ABSTRACT

Title: UNCONVENTIONAL MYOSINS IN FISH EARS

Allison B. Coffin, Doctor of Philosophy, 2005

Directed By: Professor Arthur N. Popper, Department of Biology, and Dr. Matthew W. Kelley, National Institute on Deafness and Other Communication Disorders, National Institutes of Health

Unconventional myosins are critical motor proteins in the vertebrate inner ear. Mutations in both myosins VI and VIIa cause multiple forms of human hereditary deafness but the precise function of these proteins is unknown. This dissertation uses a comparative approach to better understand the role of myosins VI and VIIa in vertebrate ears. Gene expression and protein distribution for these two myosins is examined in the ears of evolutionarily diverse fishes. RT-PCR data shows that *myo7a* is expressed in the ears of all taxonomically diverse fish species examined here, and immunofluorescence reveals that myo7a protein is distributed throughout the sensory hair bundles of all inner ear regions. Myosin VI expression and distribution is more complex. Studies in other laboratories show that zebrafish (*Danio rerio*) have two *myo6* paralogs with differing gene expression patterns. This dissertation extends previous findings by showing that all teleost fishes have two *myo6* genes while non-teleost fishes and tetrapods have one, suggesting that *myo6* duplication occurred in an ancestral teleost, probably during a genome-wide duplication. RT-PCR experiments

suggest that both *myo6* paralogs are expressed in teleost ears. mRNA localization with *in situ* hybridization shows, however, that *myo6a* is not expressed in sensory epithelia. Immunocytochemical data shows that myo6 protein is distributed throughout hair bundles in all inner ear end organs of the sea lamprey (*Petromyzon marinus*) and the zebrafish but is not found in utricular hair bundles in other fishes. While protein expression studies find that the myo6 antibody used in this dissertation binds to both myo6 proteins in the zebrafish, the gene expression studies suggest that only myo6b is expressed in hair cells, and therefore that this is differential distribution of a single protein. This dissertation adds depth to current studies of myo6-associated hereditary deafness and suggests that comparative studies between zebrafish and other fishes such as shad (*Alosa sapidissima*) that differ in myo6 protein distribution will help elucidate the function of this critical hair cell protein. Comparisons between the two *myo6* paralogs will further aid in functional studies and shed light on evolutionary processes during the teleost radiation.

UNCONVENTIONAL MYOSINS IN FISH EARS

By

Allison Beth Coffin

Dissertation submitted to the Faculty of the Graduate School of the
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Advisory Committee:
Dr. Arthur N. Popper, Chair
Dr. Matthew W. Kelley, co-chair
Dr. Robert J. Dooling
Dr. Thomas B. Friedman
Dr. Matthew P. Hare
Dr. Hey-Kyoung Lee
Dr. Joelle C. Presson

Dedication

I dedicate this dissertation to my husband Cory, my family, and my friends for their support, and to God for seeing me through.

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Chapter 1: Introduction to the Dissertation

Overview

Say the word "fish" and most people picture a salmon on a dinner plate or a goldfish in a child's tank. This view does not encompass the vast variety of fishes in the world, many of which look nothing like a salmon or a goldfish. Fishes are the largest and most diverse vertebrate group (Nelson 1994). Figure 1.1 gives a commonly accepted overview of fish phylogenetics and the times of origin for major groups.

The first recognizable fishes are the jawless agnathans, long eel-like fishes that appeared in the fossil record approximately 600 million years ago (Carroll 1988). Modern lampreys are the sole extant (living) survivors of this group. Cartilaginous fishes such as sharks and skates were among the first vertebrates with jaws. The approximately 800 living species of this group show considerable diversity, ranging from the large predatory white sharks to flattened disk-like stingrays (Nelson 1994). Most living fishes (and in fact most vertebrates) are in Class Osteichthyes. This class is subdivided into two groups, the Sarcopterygii (lobe-finned vertebrates) and the Actinopterygii (ray-finned fishes), which split from one another approximately 450 million years ago (Janvier 1996; Hedges 2001). Sarcopterygian fishes include lungfishes, which are considered the sister group (closest evolutionary relative) to the land vertebrates (Venkatesh et al. 2001; Brinkmann et al. 2004). Two extant species

of coelacanths, popularly known as "living fossils," make up the other sarcopterygian fish group.

All other bony fishes are in Subclass Actinopterygii. Bichirs, sturgeons, gars, and the bowfin comprise the approximately 45 species of basal actinopterygian fishes (Nelson 1994). These fishes are generally large and slow growing predators that are often found in swampy environments. They are important for any understanding of the evolution of fishes and vertebrates in general because of their basal position in ray-finned fish phylogeny.

Most actinopterygian fishes, however, are teleosts. With over 23,000 members, teleost fishes are the largest and most diverse fish group (Nelson 1994). Teleost sizes span several orders of magnitude from the 8 mm long goby *Trimmatom nanus* to the greatly elongated (up to 11 m) ribbon-like oarfish *Regalecus glesne*. Brightly colored damselfishes and wrasses maneuver tight quarters on tropical coral reefs, while transparent icefishes inhabit frigid Antarctic waters. This astounding array of structural and functional diversity may be due to a genome-wide duplication proposed to have occurred early in fish evolution (Amores et al. 1998; Meyer and Schartl 1999; Taylor et al. 2001a). Chapter three of this dissertation looks at the timing of this genome duplication event.

Fishes are uniquely suited to their environment and show substantial sensory adaptations. Retinal specializations exist in such diverse fishes as salmonids (salmon and whitefishes), pantodontids (freshwater butterfly fishes), and poeciliids (livebearers), providing each an adaptive advantage in survival or reproduction (Archer et al. 1987; Hawryshyn et al. 1989; Endler 1991; Coughlin and Hawryshyn

1994; Saidel 2000). Fishes also show diversity in ear structure and function (Popper and Coombs 1982; Popper and Fay 1999; Ladich and Popper 2001), including specializations for both infrasound and ultrasound detection (Sand and Karlsen 1986; Mann et al. 1997). In this introductory chapter I first review fish ear structure and describe some morphological and physiological adaptations seen in various fish taxa. I then focus on the sensory hair cells, the mechanoreceptors common to all vertebrate ears and the fish lateral line. I describe how unconventional myosins, which are the focus of this dissertation, are important for hair cell structure and function. I then discuss gene duplication in myosin VI, describing how study of *myo6* duplicates in fishes can help answer questions about genome duplication during fish evolution. Finally, I briefly introduce chapters two and three, which report the results of my dissertation research.

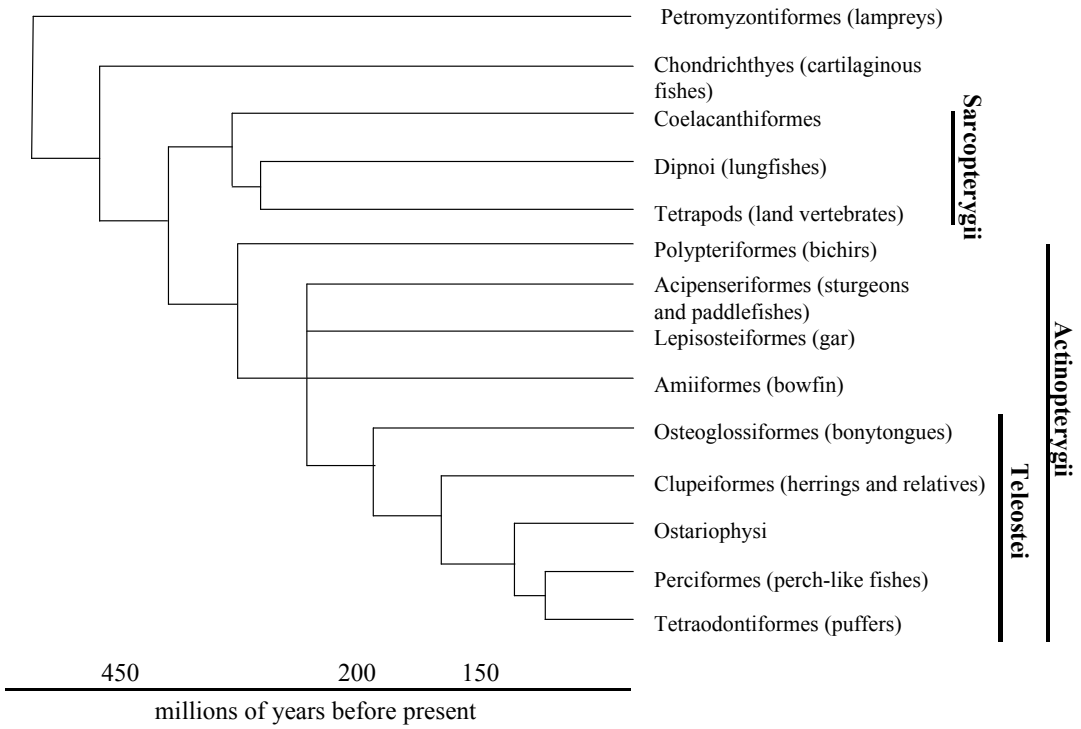

Figure 1.1. Phylogenetic relationships between major fish taxa. Tree is mostly based on Nelson (1994) with uncertainty between non-teleost actinopterygians based on Inoue et al. (2003). Dating is from Carroll (1988), Janvier (1996), and Hedges and Kumar (2002).

Fish ears show great diversity

The morphology of the ears of ancestral fishes may be inferred from anatomical studies of extant agnathan ears. Lamprey ears contain a single sensory epithelium, the macula communis, overlaid with calcareous otoconia (Fig. 1.2A; Löwenstein et al. 1968; Popper and Hoxter 1987). Two large semicircular canals, each with a sensory crista, attach to the epithelial pouch. In contrast, ears of jawed fishes have three semicircular canals (with associated cristae) and three otolithic end organs, the saccule, utricle, and lagena (Fig. 1.2B) (Popper and Fay 1999; Popper and Lu 2000). Ears of all sharks and at least some bony fishes also have a non-otolithic macula neglecta (Retzius 1881; Corwin 1989).

Different fish groups appear to use different inner ear end organs for hearing (Popper and Fay 1993; 1999). In otophysan fishes such as goldfish (*Carassius auratus*) and zebrafish (*Danio rerio*), the saccule is the primary auditory end organ (Platt and Popper 1981; Popper and Fay 1993) although the lagena is very likely to be involved in detecting sound source direction (Ladich and Popper 2001; Michaela Meyer, personal communication, 2005). Clupeid fishes such as the Pacific herring (*Clupea harengus*) have a specialized three-part utricle that appears to sub-serve hearing, but this has yet to be proven conclusively (Popper and Platt 1979; Blaxter et al. 1981). In no fish, however, is the precise contribution of all three epithelia well understood as it pertains to hearing. Work in the past few decades suggests that each epithelium may be a mosaic, serving both auditory and vestibular function (Popper et al. 1982; Schellart and Popper 1992).

Fishes also show great variation in hearing abilities and these differences depend on accessory structures associated with the ear. The coupling of a gas-filled structure such as the swim bladder or other air bubble to the ear greatly enhances hearing sensitivity by allowing the fish to detect both the particle motion and pressure wave components of a sound stimulus (Popper and Fay 1999; Popper and Lu 2000). Fishes such as the oscar (*Astronotus ocellatus*) that have no such coupling hear in the low frequency range up to approximately 800 Hz (Fay 1988; Yan and Popper 1992). Otophysans like the zebrafish possess Weberian ossicles, a chain of modified vertebrae that couple the swim bladder to the ear, and can detect sounds up to 4000 Hz (Fay 1988; Kenyon et al. 1998). Anabantid (labyrinth) fishes have a gas bubble within the ear that also enhances hearing (Ladich and Yan 1998; Ladich 2000). Fishes in the family Sciaenidae (drums and croakers) have varying degrees of anterior swim bladder projections (horns or diverticulae), and hearing sensitivity in these fishes increases as the distance between the projections and the ear decreases (Ramcharitar 2003).

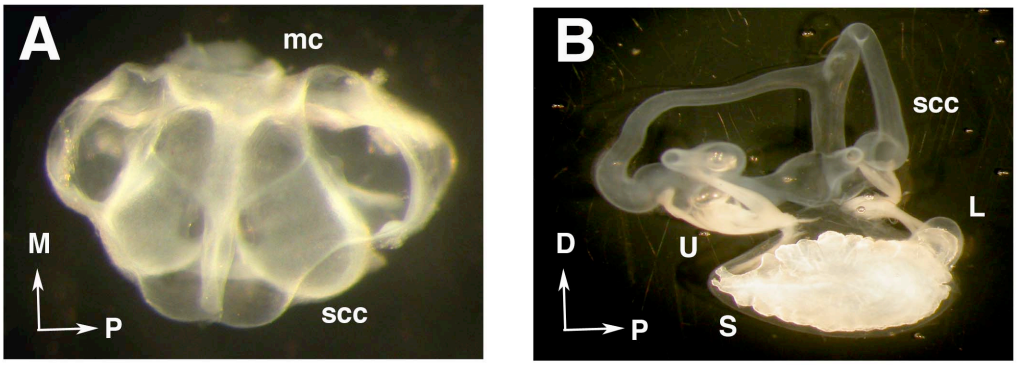

Figure 1.2. Morphology of fish ears. (A) Left ear from a sea lamprey (*Petromyzon marinus*), (B) left ear from a teleost fish, the oscar (*Astronotus ocellatus*). Abbreviations: **mc** macula communis, **scc** semicircular canal, **L** lagena, **S** saccule, **U** utricle, **M** medial, **P** posterior, **D** dorsal.

Hair cells are the fundamental sound receptors

All fish ears contain sensory hair cells (Popper and Fay 1999; Coffin et al. 2004). Mechanosensory hair cells are the fundamental sensory receptors of the vertebrate ear for the transduction of motion into bioelectrical impulses. Auditory and vestibular stimuli cause particle motion of the fluid-filled inner ear and hair cells are morphologically specialized to detect this motion. Each hair cell has an apical "hair bundle" with many actin-filled projections called stereocilia and a single true cilium called a kinocilium (although the kinocilium degenerates in mature mammalian cochlear hair cells). Stereocilia are arranged in a staircase fashion with the tallest stereocilia located adjacent to the kinocilium (Fig 1.3). Each stereocilium is joined to its neighbors by extracellular tip links, lateral links, and ankle links (Pickles et al. 1984, 1991; Pickles 1993). Mechanically gated transduction channels are located at the tips of the stereocilia in association with tip links (Fig. 1.4) (Pickles et al. 1984; Kachar et al. 2000). Mechanical gating is very fast, allowing hair cells to respond faithfully to high frequencies.

The precise organization of stereocilia and associated linkages is necessary for mechanotransduction to occur. The gating-spring hypothesis describes our current understanding of hair cell transduction (Hudspeth 1997; Gillespie and Walker 2001; Strassmaier and Gillespie 2002). In the absence of stimulation, the hair bundle projects perpendicularly from the apical surface of the cell. Stereociliary linkages maintain constant tension on the bundle and few transduction channels are open. Mechanical motion of the hair bundle reflects the incoming stimulus, leading to pivoting of the hair bundle along the axis of polarization (from the shortest stereocilia

to the kinocilium). When the hair bundle is deflected toward the kinocilium, "gating springs" that regulate the transduction channels are stretched and open the channels, letting ions (mostly K^+ and Ca^+) enter the cell. This results in graded depolarization of the cell body and release of neurotransmitter (mostly glutamate) onto afferent synapses of the eighth cranial nerve (Hudspeth 1997; Gillespie and Walker 2001; Strassmaier and Gillespie 2002; Fuchs et al. 2003). Deflection in the reverse direction (away from the kinocilium) results in closure of transduction channels and graded hyperpolarization of the cell body. Natural stimuli cause oscillation of the hair bundle between the depolarizing and hyperpolarizing positions and results in receptor potentials that code for stimulus frequency. Figure 1.4 depicts the gating spring hypothesis of hair cell transduction.

Hair bundles are molecularly complex structures, requiring suites of interacting proteins to provide the highly organized morphology necessary for mechanotransduction (reviewed in Frolenkov et al. 2004). These interactions are not fully understood but many molecular components have been identified in recent years. Transmission electron microscopy (TEM) shows that tightly packed and cross-linked actin paracrystals make up the core of each stereocilium (Tilney et al. 1983). Immunostaining for the actin-bundling proteins espin and fimbrin suggests that these two proteins are contained in cross-links within the actin paracrystal and cell transfection experiments with GFP-tagged espin suggest an additional role for espin in stereocilia elongation (Drenckhahn et al. 1991; Loomis et al. 2003; Li et al. 2004). The deaf *jerker* mouse contains a null mutation for espin and stereocilia in this mouse

degenerate early in post-natal life, further confirmation of the necessity of actin-bundling proteins in hair cells (Zheng et al. 2000).

