLCRADDCPFLMPVCGHRERRDVGRRTTLNPQSISGNAVLSAGPIITRSDETPTNNSQLGSPNEP : 363
zZPDL1 : NSVTSALISAIAILAVMSMCFFILSMSLLRGKQTPATSLSGSHNPAFS---------- : 411
hZPLD1 : PFQLNAITSALISGMVILGVTSFSLLLCSLALLHRKGPTSLVLNGIRNPVFD---------- : 415
mZPLD1 : PFQLNAVTSSLISGMVILGVLCFSLLLCSLALLHRKGSTSLVLNGVRNPVFE---------- : 415
bZPLD1 : PFQLNAVTSALISGMVILGVISFSLLLCSLALLHRKAPTSLVLNGIRNPVFD---------- : 415
identity
similarity
63%
75%
63%
73%
63%
74%

Fig. 12. Zebrafish zpDL1 amino acid sequence alignment with other species. The deduced amino acid sequences of zebrafish zpDL1 were aligned with those from human, mouse, and bovine. Identical residues in 3 or 4 proteins are highlighted. The accession numbers of each zpDL1 from different fish species are listed below: human (GenBank, accession no: AAH31261.1) , mouse (GenBank, accession no: NP_848835), and cattle (GenBank, accession no: DAA33555.1).

A


Dorsal
lateral
zpDL1
a
b
72 hpf
c
d
120 hpf

B

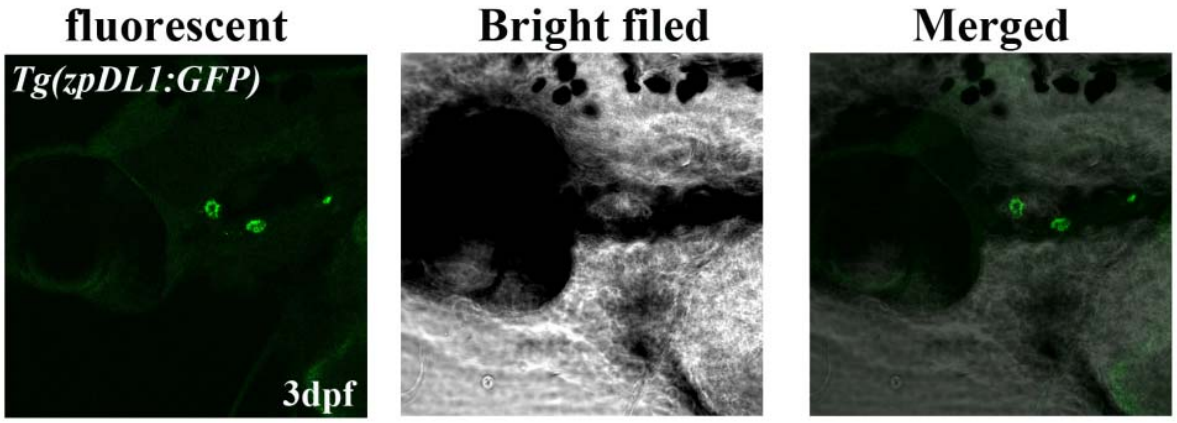

fluorescent
Bright filed
Merged
Tg(zpDL1:GFP)
3dpf

Fig. 13 . Expression profile of *zpDL1* and established *Tg(zpDL1:GFP)* stable line. (A) Whole mount *in situ* hybridization of *zpDL1* demonstrated signals in the 3 cristae of zebrafish inner ear. 72 hpf to 120 hpf. (B) *Tg(zpDL1:GFP)* stable line displays green fluroescence in the 3 cristae at 72 hpf.