Whirlin and myosin XVa also play a role in stereocilia elongation (Belyantesva et al. 2005). The myosin XVa mouse mutant, *shaker2*, has abnormally short stereocilia and this phenotype can be rescued by addition of wild-type Myo15a (Probst et al. 1998). Immunostaining shows that Myo15a and whirlin co-localize in stereociliary tips and transfection of inner ear cultures with fluorescently tagged whirlin and Myo15a shows that this myosin transports whirlin up the stereocilia (Belyantseva et al. 2005). How these two proteins influence stereocilia elongation is not yet understood.

Perhaps the most detailed understanding of protein interactions in hair bundles involves the protein products of Usher syndrome type 1 genes (Cryns and Van Camp 2004; Frolenkov et al. 2004). Usher syndrome is a human hereditary disorder marked by profound congenital deafness and progressive blindness from retinal degeneration (Cryns and Van Camp 2004). Myosin VIIa, harmonin, cadherin23, and SANS (scaffolding protein containing ankyrin repeats) form a molecular complex necessary for hair bundle cohesion and certain mutations in any of these genes result in Usher syndrome type 1 (Boëda et al. 2002; Siemens et al. 2002). Immunolocalization, gene expression, and mutant analyses suggest the following interactions (reviewed in Cryns and Van Camp 2004; Frolenkov et al. 2004). Cadherin23 is probably a component of tip links, and it binds to harmonin. Harmonin binds to stereocilia through an interaction with myosin VIIa (which binds actin). SANS also binds harmonin in this complex, although its function is unknown. In this way, tip link

proteins are tethered to the actin core of the stereocilia, providing proper bundle tension at rest. Loss of any protein in this complex eliminates gating spring tension and therefore mechanotransduction, resulting in deafness.

The proteins described above fall into many protein categories including cytoskeletal and transmembrane proteins. However, the largest group of identified hair bundle proteins is the myosins. Mutations in at least six myosin genes are known to contribute to human hereditary deafness (reviewed in Cryns and Van Camp 2004). Chapter two of this dissertation examines two important hair cell myosins, VI and VIIa, which are described in more detail below.

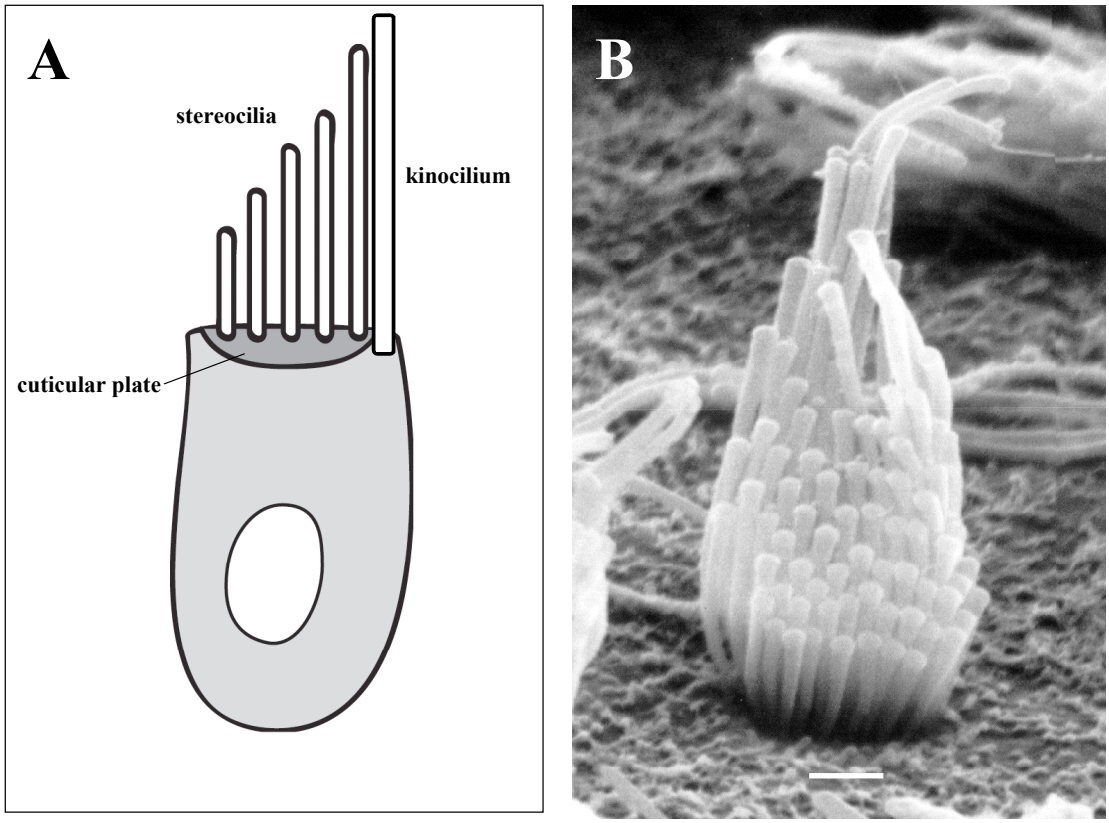

Figure 1.3. Fish hair cells. A) Cartoon of a generalized hair cell showing some of the important features. B) Scanning electron micrograph of a hair bundle from a roundnose grenadier (*Coryphaenoides rupestris*). Scale bar is 1 μm. Micrograph is courtesy of Xiaohong Deng.

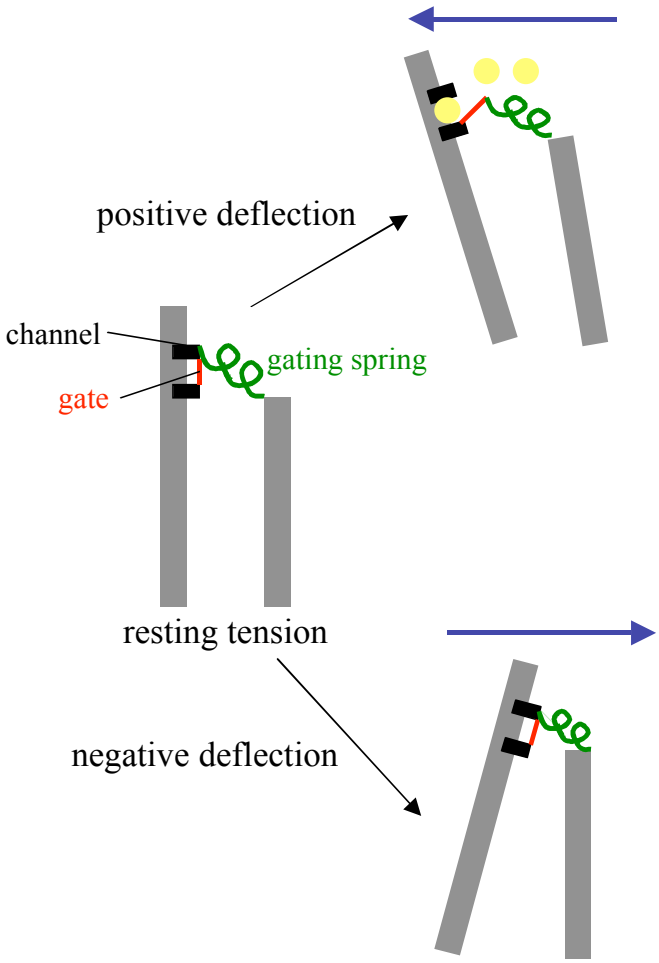

Figure 1.4. Gating-spring hypothesis of hair cell mechanotransduction. At rest, the "**gating spring**" exerts constant tension on the **transduction channel**, and the open probability of the **gate** is small. When the bundle is positively deflected toward the taller stereocilium, the spring stretches and the channel opens (top). With negative bundle deflection, the gating-spring slackens and open probability of the channel decreases still further (bottom). Blue arrows depict stimulus direction. Yellow circles represent ions entering the open transduction channel. Figure based on Hudspeth et al. 2000, Gillespie 2004.

Unconventional myosins are important motor proteins in hair cells

Myosins are motor proteins that hydrolyze ATP to slide along actin filaments (Sellers 2000; Berg et al. 2001). There are currently 18 known classes of myosins in this growing protein superfamily, named in the order for which they were discovered (Berg et al. 2001). Type II or "conventional" myosins form filaments and are a large protein component of striated, smooth, and cardiac muscle. All other myosins are called "unconventional" because they do not form filaments. Much of the molecular and biochemical research on myosins has been performed on type II myosins (Sellers 2000).

Myosin proteins are commonly divided into three protein domains, the head or motor domain, the neck, and the tail domain (Sellers 2000). The head domain contains the actin and ATP binding regions and is the most conserved region of any myosin protein. New myosins are classified based on the sequence of the head domain. The neck domain contains a variable number of IQ motifs (named for their characteristic isoleucine, glutamine amino acid sequence) that bind calcium and play a regulatory role in actin and ATP binding. Tail domains are the least conserved between myosins and are thought to bind cargo. Tail domains of some myosins contain a coiled-coil protein motif that facilitates dimerization.

Genetic and immunocytochemical research from the past decade describes the presence and distribution of several unconventional myosins within the vertebrate inner ear (e.g., Solc et al. 1994; Hasson et al. 1997). These studies showed that myosins IC, VI and VIIa are hair cell-specific within the inner ear sensory epithelia

and that there are differences in the distribution of both myosins VI and VIIa within hair cells of different vertebrates (e.g., mammals and frogs) (Hasson et al. 1997). Additional immunocytochemical work demonstrated that myosin XV is found in the stereocilia and cuticular plate of cochlear hair cells in mice (Liang et al. 1999).

The precise function of myosins in hair cells is unknown. However, mutations in three of these proteins (all but IC) have been linked to genetic forms of deafness in both humans (Weil et al. 1995; Wang et al. 1998; Melchionda et al. 2001) and mice (Gibson et al. 1995; Probst et al. 1998; Self et al. 1999). For example, recent work on myosin VIIa mouse mutants shows that abnormally large hair bundle deflections (beyond the physiological range) are required to open transduction channels in these mutants, implying that myosin VIIa operates in series with the transduction channel and explaining the deafness seen in myosin VIIa mutants (Kros et al. 2002). The mouse mutant *shaker1* has highly disorganized stereocilia, thereby providing further evidence of the importance of myosin VIIa for hair bundle cohesion (through its interaction with other hair bundle proteins described above) (Gibson et al. 1995; Frolenkov et al. 2004).

Mutations in other myosins produce different hair cell phenotypes. In the myosin VI mouse mutant *Snell's waltzer*, stereocilia develop normally but fuse and degenerate soon after birth (Self et al. 1999). Myosin VI localizes to the base of the hair bundle in wild-type rodent cochleae (Hasson et al. 1997). Myosin VI is a "backwards" directed myosin, moving toward the base of the hair bundle rather than the tips as in other myosins (Wells et al. 1999). Collectively, these three studies

implicate myosin VI in anchoring the plasma membrane surrounding each stereocilium to the cuticular plate (Hasson et al. 1997; Self et al. 1999).

Unconventional myosins are important in fish hair cells as well. Zebrafish *myo6b* (*satellite*) and *myo7a* (*mariner*) mutants exhibit phenotypes similar to corresponding mammalian myosin mutants and show defects in auditory and vestibular function (Ernest et al. 2000; Kappler et al. 2004; Seiler et al. 2004). Given the diversity in fishes, however, studies in other species are needed to confirm a role for myosins in the largest group of vertebrates. If myosins are found in all vertebrate hair cells, comparative studies between species that differ in intracellular protein localization may point to functional distinctions between hair cells. Chapter two of this dissertation looks at myosins VI and VIIa in fish hair cells.

Fish myosin VI and genome duplication

Studies of the zebrafish *satellite* mutant (described above) showed that zebrafish have two myosin VI genes (Kappler et al. 2004; Seiler et al. 2004). This duplication is not unique to myosin VI, as fish genomes have two copies of many mammalian genes scattered throughout the entire genome (Taylor et al. 2003; Jaillon et al. 2004). This duplication phenomenon was first discovered in zebrafish *hox* gene clusters and is now well documented in model fishes (Amores et al. 1998; Naruse et al. 2000; Aparicio et al. 2002).

These findings sparked the fish-specific genome duplication hypothesis, which proposes that a complete genome duplication occurred in an ancestral actinopterygian (ray-finned) fish (Amores et al. 1998; Taylor et al. 2001a). Genome

duplications are essentially tetraploidization events and have occurred many times throughout evolution in such diverse organisms as yeast (*Saccharomyces cerevisiae*), the model plant *Arabidopsis thaliana*, and the common carp (*Cyprinus carpio*) (Wolfe 2001; David et al. 2003; Seoighe 2003).

There are three lines of evidence favoring the fish-specific genome duplication hypothesis. The first is that the zebrafish genome contains duplicates of approximately 20% of mammalian genes (Postlethwait et al. 2004). Duplicates exist in blocks of syntenic regions (regions of conserved gene order), suggesting these are not isolated tandem gene duplications (Postlethwait et al. 1998; 2000). Finally, molecular clock approaches to dating these duplicate genes show that many arose during the same period, between 200-400 million years ago (Christoffels et al. 2004; Vandepoele et al. 2004).

Critics of the fish-specific duplication hypothesis use some of the same evidence discussed in the previous paragraph to argue against complete genome duplication in fishes. Robinson-Rechavi et al. (2001) analyzed 37 gene families in at least three actinopterygian lineages and found that 19% of the resulting gene phylogenies showed a topology consistent with whole genome duplication in the last common ancestor of the study species. Independent duplications in specific lineages were noted in 30% of the gene phylogenies. They argue that these data support many tandem (single gene) duplication events in fishes rather than an ancestral genome wide duplication.

The recent sequencing and annotation of the pufferfish (*Tetraodon nigroviridis)* genome provides critical evidence supporting a genome wide

duplication in fishes (Jaillon et al. 2004). *Tetraodon* and fugu (*Takifugu rubripes*, another pufferfish) share at least 675 paralogous (related via duplication) gene pairs and these genes are found on every chromosome. The presence of hundreds of gene pairs scattered throughout the genome confirms a complete duplication in at least the pufferfish ancestor.

While this evidence for genome duplication is compelling, molecular clock approaches to dating the duplication provide only a rough estimate of the timing (Christoffels et al. 2004; Vandepoele et al. 2004). Phylogenetic approaches are then used to narrow this window. Studies of *Hox* gene clusters in sarcopterygian (lobe-finned) fishes and the bichir, an ancestral actinopterygian, confirm that the duplication is confined to ray-finned fishes (Longhurst and Joss 1999; Koh et al. 2003; Chiu et al. 2004). Signatures of duplication are found in diverse teleosts such as zebrafish, medaka (*Oryzias latipes*), and pufferfishes, indicating duplication occurred at least 140 million years ago and prior to the split of these groups from a common ancestor (Amores et al. 1998, 2004; Naruse et al. 2000; Aparicio et al. 2002; Hedges and Kumar 2002). Figure 1.5 shows the evolutionary time spanning this duplication event.

These large-scale duplication events offer unique opportunities for evolution, as evolutionary rates in one or both paralogs often increase for a short time following duplication (Lynch and Conery 2000). While this often leads to a loss of function due to deleterious mutations, some duplicates take on novel functions (neofunctionalization) or partition functions across both paralogs (Ohno 1970; Force

et al. 1999; Postlethwait et al. 2004). This subfunctionalization can lead to subtle adaptive changes in spatial or temporal gene expression patterns (Force et al. 1999).

Loss of a duplicate also has evolutionary implications through the process of divergent resolution. Divergent resolution occurs when genome duplication takes place prior to a geographic split in two populations (Lynch and Conery 2000; Lynch and Force 2000; Taylor et al. 2001b). Different duplicates will be lost in each population so that when the populations are reunited, hybridization results in offspring with one functional allele and one null allele. In this way, genome duplication leads to reproductive isolation and therefore speciation. Divergent resolution following a fish-specific genome duplication may have driven the teleost radiation if this duplication is confined to teleosts. Chapter three of this dissertation looks at the timing of the fish-specific genome duplication.

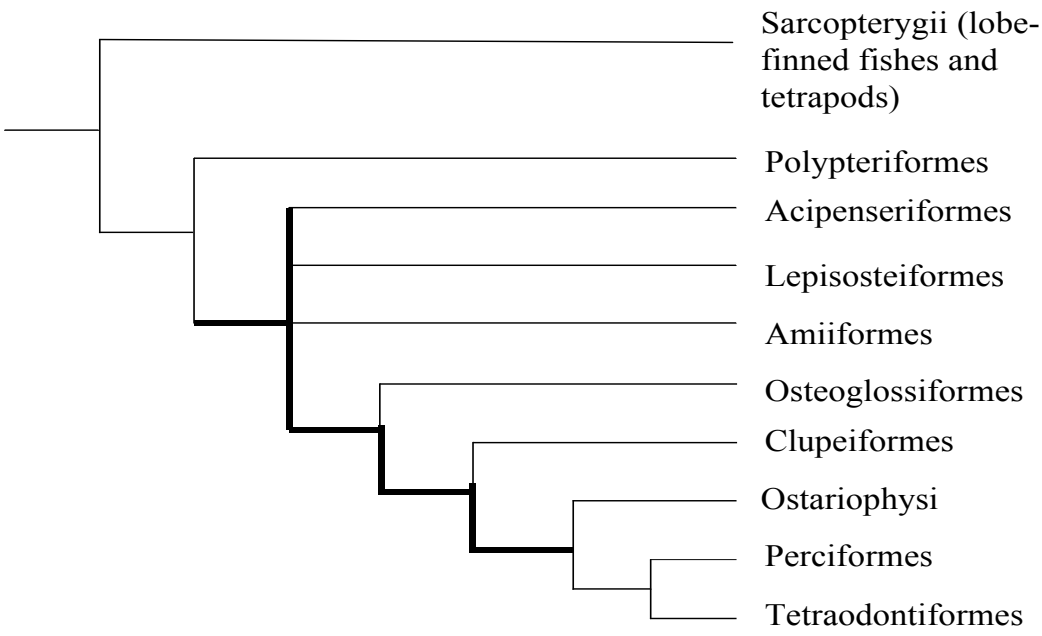

Figure 1.5. Timing of the fish-specific genome duplication. The bold line represents the ancestral lineages where this duplication may have occurred.

Chapter overview

This dissertation looks at unconventional myosins in fishes. Chapter two examines gene expression and protein distribution of myosins VI and VIIa in the ears of evolutionarily diverse fishes. Gene expression was studied with reverse transcription-polymerase chain reaction (RT-PCR) and in some cases with *in situ* hybridization when accurate localization was necessary. Protein distribution was examined with indirect immunofluorescence. We hypothesize that both myosins are expressed in all fish inner ears but that differences in protein distribution underlie functional differences between species and/or epithelia.

Chapter three takes advantage of the duplicated *myo6* paralogs in zebrafish to examine the timing of genome duplication in fishes. RT-PCR was used to amplify *myo6* genes from both teleost and non-teleost ray-finned fishes. These genes were then cloned and sequenced and the sequences were used for phylogenetic analysis. We predict that all teleost fishes have two *myo6* paralogs while non-teleosts have only one.

Chapter four offers a summary of the major findings in the two primary chapters and fits this dissertation work into the broader contexts of myosin function, genome duplication, and fish evolution.

Chapter 2: Non-mammalian models for human hereditary deafness: Myosin VI and VIIa distribution in the ears of anamniotes

Introduction

Congenital deafness affects one out of every 1000 births in the U.S., making this the most prevalent of genetic sensory disorders (reviewed in Cryns and Van Camp 2004). Hereditary deafness is highly heterogeneous, with over 70 non-syndromic deafness loci identified to date (Cryns and Van Camp 2004). Mouse models of human deafness are extremely useful for mapping deafness loci and for understanding the expression profiles and function of deafness genes (e.g., Avraham et al. 1995; Littlewood Evans and Müller 2000; Karolyi et al. 2003).

Recently, the zebrafish (*Danio rerio*) has been added as a valuable model for hereditary deafness studies, with large-scale mutant screens underway and many interesting mutations currently under study (Nicolson et al.1998; reviewed in Whitfield 2002). Fishes are the largest and most diverse vertebrate group and show substantial diversity in ear structure across taxa (Popper and Coombs 1982; Popper and Fay 1999; Ladich and Popper 2001). Therefore, restricting the choice of fish models to the zebrafish bypasses the diversity in fish ears (and other structures) that is potentially useful in understanding ear structure and function and in interpreting mutant phenotypes. Comparative studies between diverse fishes provide novel opportunities to understand structure, function, and evolution in vertebrate systems.

Fish inner ears are similar to mammalian ears in many ways. Ears of all gnathostomes (jawed vertebrates, including fish and mammals) have three

semicircular canals with sensory cristae and at least two sensory maculae overlaid with calcareous structures (otoliths or otoconia). Importantly, all vertebrate sensory epithelia contain mechanosensory hair cells for sensing vestibular and auditory stimuli. Hair cell morphology is also generally conserved in vertebrates, as all hair cells have an apical tuft of stereocilia (the hair bundle) that serves as the mechanosensory organelle (see Hudspeth 1985; Coffin et al. 2004). Hair cells synapse with either afferent or efferent fibers (or both) from the eighth cranial nerve (Simmons 2002; Fuchs et al. 2003).

The vestibular labyrinth (sensory maculae and semicircular canals) is probably homologous across vertebrates (Wever 1974; Popper et al. 1992). However, auditory function depends on different structures in different taxa. Amniotes (reptiles, birds, and mammals) have a basilar papilla (organ of Corti in mammals) devoted to auditory function. Fish have no distinct hearing organ such as a cochlea, and different groups of fishes employ different otolithic end organs for auditory function (reviewed in Popper and Fay 1999; Popper et al. 2003; Ladich and Popper 2001). Otophysan fishes such as the zebrafish and goldfish (*Carassius auratus*) are thought to primarily use the saccule for hearing (Furukawa and Ishii 1967), while clupeid fishes (including the American shad, *Alosa sapidissima*) have an unusual utricle that serves as the primary auditory organ, at least for ultrasonic frequencies (Mann et al. 2001; Higgs et al. 2004; Plachta et al. 2004).

This chapter looks at phylogenetically diverse fish ears from a cellular and molecular point of view. The focus is on fish hair cells, with particular attention to the stereociliary bundle. Hair bundle stereocilia are comprised of an actin

paracrystalline core with associated actin-binding proteins (Tilney et al. 1983; Belyantseva et al. 2003a). Myosins, as actin motors, play an important role in bundle structure and function and hair cells are exceptionally rich in myosins (reviewed in Friedman et al. 1999).

Myosins are mechanoenzymes that hydrolyze ATP to move along actin filaments. There are currently 18 classes of myosins; the type II, or "conventional" myosins that form filaments, and the remaining unconventional (non-filament forming) myosins (Sellers 2000; Berg et al 2001). At least six myosins are expressed in hair cells (Gillespie et al. 1993; Hasson et al. 1997; Liang et al. 1999; Lalwani et al. 2000; Walsh et al. 2002; Donaudy et al. 2003). Three of these (VI, VIIa, and XVa) have been intensely studied for their roles in hair bundle maturation and maintenance (Belyantseva et al. 2003b; Self et al. 1998, 1999). Mice with mutations in any of these three myosins exhibit stereocilia abnormality, congenital deafness, and vestibular dysfunction, demonstrating the critical nature of myosins in hair bundle function (Avraham et al. 1995; Gibson et al. 1995; Probst et al. 1998). Mutations in each of these three myosins have also been identified in human families with congenital deafness (Weil et al. 1995; Wang et al. 1998; Melchionda et al. 2001). Therefore, a better understanding of unconventional myosins in hair cells may lead to treatments for human hereditary deafness.

In the present study, we examine myosin VI and VIIa distribution in the ears of evolutionarily diverse fishes. We have selected the jawless sea lamprey (*Petromyzon marinus*), the cartilaginous clearnose skate (*Raja eglanteria*), and the "primitive" bony lake sturgeon (*Acipenser fulvescens*) to represent specific points in

fish evolution. We also look at three diverse teleost fishes, the zebrafish, American shad, and oscar (*Astronotus ocellatus*), species that are thought to use different end organs for hearing, in order to better understand myosin distribution, and therefore function, in vertebrate hair cells. We then extend the comparison by using the aquatic African clawed frog *Xenopus laevis* as a representative anamniotic tetrapod. Phylogenetic relationships between the study species are shown in Figure 2.1

Comparative immunocytochemical studies are critical for uncovering important structural differences with functional implications (e.g., Hasson et al. 1997). As the precise functions of myosins VI and VIIa in hair cells are not fully understood, the present study will deepen our understanding of these proteins that can be associated with deafness. Both myosins are found in all hair cell examined but intracellular protein localization differs in a species and end organ-specific manner, suggesting both functional and evolutionary implications for these important hair cell proteins.

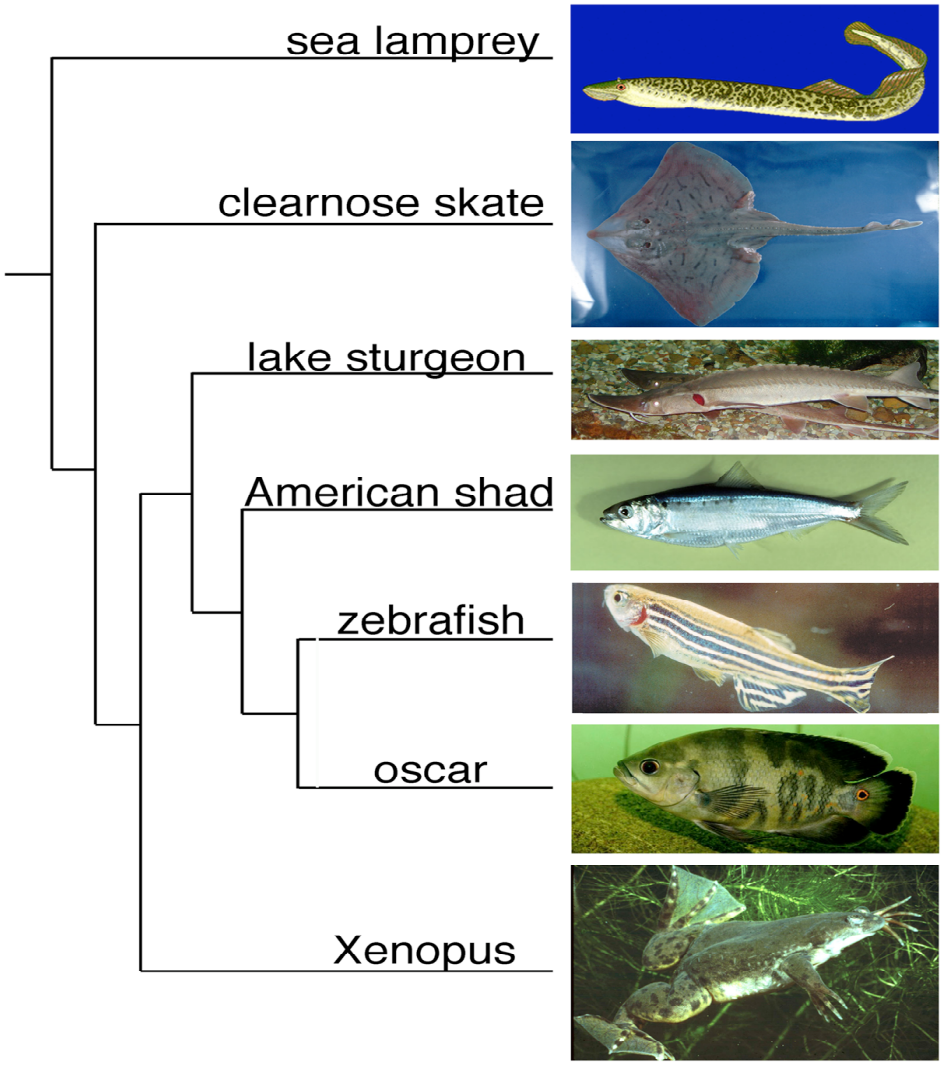

Figure 2.1. Simplified phylogenetic relationships between the species used in this study. The lineage leading to *Xenopus* also contains all other tetrapods (land vertebrates) as well as the sarcopterygian (lobe-finned) fishes. Based on Nelson (1994). Lamprey, sturgeon, shad, zebrafish, and oscar pictures are from fishbase.org.

Materials and Methods

Animals

Sea lamprey (*Petromyzon marinus*), lake sturgeon (*Acipenser fulvescens*), American shad, and *Xenopus* were all gifts from private sources. Skates were purchased from the Marine Biological Laboratory, Woods Hole, MA. Zebrafish and oscars were purchased commercially. All animals were sacrificed with an overdose of buffered MS-222 (Sigma-Aldrich) followed by decapitation. All procedures were approved by the University of Maryland Institutional Animal Care and Use Committee.

RT-PCR

Inner ears were dissected from freshly killed animals (at least three animals per species) and stored in RNAlater (Ambion). Total RNA was extracted with Stratagene's Absolutely RNA RT-PCR Miniprep kit and reverse transcribed with M-MLV reverse transcriptase (Invitrogen). PCR primers were designed to highly conserved regions of each myosin. Primer sequences are shown in Table 2.1.

All PCR was performed using either Platinum Taq (Invitrogen) or Easy-A PCR enzyme (Stratagene) with equal success. PCR programs used a 94 °C melting temperature for 30 sec, an initial 60° C annealing temperature for 45 sec, and a 72° C elongation temperature for 45 sec. After five rounds of this program, 35 additional rounds of PCR were performed with a 55° C annealing temperature (all other PCR parameters were unchanged).

PCR products were purified with Qiagen's PCR purification kit and cloned into the pCR2.1 vector using TOPO cloning (Invitrogen). Plasmid DNA was purified using Eppendorf's FastPlasmid Mini kit (Brinkmann Instruments) and sequenced on an ABI 3100 automated sequencer using the Dye Terminator reaction. At least four clones were sequenced for each product and analyzed with LaserGene version 5.5 (DNASTAR Inc.) to create one consensus sequence for each gene in each species. Sequence identity was confirmed with BLAST searches (Altschul et al. 1997).

Immunohistochemistry

Whole-mount epithelia. Myosins VI and VIIa were immunolabeled using polyclonal antibodies. Ears from four animals of each species were used for each antibody. Tissue was fixed in 4% paraformaldehyde (PFA) for 1 hour at 4ºC and sensory epithelia were dissected into 0.1 M phosphate buffer (PB). All solutions for tissue processing were made using PB. Tissue was briefly digested with type XI collagenase and permeated with 1% Triton-X (both from Sigma-Aldrich), blocked in 10% normal goat serum (Sigma-Aldrich), and exposed overnight to primary antibody at a concentration of 1:150 (myosin VI) or 1:250 (myosin VIIa) in 1% goat serum. Primary antibodies were provided by Dr. Tama Hasson at the University of California, San Diego.

Following incubation in primary antibody, tissue was rinsed in 1% goat serum and exposed to Alexa 594 goat anti-rabbit (Molecular Probes). Tissue was then rinsed in PB, double labeled with Alexa 488 phalloidin (Molecular Probes) to label actin-

rich stereocilia, and coverslipped with Prolong-Antifade (Molecular Probes). The primary antibody was omitted for negative controls.

Cryosections. Ears were fixed and dissected as described for whole mounts. Epithelia were saturated in increasing sucrose solutions (5%-25%), and embedded in Tissue Tek® OCT embedding medium (Sakura). Sections of 12 μm thickness were cut using a Leica CM3050S cryostat. Post-embedding immunocytochemistry was the same as used for whole epithelia except that the collagenase/triton step was omitted, and all PB rinses included 0.5% Tween-20 (Sigma-Aldrich).

Image acquisition and processing

A Zeiss LSM 510 confocal microscope was used to image all samples. Optical sections were taken at varying intervals (0.1-2μm) and analyzed with Adobe Photoshop (Macintosh, v. 7.0) software. Adobe Photoshop was used to adjust brightness and contrast but not to alter image content. Three-dimensional reconstructions were performed with LSM software 5.0 from Zeiss.

Western blotting

Ears and brains from each species (minimum two animals per species, more for small animals such as zebrafish) were dissected in protease inhibitors (Sigma-Aldrich) and homogenized in Laemmlli sample buffer (Bio-Rad) with 5% β-mercaptoethanol (Calbiochem). While sufficient tissue was not available from all species, the use of zebrafish, clearnose skate, American shad, and *Xenopus* represents sufficient phylogenetic diversity that differences in protein weight or antibody binding affinity should be detected. Proteins were separated on a 10% denaturing

tris-glycine gel (Invitrogen), transferred to a PVDF transfer membrane (Amersham Biosciences) and probed with primary antibody (same antibodies as for immunocytochemistry). Membranes were then exposed to donkey anti-rabbit horseradish peroxidase and developed using an ECL+Plus detection kit (all from Amersham Biosciences). As both myosins VI and VIIa are in mouse inner ear (Avraham et al. 1995; Gibson et al. 1995), mouse cochlear tissue was used for positive control protein (mouse cochlear protein was a gift from Dr. Mireille Montcouquiol). Primary antibody was omitted for negative controls. Anti-GAPDH, an antibody to a housekeeping gene, was used for positive control reactions.