Appendices

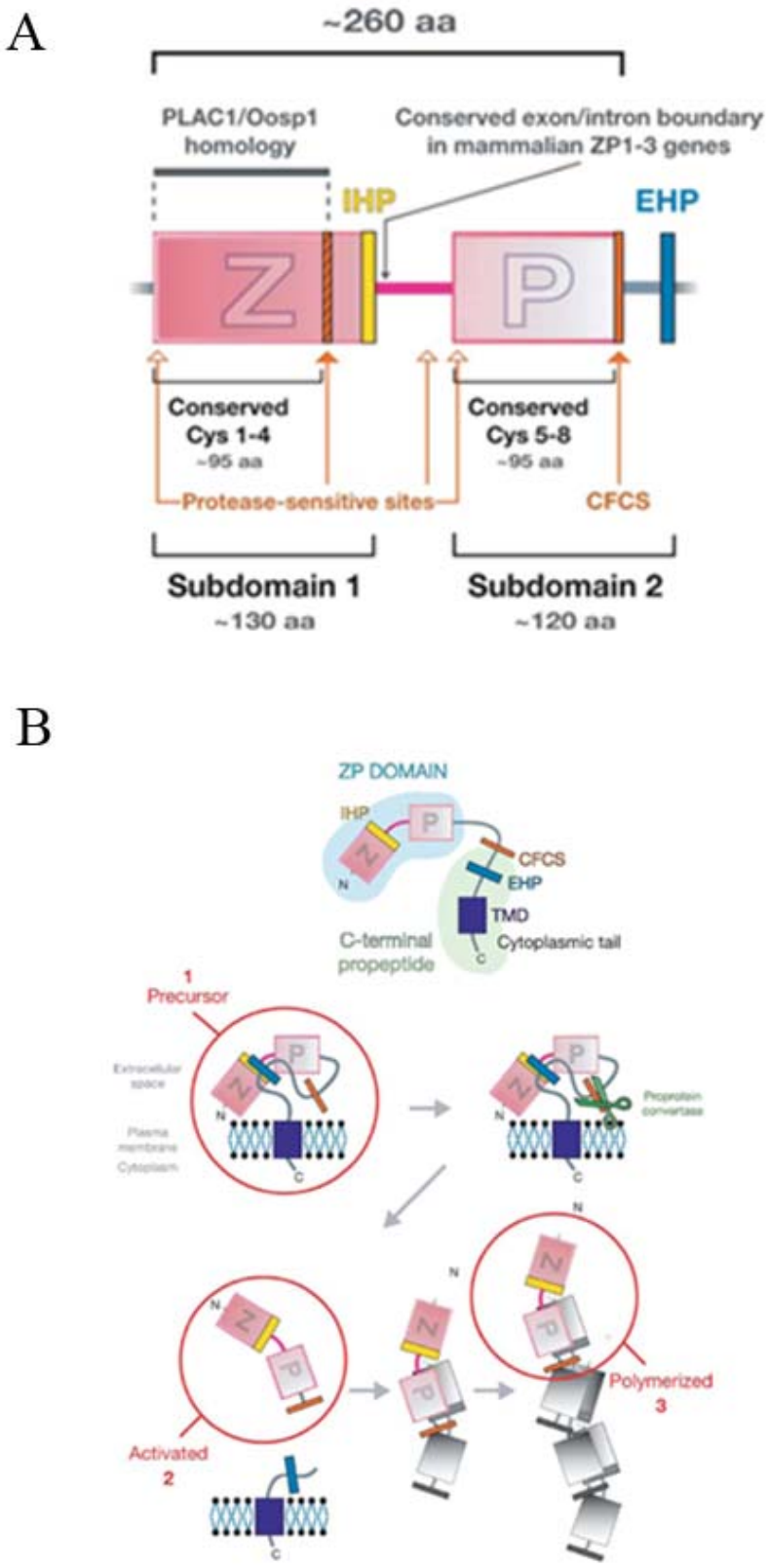


Fig.1 Structure and polymerization of ZP domain containing protein. (Luca Jovine et al., 2005)