Epitope expression

Both myosin antibodies were raised to mammalian antigens (Hasson et al. 1997). Therefore, these antibodies may not properly cross-react with fish tissue. Additionally, recent work shows that zebrafish possess two myosin VI genes, *myo6a* and *myo6b* (Kappler et al. 2004; Seiler et al. 2004). It is not known whether the myosin VI antibody used here binds to zebrafish myo6a, myo6b, or to both proteins. To determine which protein the antibody detects we expressed zebrafish myosins in mammalian cell cultures. PCR primers were designed to amplify the cDNA region corresponding to the antigenic epitope for myo6a, myo6b, or myo7a. Forward primers included an EcoR1 (myo6a and 6b) or Xho1 (myo7a) restriction site followed by a start codon. Reverse primers included a stop codon and Sal1 (myo6a and 6b) or BamH1 (myo7a) site. Expression primer sequences are shown in Table 2.2.

Myo6a or *6b* RT-PCR products were directionally cloned into the pIRES2-EGFP vector (BD Biosciences) and used to transfect NIH 3T3 cells. *Myo7a* products were cloned into the same vector and transfected into HEK293 cells. Cells that expressed EGFP were successfully transfected, while EGFP-negative cells served as internal negative controls. Transfected cells were plated on coverslips, fixed in 4% paraformaldehyde, and processed for immunocytochemistry as described for tissue sections (above).

In situ hybridization

Myo6b expression in zebrafish hair cells was confirmed by Seiler et al. (2004). However, in situ hybridization for *myo6a* in that paper did not yield clear results. Gene expression localization for *myo6a* was therefore repeated here. Zebrafish *myo6a* RT-PCR products were enzymatically cut out of the TOPO cloning vector (see RT-PCR, above), cloned into Invitrogen's pBlueScript KS+ vector, and used to construct digoxigenin-labeled probes for *in situ* hybridization.

Whole-mount *in situ* hybridization was performed on zebrafish ears (six animals) following 1 hour of fixation in 4% PFA. Epithelia were digested in proteinase K (Invitrogen), briefly post-fixed in fresh 4% PFA, blocked in hybridization solution, and hybridized overnight at 65° C. Hybridization was detected with alkaline phosphatase-conjugated anti-digoxigenin antibody and colorized with NBT and BCIP. Negative control reactions used a sense RNA probe.

Following color development, epithelia were embedded in Immunobed plastic

resin (Polysciences) and cut into 3-5 μm thick sections for viewing with DIC microscopy. Zebrafish brain sections were used as a positive control.

Table 2.1. Primers for RT-PCR. Primers were designed to regions of *myo6* and *myo7a* that are conserved between zebrafish and mammals.

Gene	Forward primer	Reverse primer
myo6a	CAAGCTGATACCTCAAAACC	CTATACAGTCCTGATTATCAA
myo6b	CTATGCAGTCCTGATTATCA	GAACCAGTCAAGTCAGACTCT
myo7a	AGATGTTCGGCTTCCTGGGA	TTCCGAGTGTCTCGTAGATT

Table 2.2. Primers for epitope expression in cultured cells. Start codons in forward primers and stop codons in reverse primers are indicated in bold. Restriction sites are in gray.

Gene	Primer
myo6a	For: GATCGAATTC**ATG**GTTGCTCCACCACAAAAGCTCAAGAGCTT
	Rev: GATCGTCG**ACT**CAGTTCCTCAGGTACTGGATTCCC
myo6b	For: GATCGAATTC**ATG**GCCCAGAACGAGGCAGAACT
	Rev: GATCGTCG**ACT**CAAGCATTCTTCAAGTATTGGAT
myo7a	For: CTCGAG**ATG**TACAAACGACTCAAAGGAGAGTAC
	Rev: GGATCC**TCA**GTAGGTTTTCTTTCCGAGTG

Results

Gene Expression

We examined unconventional myosin expression and distribution in the inner ears of evolutionarily diverse fishes as well as the aquatic frog *Xenopus* (see Fig. 2.1 for the phylogenetic relationships between study species). Ears of all species express *myo6* and *myo7a* as determined by RT-PCR. Teleost (*Alosa*, *Astronotus*, and *Danio*) ears express both *myo6* paralogs (*myo6a* and *myo6b*). RT-PCR results are shown in Figure 2.2. As Seiler et al. (2004) found that only *myo6b* was expressed in larval zebrafish ears, we used *in situ* hybridization to examine *myo6a* expression patterns in the adult ear (Fig. 2.3). *Myo6a* is not expressed in sensory hair cells in adult zebrafish (Fig. 2.3A). No labeling is detected when sense probed was used as a negative control (Fig. 2.3B). The same antisense probe labels zebrafish brain, a tissue known to express *myo6a*. Zebrafish myo6a and myo6b are 83% identical at the amino acid level. Alignment of full-length zebrafish sequences is shown in Figure 2.4

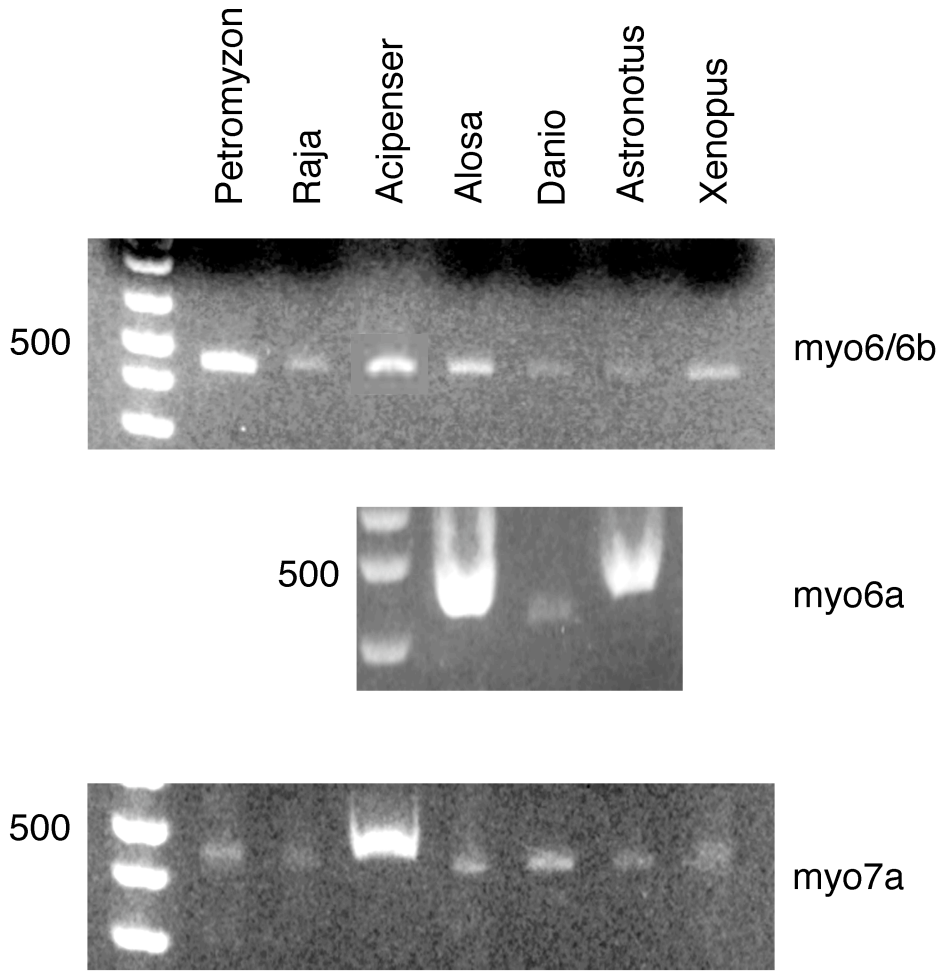

Figure 2.2. Expression of unconventional myosin genes in fish and *Xenopus* inner ears. Each band is a PCR-amplified cDNA sequence. Top: *Myo6b* and ancestral *myo6* expression. Middle: *Myo6a* expression in teleosts only. Bottom: *Myo7a* expression. PCR products were cloned and sequenced for verification.

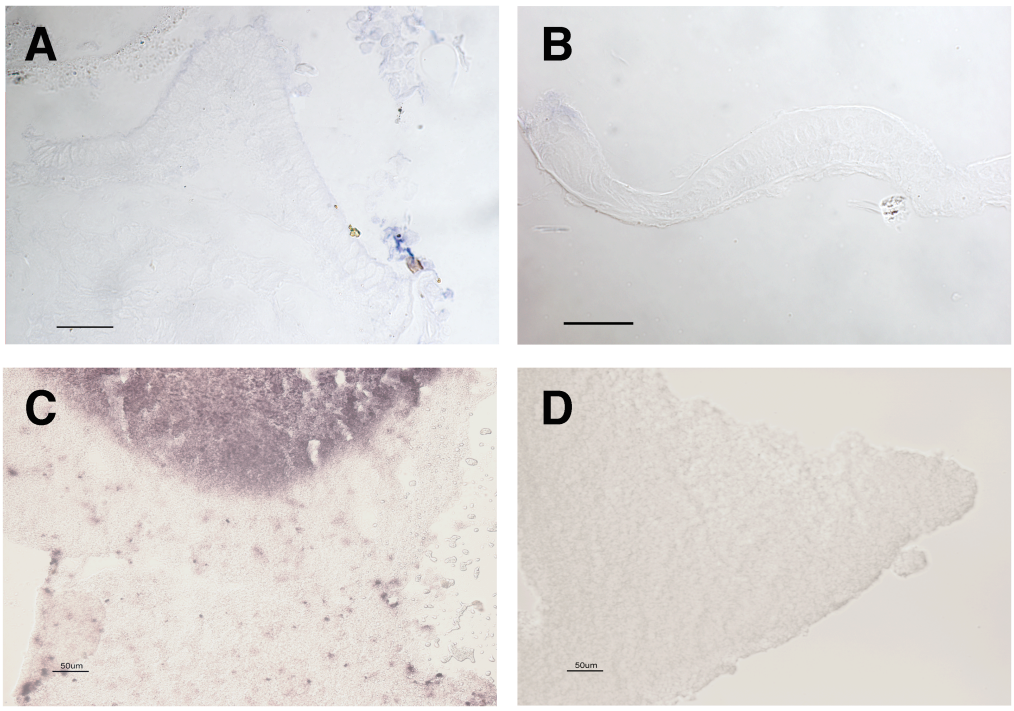

Figure 2.3. Localization of *myo6a* expression in the zebrafish ear as shown by *in situ* hybridization. A) Antisense probe labeling in a semicircular canal crista. B) Sense (negative control) probe labeling in the utricle. C) Antisense probe and D) sense probe labeling of zebrafish brain sections. Scale bars in A and B are 25 μm.

```
MDDGKPVWAPHPTDGFQLGRIIDISADSLTIEPLNQRGKNFQAPVDQVFP 50
MDDGKLVWAPHPTDGFQLGMIVDIGADALTIEPLHQRGKTFLAPISQVFP 50
***** ************* *:**.**:******:****.* **:.****

AEDDVNKHVEDNCSLMYLNEATLLNNVRVRYSKDKIYTFVANILIAVNPY 100
AEDDVNKHVEDNCSLMYLNEATLLNNIRIRYSKDMIYTFVANILIAVNPY 100
**************************:*:***** ***************

CEIPKLYSPETIKQYQGRSLGTLPPHVYAIADKAYSDMKVLKMSQSIIVS 150
FDIPKLYSPESIKSYQGRSLGTLPPHVYAIADKAYRDMRVLKMSQSIIVS 150
 :*********:**.********************** **:***********

GESGAGKTENTKFVLRYLTTSYGTGQDIDERIVEAKPLLEAFGNAKTVRN 200
GESGAGKTENTKFVLRYLTTSYGTGQDIDERIVEANPLLEAFGNAKTVRN 200
************************************:**************

NNSSRFGKFVEIHFNEKNAVVGGFVSHYLLEKSRICTQGQEERNYHIFYR 250
NNSSRFGKFVEIHFNEKNAVVGGFVSHYLLEKSRICMQSQEERNYHIFYR 250
********************************** *.***********

LCAGAPEDIREKFHLSSPDCFRYLCRGCTRYFSTKDSDKLIPQNRKSPE- 299
LCAGASEDIRNMLHLNSPDSFRYLNRGCTRYFANKDSDKQIMQNXKSPED 300
*****.****: :**.***.**** *******:.***** * ** ****

--LLKAGPLKDPLLDDHADFNRMSVAMKKIGLDDTEKLNLFRVVAGVLHL 347
HKHGKVGALKDPLLDDLGDFNRMVVAMKKIGLDDTEKLNLFRVVAGVLHL 350
    *.*.******** .***** **************************

GNIDFEEAGSTSGGCVLKKTCGQSLQFCAELLGLDEEDLRVSLTSRVMLT 397
GNIDFEETGSTSGGCILKNQSSQTLEYCADLLGLDQDDLRVSLTTRVMLT 400
*******:*******:**: ..*:*::**:*****::*******:*****

TAGGTKGTVIKVPLKVEQASSARDALAKAIYSRLFDHVVTRINQCFPFDS 447
TAGGAKGTVIKVPLKVEQANNARDALAKAVYSRLFDHVVKRVNQCFPFDT 450
****:**************..********:*********.*:*******:

SAHFIGVLDIAGFEYFEHNSFEQFCINYCNEKLQQFFNERILKEEQELYQ 497
SSNFIGVPDIAGFEYFEHNSFEQFCINYCNEKLQQFFNERILKEEQELYQ 500
*::**** ****************************************

REGLGVNEVHYVDNQDCIDLVESKVVGILDILDEENRLPQPSDQHFTETV 547
REGLGVNEVHYVDNQDCIDLVEAKLVGVLDILDEENRLPQPSDQHFAEAV 550
**********************:*:**:********************:*:*

HSKHKDHFRLTVPRKSKLQVHRNVRDDEGFIIRHFAGAVCYETTQFVEKN 597
HSKHKDHFRLTVPRKSKLTIHRNLRDDEGFIIRHFAGAVCYETTQFVEKN 600
****************** :***:**************************
```

```
NDALHMSLACLVSESKDKFIGELFENSNHSKDTKQKAGKLSFISVGNKFK 647
NDALHMSLESLVCESKDKFVRDLFENNSNSKDSKQKAGKLSFISVGNKFK 650
******** .**.******: :****..:***:****************

TQLNILLEKLHSTGSSFVRCVKPNLKMVGHHFEGAQILSQLQCSGMVSVL 697
TQLNLLLEKLRSTGSSFIRCVKPNLKMVSHQFEGAQILSQLQCSGMVSVL 700
****:*****:******:**********.*:******************

DLMQGGFPSRAPFHELYNMYKQYMPAKLTRLDPRLFCKALFKALGLNEND 747
DLMQGGFPSRAPFHELYNMYKQYMPNKLTRLDPRLFCKALFKALGLNEND 750
************************ ************************

YKFGLTKVVFRPGKFAEFDQIMKSDPDHLAELVKRVNKWLICSRWKKVQW 797
YKFGLTRVFFRPGKFAEFDQIMKSDPDHLAELVKRVNKWLVCSRWKKVQW 800
******:*.*********************************:*********

CALSVIKLKNKMLYRAQACVQMQKTVRMWLCRRKHKPRIDGLVKAQNLKK 847
CTLSVIKLRNKMSYRASACIRIQKTVRMWLCRRRHKPRVDGLVKVKNLRK 850
*:******:*** ***.**:::***********:****:*****.:**:*

RMEKLNEVVSGLKEGKQEMSKHMQDLDSSIDAHIRKIKSIVMSRMDIDHE 897
RMERFNEAVNGLKEGKAEMSKQIEELAASTDALMAKIKTTVMSRKEIEQE 900
***::**.*.****** ****:::::* :* ** : ***: **** :*::*

HQALVTRSQELLSAMQKKKQEEEEMERLKRIQEEMEKERKRREEEEQKRK 947
YEGLVKRSEQLLSSMQKKKQEQEETERLKHIQEZMEKERKRHEEEEQLRK 950
::.**.**::***:*******:** ****:*** *******:***** **

REEEERRQKAEMELKRKQEEEERKKREEEERKLQEEMELQLEAEREQETS 997
QEEEDRRMKSEMEQKRKQEEEERKKREEEERVLQAELEMQLALDREEETQ 1000
:***:** *:*** ***************** ** *:*:**  :**:**.

RQAVLEQERRDRELALRIAQSEAELIPEETPPDAGLRSVAPPQKLKSLTM 1047
RQTILEQERRDRELAMRIAQNEAELIQDEAQMDPILR------------- 1037
**::***********:****.***** :*:  *. **

EEMAKEMSDLLARGPQVSANNAQADVKKYELSKWKYAEVRDAINTSCDIE 1097
RDATTGVWFFTEMGAQVQANKVAAGVKKYDLSKWKYAELRDAINTSCDIE 1087
.: :. :  :   *.**.**:. *.****:********:***********

LLAACREEFHRRLKVYHAWKSKNKKRNVQEEQRAPKAITDYVGIIGSQAF 1147
LLAACREEFHRRLKVYHAWKSKNKKRNTDTEMRAPKSVTDYA-------- 1129
**************************.: * ****::***.

IAQQNPVVPAAVPRQHEIVMNRQQRFFRIPFIRPGDQYKDPQSKKKGWWY 1197
--QQNPAPPVPA-RQQEIAMNRQQRYFRIPFIRPADQYKDPQNKKKGWWY 1176
  ****. *... **:**.******:********.*******.*******
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```
AHFDGPWIARQMELHPDKHPILLVAGKDDMEMCELSLEETGLTRKRGAEI 1247
AHFDGPWIARQMELHPDKQPILLVAGKDDMEMCELSLEETGLTRKRGAEI 1226
******************:********************************

LPRQFEEIWERCGGIQYLRNAIESRQARPTYATAMLQSMLQSMLK 1292
LPRQFEEIWERCGGIQYLKNAIESKQARPTYATAMLQNLLK---- 1267
******************:*****:************.:*:
```

Figure 2.4. Amino acid alignment of zebrafish myo6a (top sequence in each pair, accession no NP_001004111) and myo6b (bottom sequence, accession no: NP_001004110) generated with ClustalW. Color and symbol key: *identical residue, : conserved, . semi-conserved, not conserved.

Immunohistochemistry

All hair cells examined in each of the species contain both myosins, while other inner ear cell types do not contain myosins VI or VIIa (Fig. 2.5-2.7). This labeling is specific, as controls that lacked primary antibody do not show myosin immunolabeling (Fig. 2.8). Myosin VIIa is present in the cytoplasm and stereocilia of all hair cells examined in this study (Fig. 2.7). Labeling appears evenly distributed along the length of the stereocilia. This pattern is consistent in all species examined here, although there is some variability in fluorescence intensity that was not quantified. Myosin VI distribution, however, differs between epithelia within an organism and between species.

Myosin VI is present in the cytoplasm, but not the nucleus, of all hair cells examined (Figs. 2.5-2.6). Labeling is enhanced in the cuticular plate at the apical surface of the cell. These findings are consistent with studies in frogs and mammals (Hasson et al. 1997). However, myosin VI labeling in the stereocilia is not identical across species. Myosin VI is present throughout the length of the stereocilia in sea lamprey hair cells of the macula communis (the single otolithic epithelium in this fish) and canal cristae (Fig. 2.9). Myosin VI is also found in stereocilia of zebrafish inner ear hair cells in every end organ (Fig. 2.5). In all other jawed fishes examined here (clearnose skate, lake sturgeon, American shad, and oscar), myosin VI is present in saccular and lagenar hair bundles but is not seen in utricular hair bundles (Fig 2.5).

Myosin VI distribution in *Xenopus* is different from that of the fishes studied. Once again, the protein is present throughout the cytoplasm, and while the stereocilia

of the utricle contain myosin VI, those in the saccule do not (Fig. 2.6). Hair cells in both the amphibian and basilar papillae resemble those of the *Xenopus* utricle and lagena in that they also contain myosin VI in the stereocilia (data not shown).

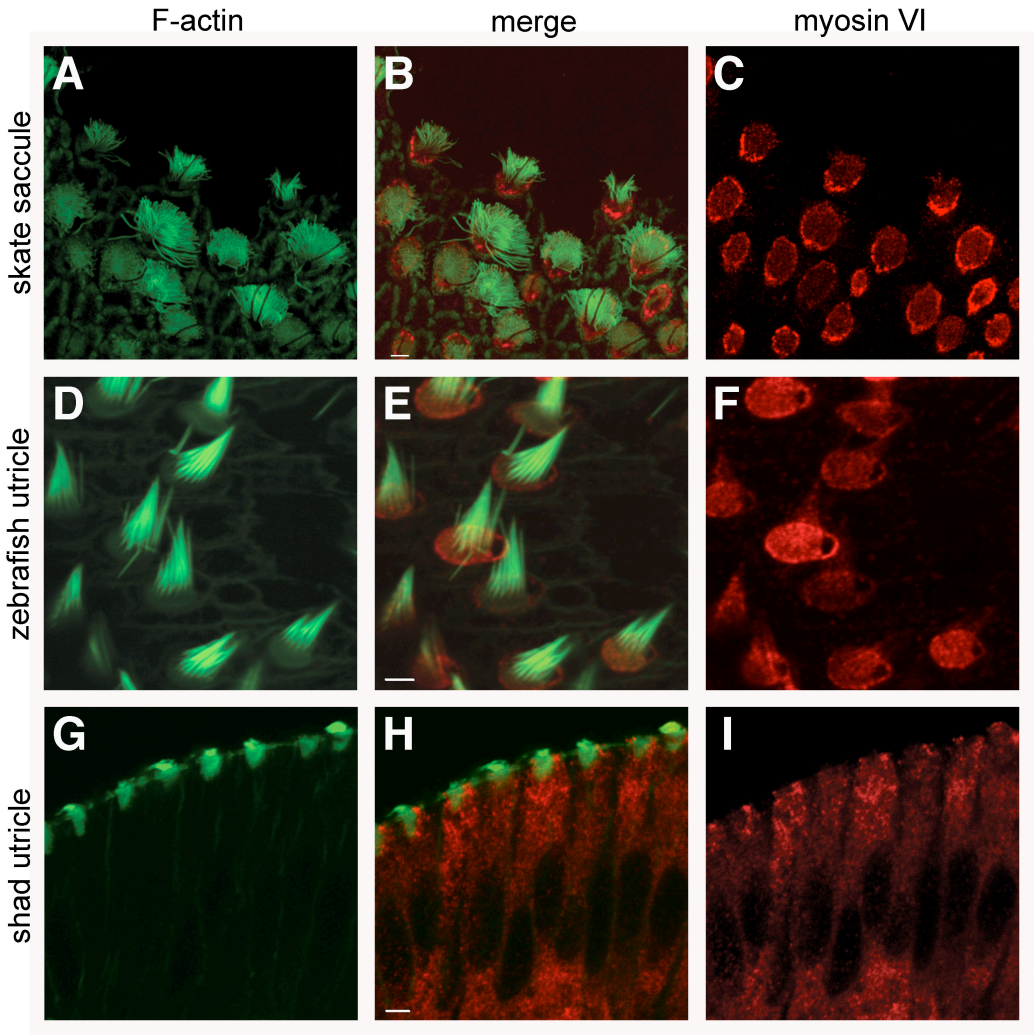


Figure 2.5. Myosin VI distribution in fish hair cells. The left column is phalloidin label (green), the right column in myosin VI immunofluorescence (red), and the middle column is the merged image. A-C) clearnose skate saccule, (D-F) zebrafish utricle, (G-I) American shad utricle. Skate and zebrafish images are whole-mount epithelia, shad images are from a 12 µm thick cryosection. All scale bars are 2 µm.

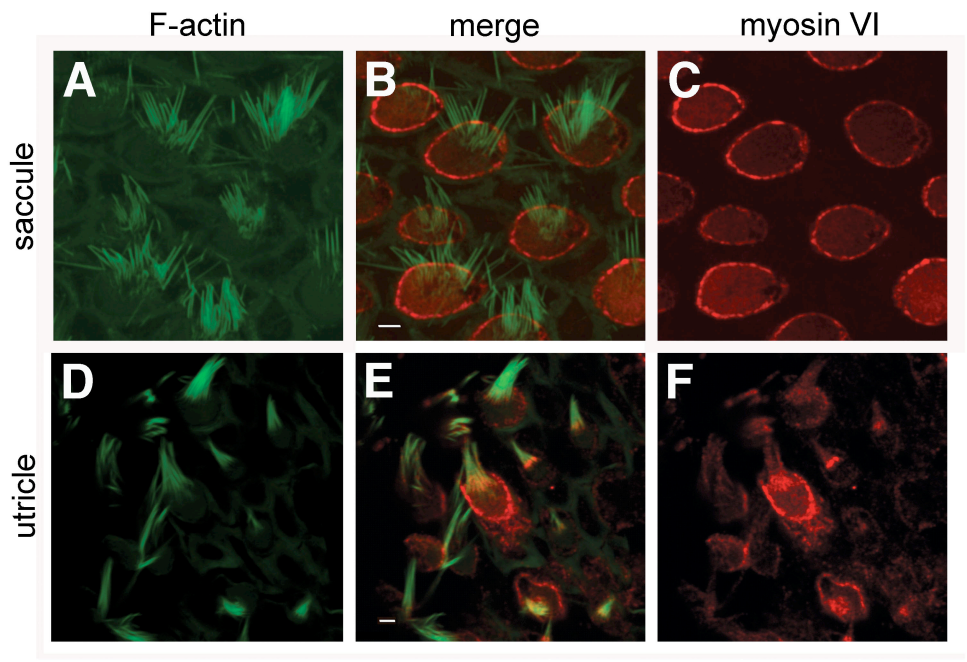


Figure 2.6. *Xenopus* hair cells immunolabeled for myosin VI. A-C) saccular hair bundles lack myosin VI, which is present in (D-F) utricular hair bundles. Scale bars are 2 μm.

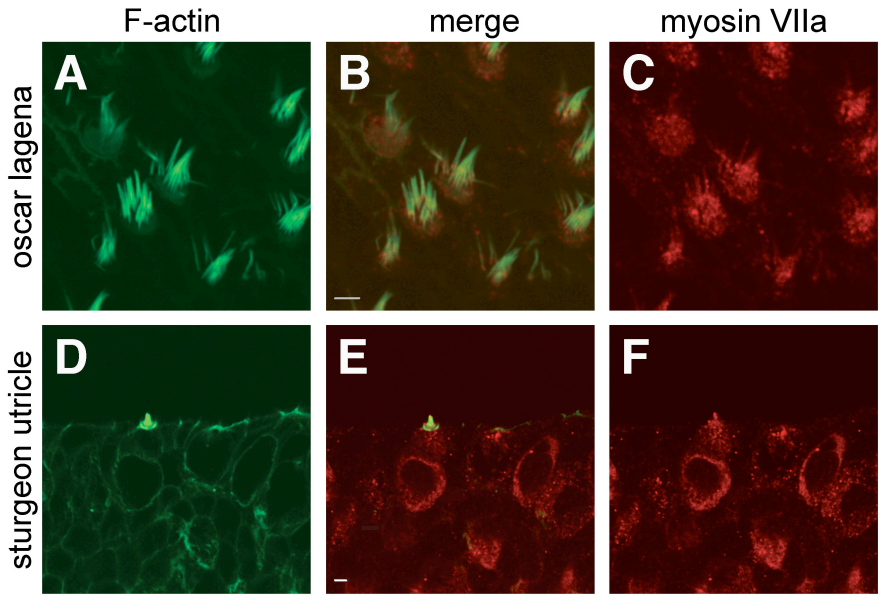


Figure 2.7. Myosin VIIa distribution in hair cells of (A-C) oscar lagena and (D-F) lake sturgeon utricle. Scale bars are 2 µm.

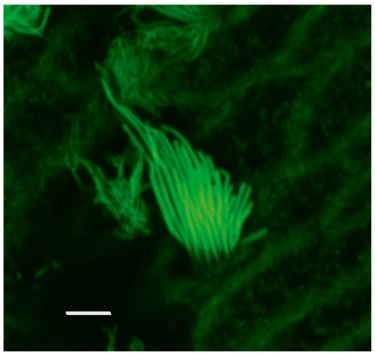


Figure 2.8. Immunocytochemistry control image of a clearnose skate utricle (primary antibody was omitted). The image is the merge of both phalloidin and myosin labeling. Scale bar is 2 µm.

Sea lamprey hair cells

Lamprey hair cells are unusual in that they contain a unique cytoplasmic organelle. This organelle was first noted in *Lampetra fluviatilis* by Löwenstein and Osborne (1964) and was suggested to be endoplasmic reticulum based on its striated appearance. As described by Popper and Hoxter (1987), this organelle extends basally from just below the cuticular plate at the apical surface of the cell to the basal region of the cell body. The present study shows that this unusual organelle labels distinctly with phalloidin (Fig. 2.9), demonstrating that it contains a large quantity of actin. However, myosin labeling (both VI and VIIa) is not higher in this structure than in the surrounding cytoplasm, suggesting that these myosins do not specifically associate with the lamprey actin organelle.

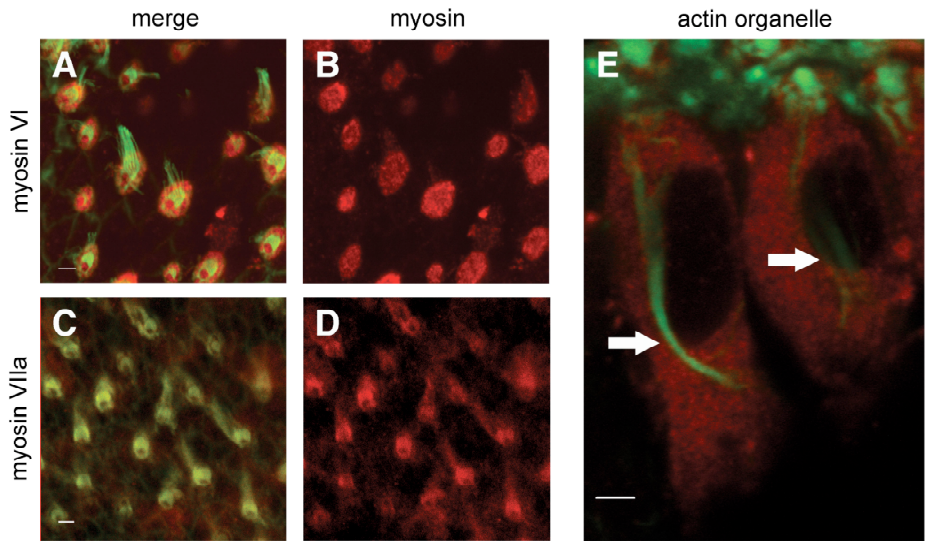


Figure 2.9. Myosin distribution in sea lamprey hair cells. A-B) myosin VI and (C-D) myosin VIIa labeling in the macula communis. Only the merged phalloidin/myosin images (panels A and C) and myosin labeling (panels B and D) are shown. E) Intracellular actin-rich projection (arrows) in lamprey hair cells. Cell bodies are labeled with anti-myosin VI. All scale bars are 2 μm.

Antibody specificity

Immunoblots show that the myosin VI antibody binds to a single approximately 150 kDa band from zebrafish ear, *Xenopus* brain, and mouse cochlea (Fig. 2.10A). Immunoblotting with skate brain and American shad ear produces identical results (data not shown). Anti-myosin VIIa binds two bands in mouse cochlea, one at 250 kDa and the other slightly smaller. Myosin VIIa antibody binds to a single band from zebrafish and American shad ears that is the same size as the smaller band in mouse (Fig. 2.10B).

Peptide expression studies in cultured cells show that both antibodies have high affinity for fish myosins (Fig. 2.11). HEK293 cells transfected with GFP-tagged myo7a clearly immunolabel with myosin VIIa antibody. Transfection of NIH3T3 cells with zebrafish myo6a-GFP and myo6b-GFP shows that the myosin VI antibody binds to both myo6 paralogs in zebrafish. Binding affinity appears higher for myo6b but this effect was not quantified.

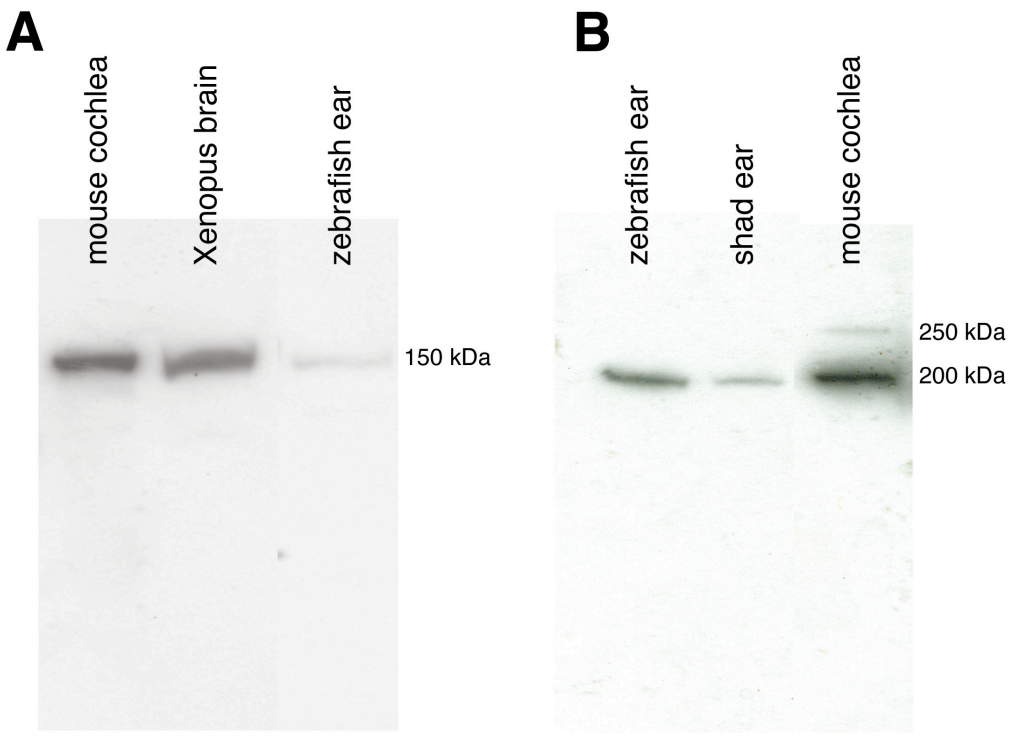


Figure 2.10. Western blots for A) myosin VI and B) myosin VIIa. A) Myo6 antibody labels one band of the expected molecular weight in fishes. B) Myo7a in fishes corresponds to the smaller of the two mammalian isotopes.