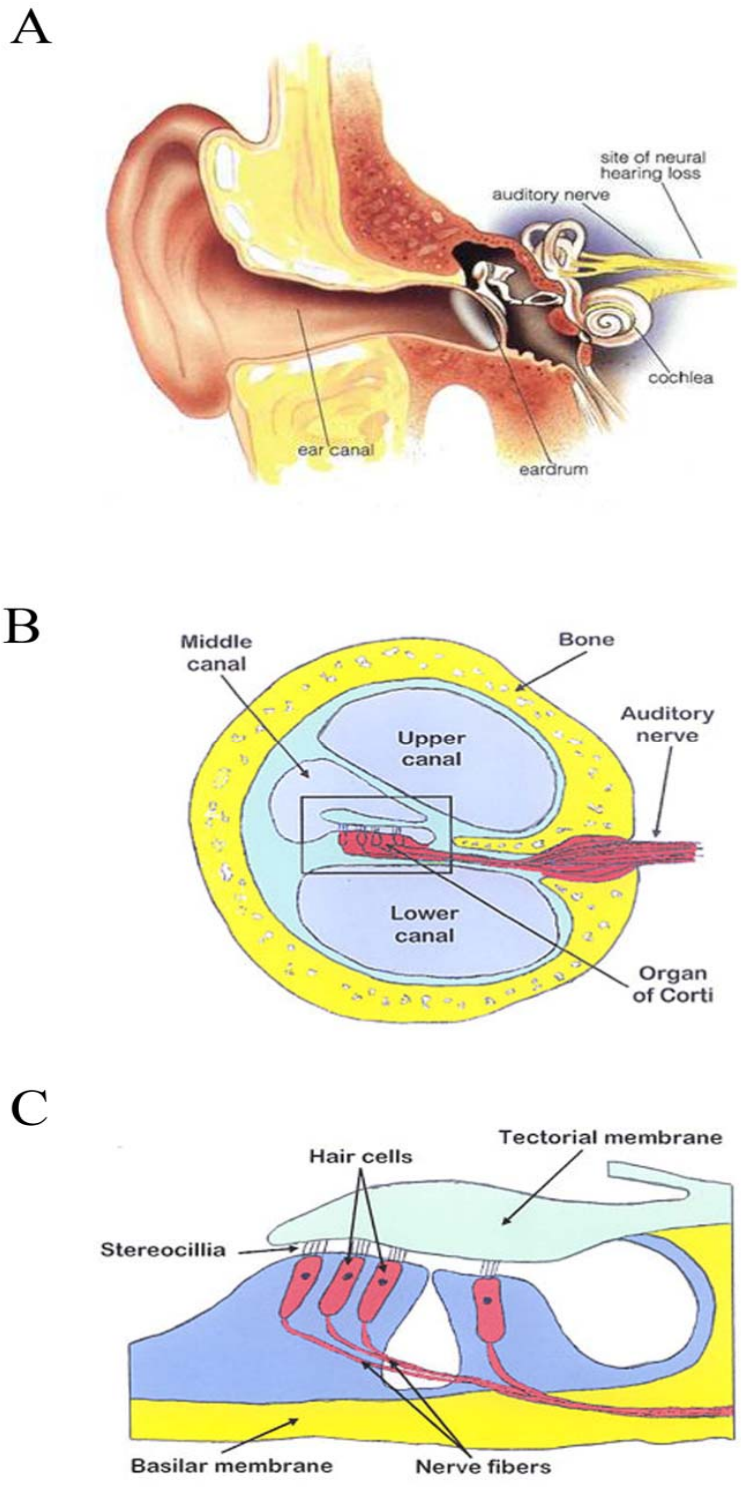


Fig. 2. Structure of human inner ear.(A) human ear. (B) Inside the cochlear and diagram showing the organ of Corti. (C) Structure of tectorial membrane coupling to hair cells in the organ of Corti. (Roger Russell et al., 2008)

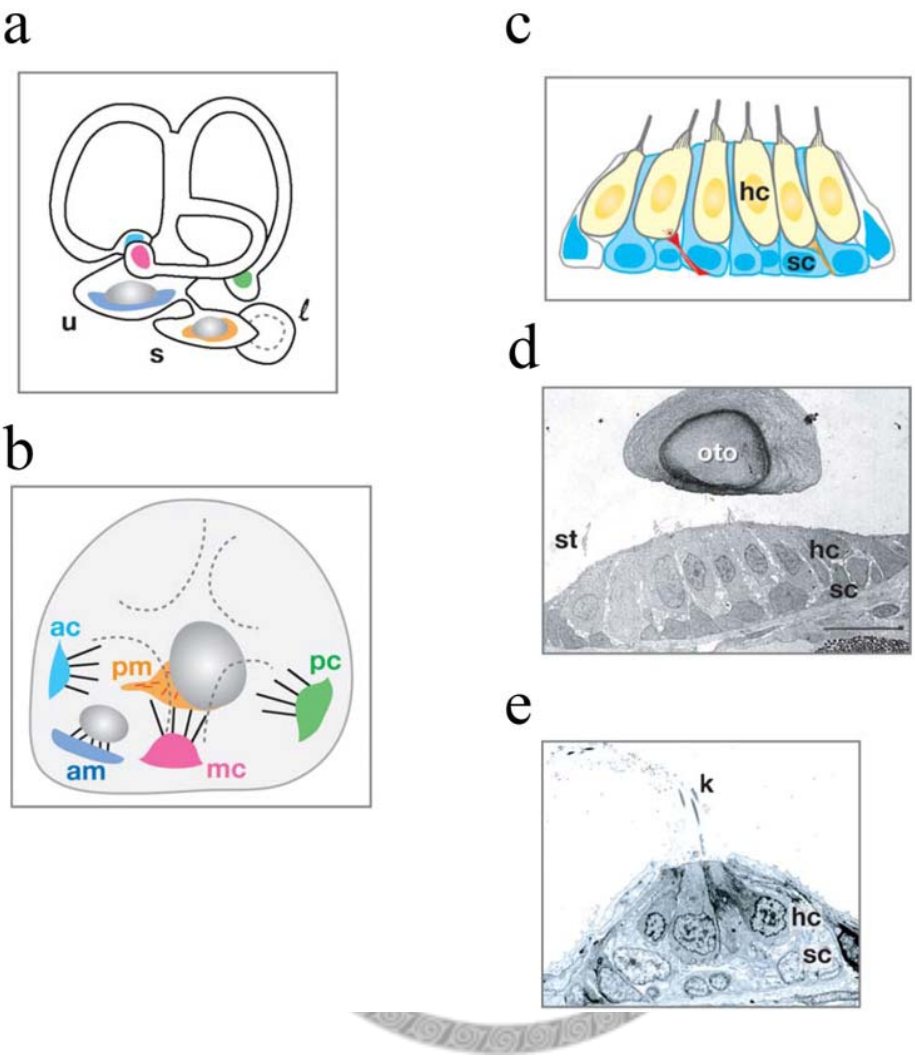


Fig 3. Structure of zebrafish inner ear , otoliths and hair cells. (a) mature zebrafish inner ear. (b) larval zebrafish inner ear (c) hair cell patch arrangement. (d)&(e) EM of hair patch. ac, anterior crista; am, anterior macula; l, lagena; mc, medial crista; o, otolith; pc, posterior crista; pm, posterior macula; s, saccule; u, utricle; hc, hair cell; k, kinocillia; sc, supporting cells.(Teresa Nicolson et al., 2005)