Figure 2.11. Epitope expression in cultured cells. Anti-myosin VI binds to both myo6a and myo6b. Epitopes from each myo6 paralog were expressed in NIH3T3 cells. A-D) myo6a, E-F) myo6b, G) myo7a, H) control. A) NIH-3T3 cell expressing GFP B) Anti-myo6 labels only the GFP-tagged cell. C) DAPI labeling of all viable nuclei. D) DIC image showing many cell bodies. E) GFP expression in myo6b-transfected NIH3T3 cells. F) Anti-myo6 labeling of a successfully transfected cell, double-labeled with DAPI. Surrounding non-transfected cells (blue nuclei) do not bind anti-myo6. G) Myo7a expression; merged image of GFP tag, anti-myo6 label, and DAPI, showing many cells but only the GFP (green) cell binds the antibody (red). H) Negative control (no primary antibody) showing that GFP-tagged cells do not bleed-through to red fluorescent channels. Merged image of DAPI, GFP, and immunocytochemistry.

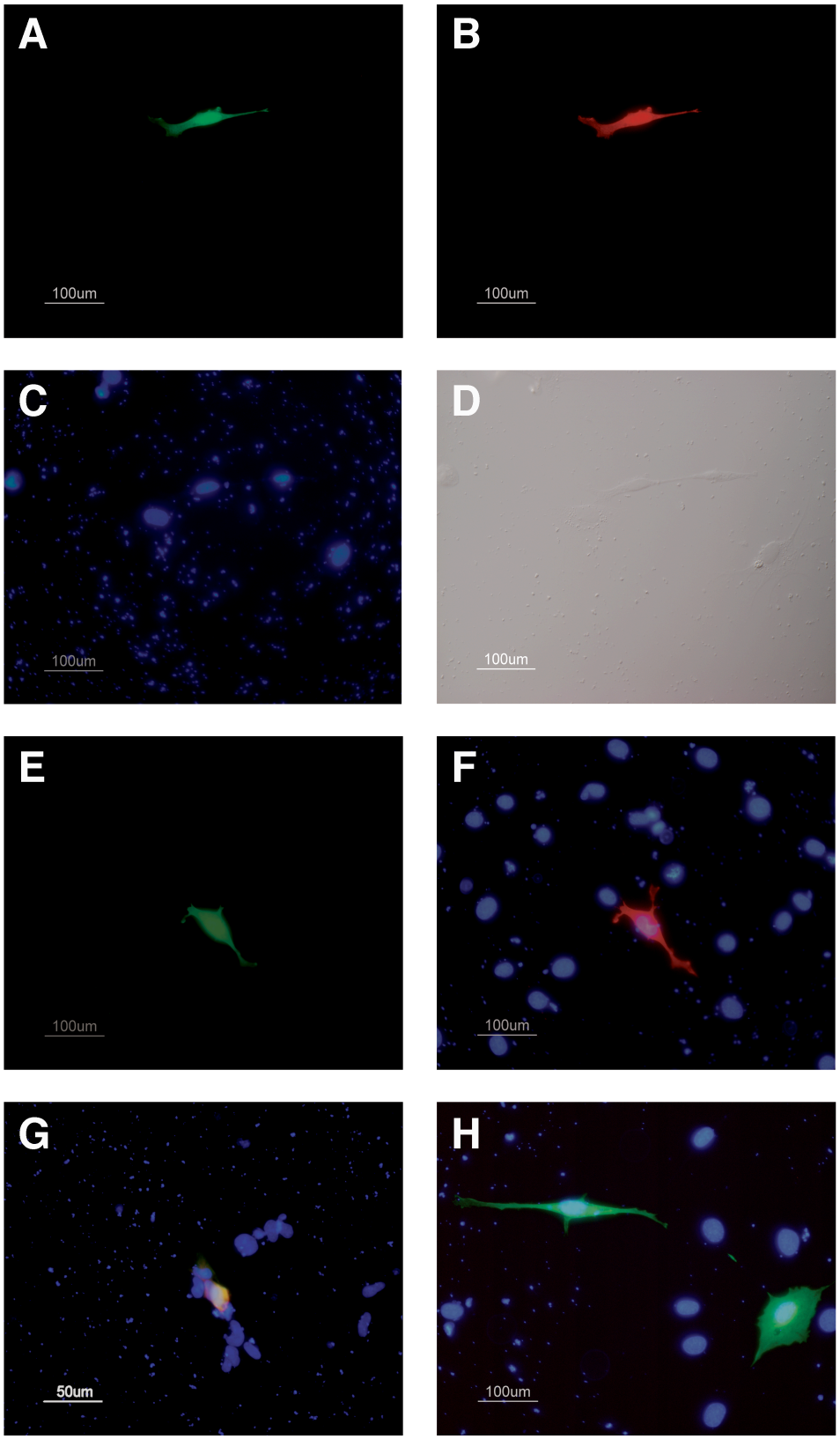
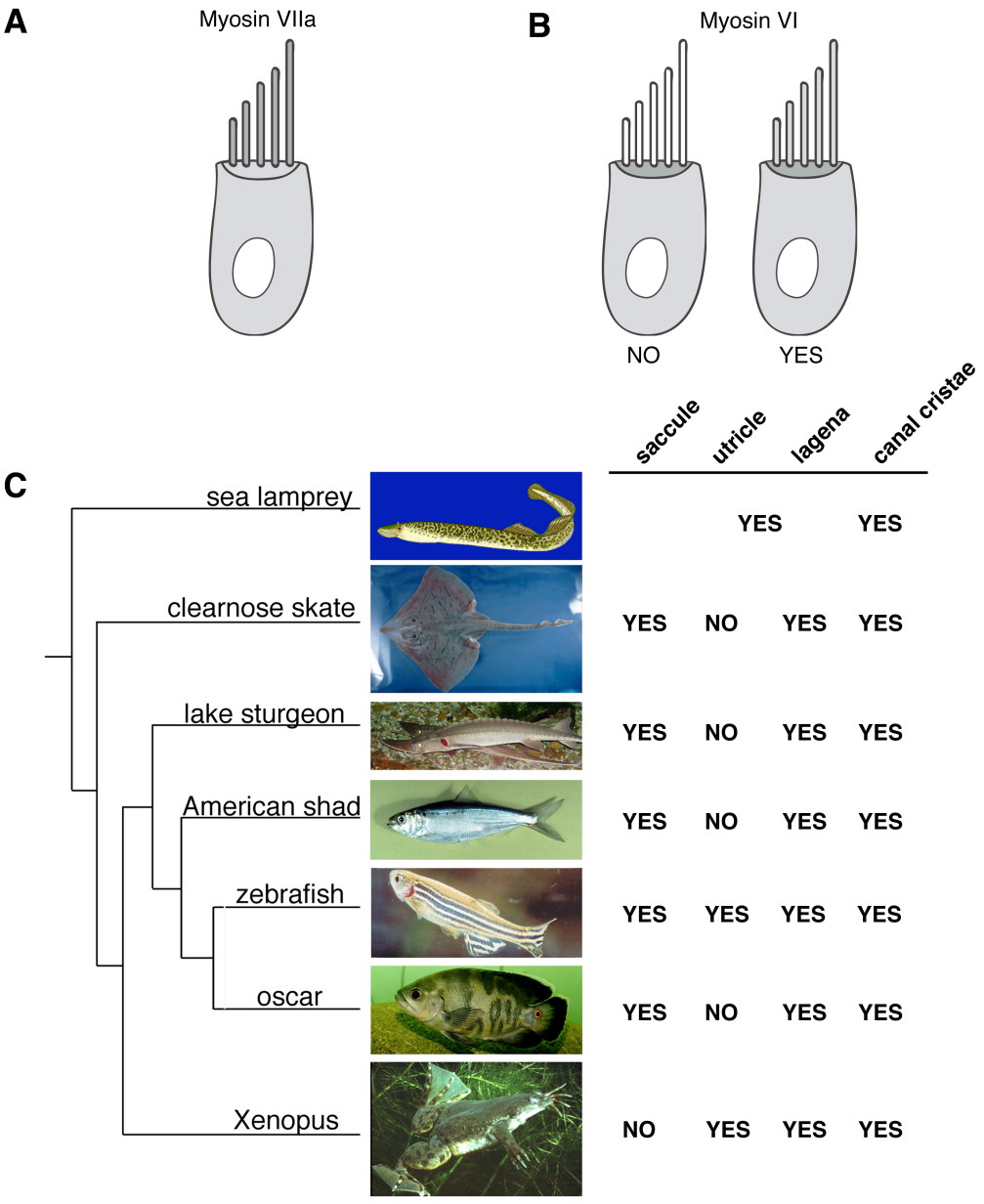

A
100um
B
100um
C
100um
D
100um
E
100um
F
100um
G
50um
H
100um

Figure 2.12. Summary of myosin distribution in fish hair cells. Shaded areas in A and B contain myosin VI or VIIa, white areas do not. A) Myosin VIIa distribution in hair bundles and cell bodies of all hair cell observed in this study. B) Myosin VI distribution patterns partition into two primary categories. Myosin VI localizes to cell bodies and the cuticular plate, while it is either absent (NO) or present (YES) in hair bundles. C) Phylogenetic distribution of myosin VI protein localization patterns. "NO" and "YES" correspond to the bundle distribution patterns shown in B. Hair cell drawings are courtesy of Dr. Michele Halvorsen.


A
Myosin VIIa
B
Myosin VI
NO
YES
C
saccule
utricle
lagena
canal cristae
sea lamprey
YES
YES
clearnose skate
YES NO YES YES
lake sturgeon
YES NO YES YES
American shad
YES NO YES YES
zebrafish
YES YES YES YES
oscar
YES NO YES YES
Xenopus
NO YES YES YES

Discussion

All vertebrate hair cells investigated herein contain both myosins VI and VIIa. The presence of both proteins in evolutionarily distant species such as the ancestral sea lamprey and the more recently evolved oscar suggest that early vertebrate ears contained hair cells with these proteins. This is expected, as these proteins are considered critical for normal hair cell function (reviewed in Friedman et al. 1999). However, myosin VI distribution differs between species and end organs (summarized in Figure 2.12), suggesting multiple roles for this protein in hair cells of some vertebrate species.

Myosin VI

Myosin VI distribution in fishes and *Xenopus* differs in a species and end organ-specific manner. Myosin VI is found throughout the length of the stereocilia in all fish saccules and lagenae examined in this study as well as in hair bundles of the zebrafish utricle. However, myosin VI is lacking in utricular stereocilia of skates, sturgeon, shad, and oscars, suggesting that this distribution is the plesiomorphic (inherited from the common ancestor) condition in fish utricles. Myosin VI distribution in utricular stereocilia of most fishes is the same as myosin VI distribution in mammalian hair cells, where myosin VI is enriched in the stereocilia rootlets and the cuticular plate but is absent in the stereocilia (Hasson et al. 1997).

These differences in myosin VI distribution suggest that this protein may play multiple roles in vertebrate hair cells. In the myosin VI mutant mouse, *Snell's waltzer*, hair bundles form normally during development but then fuse shortly after

birth and eventually degenerate (Avraham et al. 1995; Self et al. 1999). It is hypothesized that myosin VI functions to anchor the plasma membrane in between individual stereocilia, and that in the absence of normal myosin VI, the membrane "zips up," forming giant stereocilia which then degenerate (Hasson et al. 1997; Self et al. 1999). Myosin VI is uniquely qualified to perform this anchoring function because it is one of the few myosins known to move backwards (toward the minus end) along actin filaments (Wells et al. 1999). It could therefore move toward the base of the stereocilia and exert constant tension on the membrane.

While evidence from the *Snell's waltzer* mutant supports this role for myosin VI, the upregulation of myo6 throughout the bundle in some fish end organs implies that it may have an additional role in many fish hair cells. Myosin VI plays multiple roles in endocytosis in some cells (Hasson 2003) and may have distinct structural and functional roles in the Golgi complex of fibroblasts (Warner et al. 2003). It is therefore reasonable that myosin VI may have a functional as well as a structural role, possibly membrane trafficking or receptor-mediated endocytosis, in hair bundles of the saccule and lagena in most fishes and in all hair bundles of zebrafish. Little is known about receptor-mediated endocytosis in stereocilia but there is evidence for this form of endocytosis in other microvillar cells. For example, myosin VI localizes to microvilli in brush border cells of the kidney proximal tubule, where it probably transports membrane-bound receptors from the microvillar surface to the apical intermicrovillar (IMV) region (Biemesderfer et al. 2002). Clathrin-dependent endocytosis then occurs in the IMV region, and this process also requires myosin VI (Biemesderfer et al. 2002). It is possible that myosin VI may have a similar role in

transport of membrane-bound receptors to the cuticular plate of hair cells since this region of hair cells is endocytically active (Kachar et al. 1997).

Absense of myo6 in some fish hair bundles and in all mammalian hair bundles has several implications for stereocilia function. Protein transport from stereocilia to the cuticular plate may not be necessary in these hair cells, although it is more likely that a different protein has taken over this transport role. Griesinger et al. (2004) suggest that protein transport down the stereocilia for apical endocytosis at the cuticular plate may be important in mammalian cochlear outer hair cells. These authors do not suggest a transport mechanism, but another backwards-directed myosin could compensate for a lack of myosin VI. Myosin IX is the only other backwards myosin identified to date (Inoue et al. 2002a). RT-PCR studies show that *myo9b* is expressed in mouse inner ear but specific localization has not been studied (Grewal et al. 1999). Therefore, myosin VI and myosin IX may have complementary roles in stereocilia protein transport. Immunolocalization of myo9 in mammalian and fish inner ears is necessary to substantiate this hypothesis.

Studies in chick (*Gallus gallus*) show that myo6 is absent from mature hair bundles but present in hair bundles of developing hair cells (Luke Duncan, personal communication, 2005). Hair cell proliferation in fishes continues throughout the lifetime of the animal (Lombarte and Popper 1994; Lanford et al. 1996), leading to the suggestion that those hair bundles that contain myo6 may be immature. However, this is unlikely for two reasons. First, immature fish hair bundles have a specific morphology with very short stereocilia (Lombarte and Popper 1994), while many myo6-containing bundles seen in this study are morphologically mature. Second, hair

cell proliferation in fishes occurs throughout the epithelium such that new hair cells are surrounded by mature ones (Popper and Hoxter 1990). If only immature bundles in fishes contain myo6, one would expect a single myo6-containing bundle neighbored by bundles that lack myo6. Myo6-containing hair bundles are seen in great quantity throughout fish epithelia rather than in a spotty pattern, additional evidence that myo6 localizes to mature fish hair bundles. At the same time, we did observe some variation in immunofluorescence intensity in hair bundles and this variation may be correlated to the developmental stage of the hair cell. This variation was not quantified. Future studies using a BrdU (a marker of mitotic cells) and myo6 immunocytochemistry would be helpful to examine the relationship between myo6 distribution and hair cell development.

Peptide expression experiments show that the myosin VI antibody used in this study binds to both teleost myo6a and myo6b (Fig. 2.11). However, *in situ* hybridization data (Fig. 2.3 and Seiler et al. 2004) reveal that *myo6a* is not expressed in zebrafish hair cells. This may apply to other teleosts as well, so the differences in myo6 distribution shown in Figure 2.5 are probably due to differential distribution of a single protein rather than differences in expression of two related proteins.

It is possible that the observed differential distribution of myo6 in hair bundles may be due to epitope masking rather than actual differences in protein distribution. The myo6 antibody used in this study was raised to a fusion protein from myo6 tail, and myosin tail regions are thought to bind cargo. Binding of cargo proteins to hair bundle myo6 could potentially mask the antibody-binding site in utricular hair

bundles. Localization of myo6 binding partners in fish stereocilia would resolve this issue.

Future studies should look at myo6 distribution in lateral line hair cells of fishes such as American shad and oscar. Hair cells in superficial and canal neuromasts differ from one another in sensitivity to aminoglycoside antibiotics, substances that may be taken up by apical endocytosis (Song et al. 1995; Seiler and Nicolson 1999). As myo6 may be involved in apical endocytosis in hair bundles, lateral line hair cells from superficial vs. canal neuromasts may differ from one another in myo6 distribution.

Myosin VIIA

Myosin VIIa is located throughout the stereocilia in all fishes and in the aquatic frog *Xenopus*. This distribution is also seen in both cochlear and vestibular hair cells of mammals (Hasson et al. 1995, 1997) but differs from that of bullfrogs (*Rana catesbeiana*), where myosin VIIa is concentrated in the proximal third of the hair bundle near the basal tapers (Hasson et al. 1997). Hasson et al. (1997) suggested that myo7a associates with lateral stereociliary links. If this is true, then fishes may have lateral links all along the hair bundle in a similar pattern to mammals. Detailed electron microscopy of fish hair bundles is needed to substantiate this hypothesis.

The importance of myosin VIIa for proper hair bundle formation is illustrated in the *shaker1* mouse mutant and the *mariner* zebrafish mutant (Gibson et al. 1995; Ernest et al. 2000). In these myosin VIIa mutants, hair bundles are greatly disorganized and do not respond properly to stimulation. Myo7a is also necessary for

physiological bundle function, as recent electrophysiological studies in *shaker1* show that the stimulus magnitude necessary for transduction channel opening is altered in myo7a mutants and support the hypothesis that myo7a is associated with the transduction complex (Kros et al. 2002), perhaps by acting in series with the extracellular tip links that are believed to gate the transduction channel. Conservation of myo7a distribution among fishes and mammals suggests conservation of function as well.

Sea lamprey hair cells

All hair cells in the sea lamprey inner ear contain an unusual actin-rich cytoplasmic organelle. This structure was previously identified in hair cells of multiple lamprey species using transmission electron microscopy (TEM) but was suggested to be membranous rather than cytoskeletal. Interestingly, a similar actin-rich structure is seen in cochlear inner hair cells and vestibular hair cells of the *shaker2* mutant mouse (Probst et al. 1998). *Shaker2* is a myosin XVa mutant with extremely short stereocilia and congenital deafness. Myosin XVa localizes to the tips of normal hair cells and is proposed to function in organization of the hair bundle staircase (Belyantseva et al. 2003b). Myosin XVa mutant hair cells do not form a normal staircase, suggesting that actin filaments form improperly in these mutants (Probst et al. 1998; Anderson et al. 2000). While sea lamprey hair bundles appear to have a normal staircase shape, the presence of this unusual actin structure suggests that the hair cells in this species may lack myosin XVa, or that it may function differently in normal lamprey hair cells as compared to hair cells of jawed

vertebrates. Myosin XV characterization in the sea lamprey inner ear is an interesting direction for future study and may help pinpoint the function of this important hair cell protein.

Conclusions

Myosins VI and VIIa are present in all vertebrate hair cells, underscoring the importance of these proteins in auditory and vestibular function and suggesting ancient evolution of this hair cell feature. Future studies should focus on myosin distribution in other chordate mechanoreceptors in order to better understand the evolution of these complex cells. The recent discovery of hair cell-like structures in the coronal organ of urochordates (Burighel et al. 2003) provides a useful platform for further comparative work.

As all fish hair cells contain these critical proteins, fishes may serve as useful models for future studies of human hereditary deafness. Conservation of myosin VIIa distribution in hair cells of diverse vertebrate groups suggests that myo7a function is conserved as well. The zebrafish *mariner* (myo7a) mutant is therefore a good model for myo7a-induced hereditary deafness in humans. Differences in myo6 distribution between zebrafish and mammals, however, suggest that the zebrafish *satellite* (myo6b) mutant is not an ideal model for this form of human genetic deafness. Furthermore, studies in fishes such as the American shad and clearnose skate that have end organ-specific differences in myosin VI distribution may help uncover the function of this important hair cell protein. Inner ear studies in diverse fishes can lead

to new insights in important hair cell genes and therefore aid our understanding of genetic deafness in humans, eventually leading to novel therapies for specific forms of hereditary hearing impairment.

Chapter 3: The "fish-specific" genome duplication is confined to teleost fishes

Introduction

Increasing complexity during metazoan evolution is marked by many gene duplication events, freeing duplicated genes from selective pressures and allowing them to evolve new functions (Ohno 1970; Lynch and Conery 2000). In some cases, it appears that whole genome duplications have occurred, providing the genetic substrate for evolutionary novelty while bypassing dosage effects seen in single gene duplication events (Ohno 1970; Spring 1997; Wolfe 2001; Papp et al. 2003).

Multiple lines of evidence suggest two rounds of genome duplication have occurred during vertebrate ancestry (the 2R hypothesis) (Ohno 1970; Sidow 1996; Pebusque et al. 1998), with one duplication in the stem vertebrates and another following the evolution of the jawless vertebrates (Agnatha) (Holland and Garcia-Fernàndez 1996; Escriva et al. 2002; Robinson-Rechavi et al. 2004). Phylogenetic analysis of multi-gene families, however, disputes 2R and suggests an alternative hypothesis of many independent gene duplications (Hughes 1999; Hughes et al. 2001).

There is considerably more support for a fish-specific genome duplication after the split of the lobe-finned (sarcopterygian) and ray-finned (actinopterygian) fishes (but see Robinson-Rechavi et al. 2001 for a contradictory opinion), which took place approximately 450 million years ago (Janvier 1996; Hedges 2001). With over 23,000 species (Nelson 1994), teleost fishes are the largest and most diverse group

within the Actinopterygii. Many teleost fishes have multiple copies of single-copy mammalian genes (Cresko et al. 2003; Farber et al. 2003; Taylor et al. 2003; Vandepoele et al. 2004). While this may have occurred via duplication of many single genes or chromosomal segments during teleost evolution, the large number of functional duplicates and conserved synteny (gene order) between teleost and tetrapod chromosomal segments suggests that fishes experienced a genome-wide duplication event some time during their evolution (Amores et al. 1998; Meyer and Schartl 1999; Taylor et al. 2001a).

Gene duplication in fishes has been best characterized in *Hox* gene clusters. Tetrapods have four *Hox* clusters, while zebrafish (*Danio rerio*), medaka (*Oryzias latipes*) and fugu (*Takifugu rubripes*), three distantly related teleosts, have at least six (Amores et al. 1998; Naruse et al. 2000; Aparicio et al. 2002; Amores et al. 2004). These discoveries prompted the "fish-specific genome duplication hypothesis" which states that a complete genome duplication event occurred prior to the split of the lineages containing the zebrafish, medaka, and fugu, placing this event early in teleost evolution or in a basal non-teleost actinopterygian ancestor (Amores et al. 1998; Meyer and Schartl 1999; Taylor et al. 2003).

While much of the work on duplicated fish genes supports the idea of a whole genome duplication, the evidence was equivocal until recently. Many studies that looked at phylogenetic reconstructions of duplicate pairs found some evidence in favor of genome duplication and some that better supported tandem gene duplication events (e.g., Taylor et al. 2001a, 2003). Taylor et al. (2003) selected 53 sets of homologous genes with duplicates in fishes and found that over half of the individual

gene phylogenies were consistent with genome-wide rather than individual gene duplication events. In contrast, Robinson-Rechavi et al. (2001) suggested that fish are simply more prone to individual gene duplication events and that phylogenetic studies such as that of Taylor et al. (2003) do not overwhelmingly support the genome duplication hypothesis. However, the recent completion of the *Tetraodon nigroviridis* genome provides strong evidence for a fish-specific genome duplication in at least the ancestor of two pufferfishes, *Tetraodon* and *Takifugu* (Jaillon et al. 2004). Jaillon et al. (2004) examined over 900 paralogous pairs in both pufferfishes and found that at least 75% of the pairs were common to both species. Furthermore, evidence of duplication was found on every *Tetraodon* chromosome, suggesting genome-wide rather than tandem gene duplication (Jaillon et al. 2004).

Where in actinopterygian evolution did the proposed duplication occur? Medaka, zebrafish, and fugu shared a common ancestor approximately 140 million years ago (Hedges and Kumar 2002), placing the duplication prior to this point. Since the sarcopterygian lineage split from the actinopterygians approximately 400 million years ago (Janvier 1996; Hedges 2001), this presents a large (~200 million years) window of time for the genome duplication to take place. Within this window are the origins of the basal teleost groups Clupeomorpha (herrings and relatives), Elopomorpha (eels and tarpon), and Osteoglossomorpha (bonytongues) (Nelson 1994). The most likely extant sister taxon to the teleosts is the order Amiiformes, containing one extant species, *Amia calva* (bowfin) (Nelson 1994; Inoue et al. 2003). Fishes of the order Polypteriformes (bichirs and reedfishes) are probably the most primitive actinopterygians, with the Lepisosteidae (gars) and Chondrostei (sturgeons

and paddlefishes) in an intermediate evolutionary position (Nelson 1994; Venkatesh et al. 2001). However, conflict between morphological and molecular data has yet to fully resolve the basal actinopterygian clades (Janvier 1996; Arratia 2001; Venkatesh et al. 2001; Inoue et al. 2003). Figure 3.1 gives an overview of the phylogenetic relationships between major vertebrate taxa.

Hox gene characterization provides some additional insight into genome duplication timing. One recent study examined *hox* gene cluster number and identity in the primitive bichir (*Polypterus senegalus*) and found four clusters with apparent orthology to those in mammals (Chiu et al. 2004). Studies also showed that the sarcopterygian fish *Latimeria menadoensis* (Indonesian coelacanth) has four *hox* clusters (Koh et al. 2003), supporting the hypothesis that four clusters represents an ancestral condition and that the fish-specific genome duplication took place later in actinopterygian evolution, and possibly early within the teleost radiation (Chiu et al. 2004).

Here we examine the timing of genome duplication using a different nuclear gene, myosin VI. There are at least 18 classes of myosins, the type II or "conventional" (filament forming) myosins and the remaining 17+ classes of unconventional myosins that do not form filaments (Sellers 2000; Berg et al. 2001). We focus on myosin VI, an unconventional myosin expressed in the inner ear and which is crucial for normal hearing function in mammals and zebrafish (Hasson et al. 1997; Self et al. 1999; Melchionda et al. 2001; Kappler et al. 2004; Seiler et al. 2004). Mutations in mammalian myosin VI lead to inner ear pathology and deafness (Self et al. 1999; Melchionda et al. 2001). Tetrapods have one myosin VI gene, while recent

work showed that zebrafish and striped bass (*Morone saxatilis*) have two such genes (*myo6a* and *myo6b*) (Breckler et al. 2000; Kappler et al. 2004; Seiler et al. 2004). Zebrafish *myo6* paralogs are found on separate chromosomes (17 and 20), the same chromosomes that contain other duplicated genes such as *sox11* (de Martino et al. 2000; Seiler et al. 2004).

Preliminary phylogenetic analysis shows that myosin VI tree topology is consistent with a fish-specific genome duplication rather than a single gene duplication event (Seiler et al. 2004). In the present study we have cloned and sequenced portions of *myo6* in an evolutionarily diverse range of fishes to narrow the window of time for genome duplication in fishes. We show that genome duplication in fishes probably occurred in the teleost ancestor, supporting the hypothesis that genome duplication in an ancestral teleost provided the genetic substrate for the teleost radiation. This study narrows the time window for the fish-specific genome duplication by approximately 150 million years.

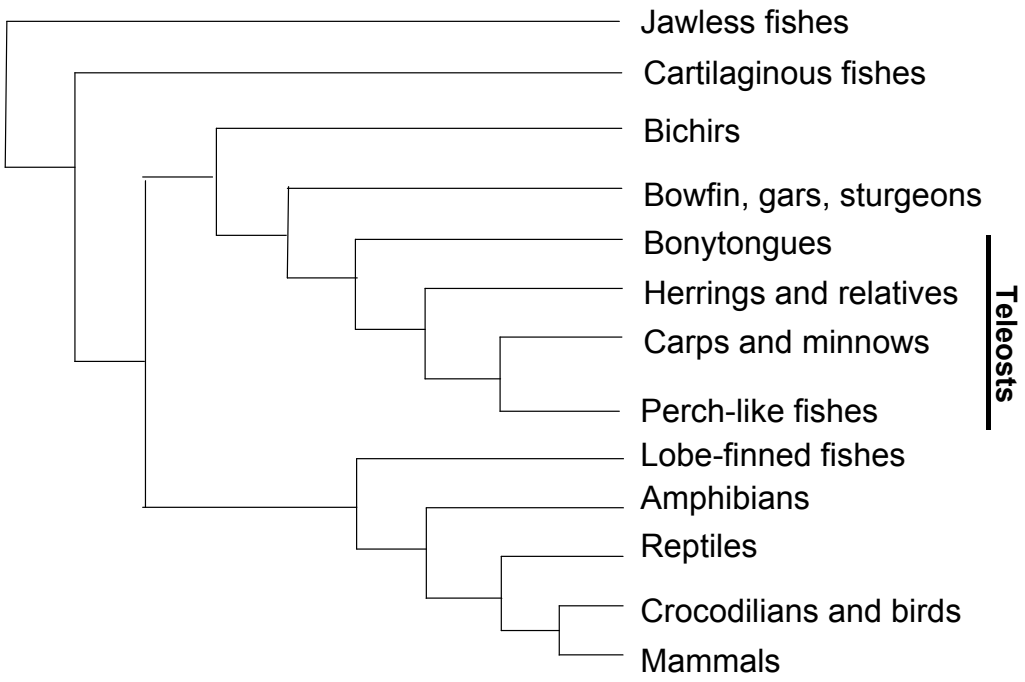


Figure 3.1 Simplified overview of vertebrate phylogenetics. Fish classification is based on Nelson (1994).

Materials and Methods

Animals

An evolutionarily diverse array of fishes was selected for this study to explore the question of timing in fish genome duplication, with a focus on those fishes that are thought to have evolved just before and just after the first teleosts (Nelson 1994). Sea lamprey (*Petromyzon marinus*) was selected to represent the ancestral vertebrate condition while clearnose skate (*Raja eglanteria*) represents cartilaginous fishes. Actinopterygians included the lake sturgeon (*Acipenser fulvescens*) and bowfin (*Amia calva*) as non-teleost bony fishes, while the freshwater butterfly fish (*Pantodon buchholzi*), American shad (*Alosa sapidissima*), zebrafish, and oscar (*Astronotus ocellatus*) represent specific teleost radiations comprised of mostly basal lineages. Phylogenetic relationships between the study species are shown in Figure 3.2. All work was done under the supervision of the University of Maryland Institutional Animal Care and Use Committee.

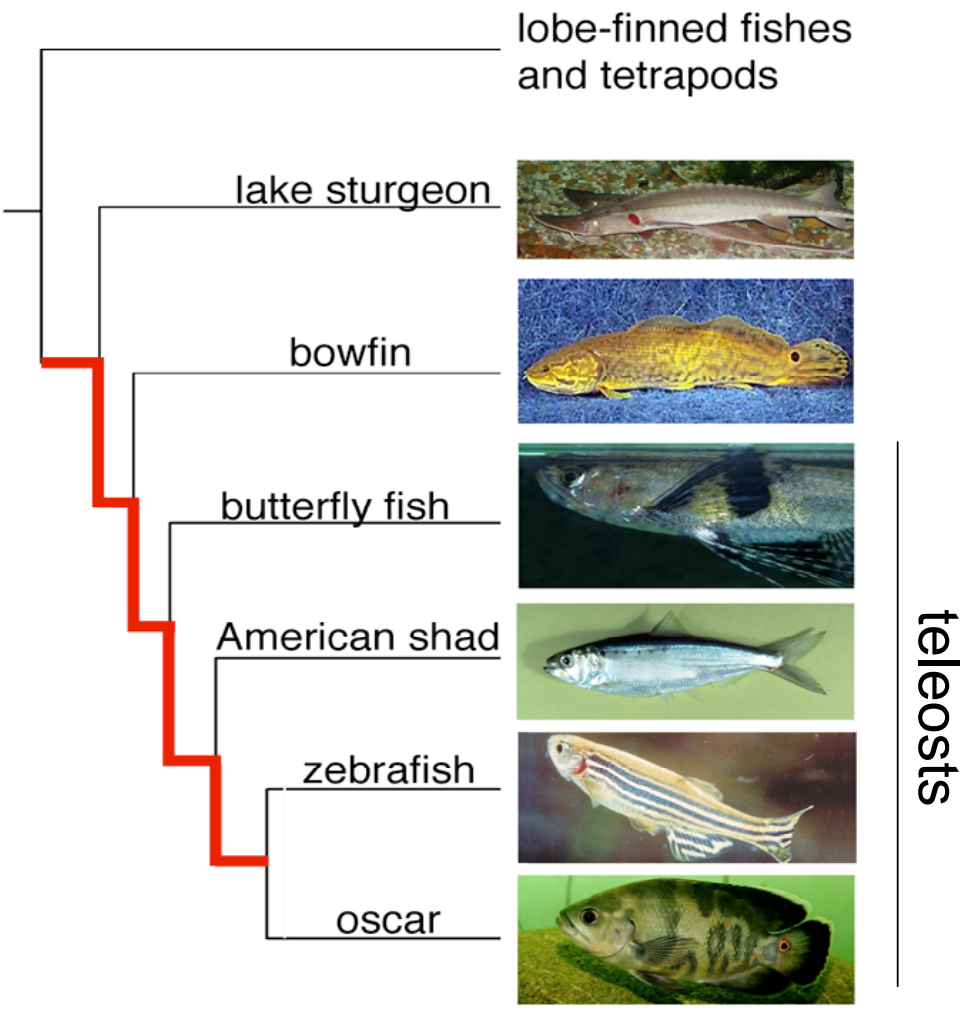


Figure 3.2. Simplified phylogenetic tree showing the relationships of species included in this study. Tree is based on Nelson (1994). The red line depicts the lineages in which the fish-specific genome duplication may have occurred, spanning approximately 250 million years. Pictures are from fishbase.org.

Tissue preparation and cloning

Animals were sacrificed with an overdose of buffered MS-222 (Sigma-Aldrich). Total RNA was isolated from tissues known to express both *myo6* paralogs (inner ear, brain, and eye; Breckler et al. 2000; Kappler et al. 2004; Seiler et al. 2004). RNA isolation and RT-PCR were performed as described in Chapter two, page 27. PCR primers were designed to amplify a conserved 684 bp region of the myosin VI motor domain (bases 912-1595 of the zebrafish *myo6a* sequence) (Table 2.1, page 32). To verify the integrity of each cDNA template, positive control primers were also designed to a housekeeping gene for which species-specific sequence was available in Genbank (Table 3.1).

Following PCR amplification, cDNAs were cloned and sequenced as described in chapter two. At least four clones were sequenced for each species and gene. Sequence identity was confirmed with BLAST searches (Altschul et al. 1997).

Phylogenetic analysis

DNA sequences and translated amino acid sequences were aligned with the ClustalW algorithm using LaserGene (version 5.5, DNASTAR Inc.). Since saturation of third codon positions can occur in DNA sequences with long divergence times, analysis was performed on both amino acid sequences and on DNA sequences with and without third codon positions (Muto and Osawa 1987; Zardoya and Meyer 1996).

In addition to the myosin VI genes cloned in this study, frog (*Xenopus tropicalis*, accession number BC079965), chicken (*Gallus gallus*, NM_204735), and mouse (*Mus musculus*, NM_008662) myosin VI sequences were included to augment the analysis.

Both maximum parsimony and neighbor-joining analyses were performed on amino acid sequences using PAUP* (version 4.0, Swofford 2000) run on a Macintosh PowerBook G4. Heuristic search algorithms were used for parsimony analyses. Bootstrap values (1000 replicates) provided statistical support for the parsimony tree (Felsenstein 1985). Maximum parsimony, neighbor-joining, and maximum likelihood methods were used to analyze DNA sequences. Hierarchical likelihood ratio tests implemented in Modeltest 3.6 (Posada and Crandall 1998) were used to find the best model of evolution for the given sequence data. In all cases sea lamprey myosin VI was used as the outgroup to root the tree.

MacClade (v. 4.03, run on a Macintosh G3) was used to compare alternate tree topologies indicative of genome vs. tandem gene duplication events (see Taylor et al. 2001a). Branch lengths, consistency indexes, and retention indexes for alternate trees were then measured using the maximum parsimony criterion.

Table 3.1. Positive control gene identities and primer sequences.

Species	**Gene**	**Forward primer**	**Reverse primer**
Petromyzon marinus	18S ribosome	GTTGGTGGAGCGATTTGTCT	CCAATCCGAGGACCTCACTA
Raja eglanteria	cytochrome oxidase	GTTACAGCCCATGCCTTTGT	GTGGGCTAGATTGCCTGATA
Acipenser fulvescens	18S ribosome	GACTCCGGTTCTGTTTTGTGG	ATCTGTCAATCCTTCCCGTG
Amia calva	cytochrome b	CCACCCTCACACGATTCTTT	TTATTTGGGATGGAGCGAAG
Pantodon buchholzi	16S ribosome	CGCGCTAAGGTAGCGTAATC	CCGAAGACAAGTGGGTCAGT
Alosa sapidissima	cytochrome c	CCAGGCTTTGGAATGATCTC	CATGATTGCAAATACCGCAC
Astronotus ocellatus	cytochrome b	TTGCAATCCTCCTTATTGCC	CTAGTAGTCCGGTGGTGGGA

Results

A single myosin VI gene was successfully amplified from *Petromyzon*, *Raja*, *Acipenser*, and *Amia*. Two myosin VI genes were amplified from *Pantodon*, *Alosa*, *Danio*, and *Astronotus*. BLAST searching confirms that these latter genes correspond to published *myo6a* and *myo6b* sequences from zebrafish and striped bass. Maximum parsimony analysis of DNA sequences shows that *myo6a* paralogs form a monophyletic group with high bootstrap support (Fig. 3.3). *Myo6b* paralogs in teleosts group with the ancestral *myo6* sequences from non-teleost fishes. A test of 1000 randomly generated trees using maximum parsimony (run in PAUP*) never uncovered a tree with a better score than the tree shown in Figure 3.3, showing that this tree topology is signficantly different from chance (best random tree length=298 steps). All other analyses including neighbor-joining on DNA and amino acid sequences and parsimony on amino acids sequences yielded trees essentially identical to that shown in Figure 3.3, so they are not presented here.

Maximum likelihood analysis using a modified general time reversible model of DNA sequence evolution (Tamura and Nei 1993) that accounts for invariable sites (54%) and unequal rates of evolution between sites (α=7.11 for the gamma distribution) also uncovered the tree shown in Figure 3.2. The rate substituion matrix is given in Table 3.2. Tree topology did not differ depending on inclusion or exclusion of third codon positions.

PCR reactions with control primers amplified the appropriate species-specific genes, showing the cDNA templates were not contaminated by DNA of other species

and were and of good quality (Fig. 3.4). Positive control identity was confirmed by sequencing.

Tests of alternate tree topologies using amino acid sequences show that the tree with the perfect genome duplication topology (Fig 3.5A) is 60 steps shorter (maximum parsimony criterion) than the tree resulting from independent gene duplication events in different teleost lineages (Fig 3.5B.). This indicates that the *myo6* paralogs seen in teleosts evolved from a single duplication event. The amino acid tree with the same topology as that in Figure 3.3 is only four steps shorter than the duplicated genome topology tree in Figure 3.5A, indicating that the grouping of teleost myo6a with tetrapod myo6 is probably an artifact (see below).

Figure 3.3. Maximum parsimony tree of *myo6* DNA sequences without third codon positions. Numbers above branches are branch lengths. Branches are not drawn to scale. Numbers below branches are percent bootstrap support (1000 replicates). Tree length is 217 steps, CI=0.885, RI=0.924.

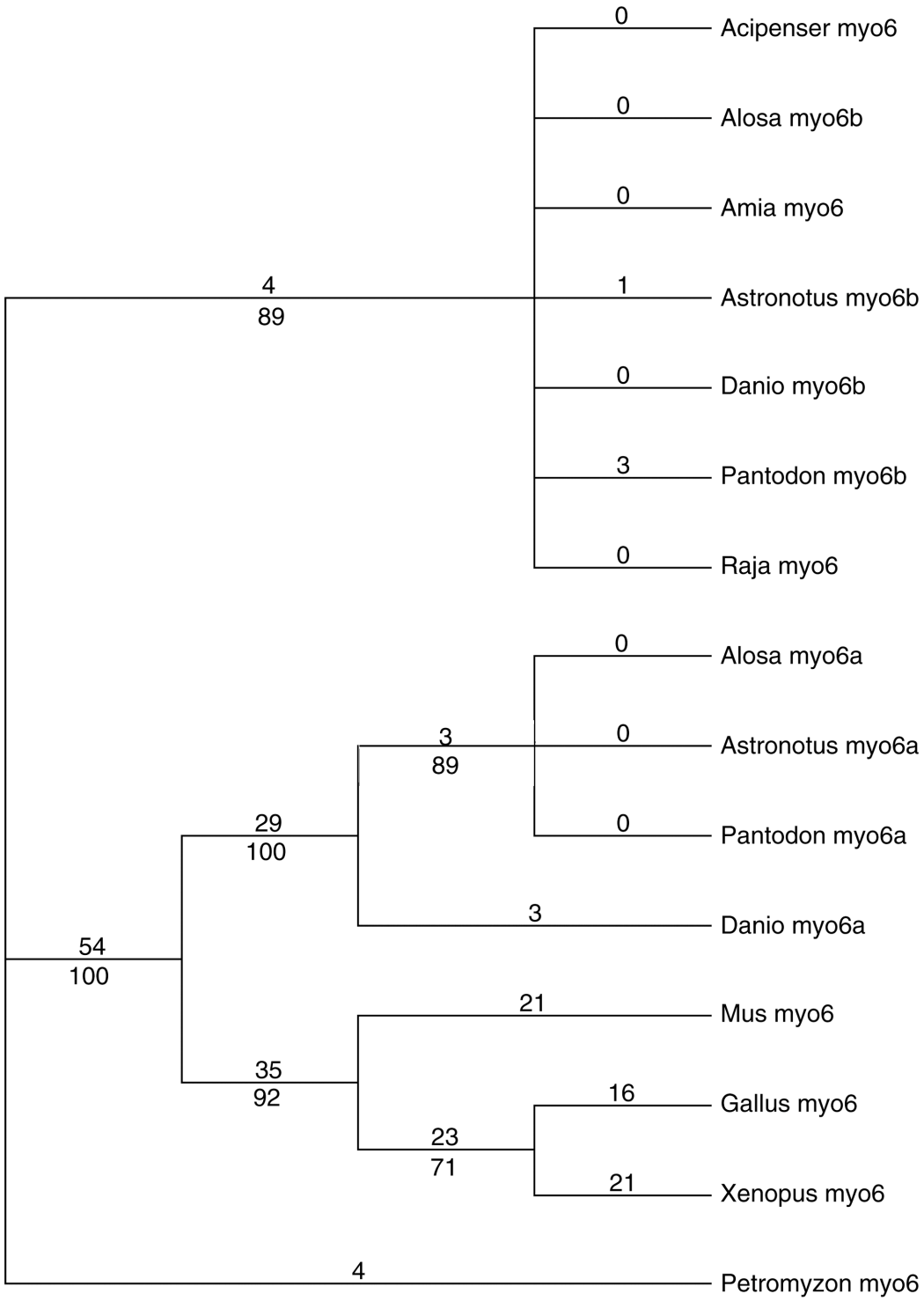

0
Acipenser myo6
0
Alosa myo6b
0
Amia myo6
4
89
1
Astronotus myo6b
0
Danio myo6b
3
Pantodon myo6b
0
Raja myo6
0
Alosa myo6a
3
89
0
Astronotus myo6a
29
100
0
Pantodon myo6a
3
Danio myo6a
54
100
21
Mus myo6
35
92
16
Gallus myo6
23
71
21
Xenopus myo6
4
Petromyzon myo6

Table 3.2. DNA substitution rate matrix used for maximum likelihood analysis.

	A	**C**	**G**	**T**
A	----------	1	3.29	1
C	1	----------	1	7.40
G	3.29	1	----------	1
T	1	7.40	1	----------

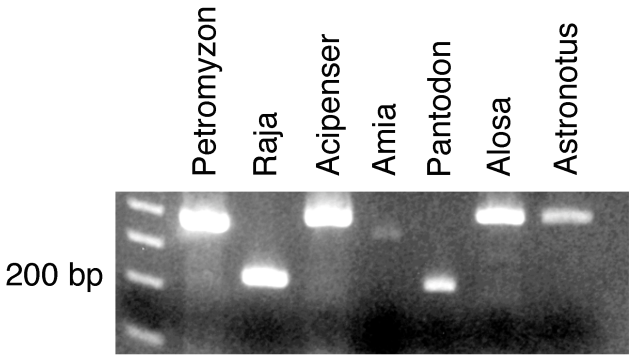


Figure 3.4. PCR products for positive control genes. Identities of each gene are given in Table 3.1. There is no positive control gene for zebrafish because *myo6* sequences were specific for this species and were confirmed by sequencing.

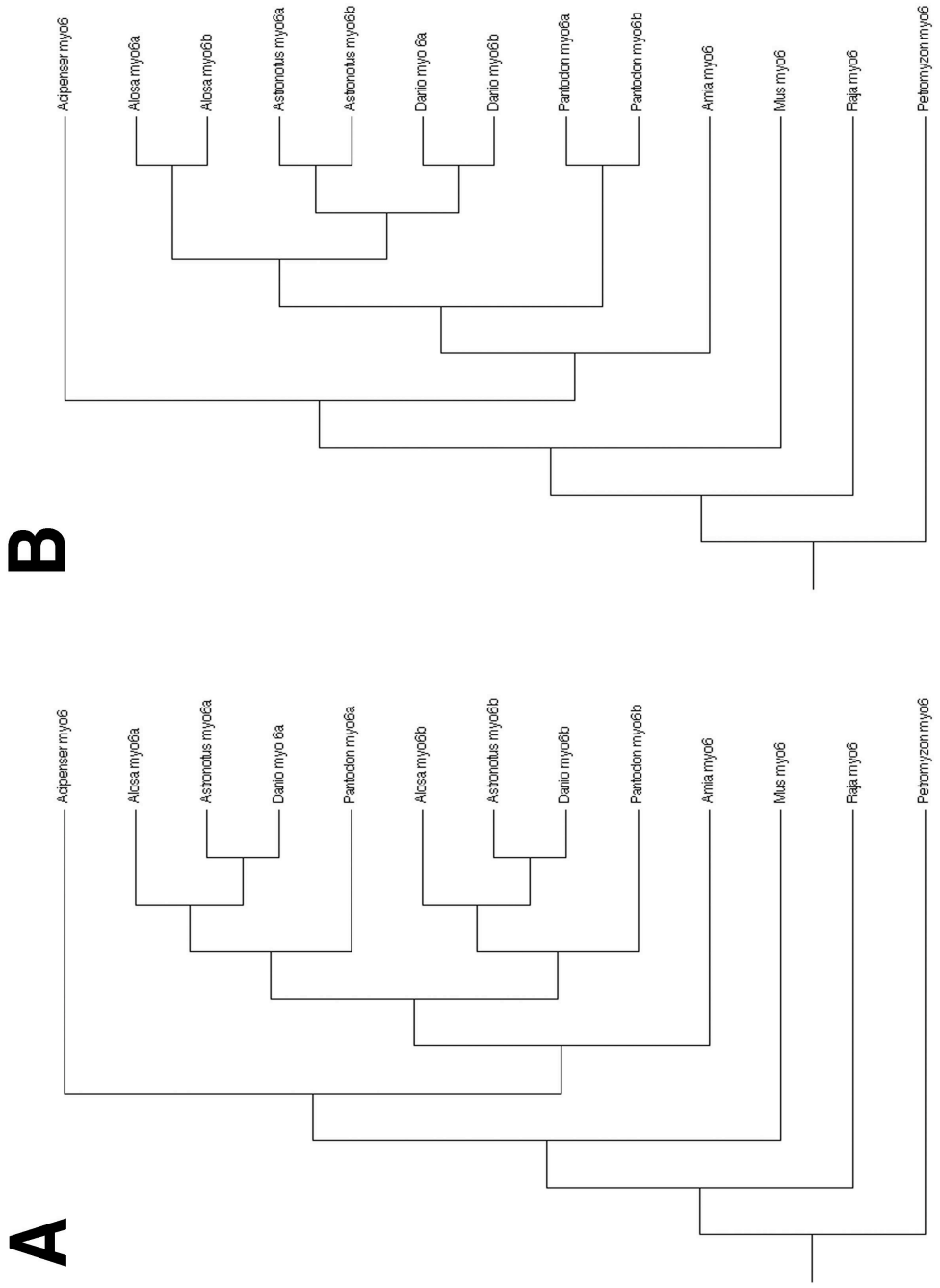


Figure 3.5. Test of competing amino acid tree topologies using maximum parsimony in MacClade. A) Tree topology consistent with one *myo6* duplication in a basal teleost (length =40, CI=0.83, RI=0.90). B) Topology indicative of independent duplication events in the different teleost lineages (length=100, CI=0.33, RI=0.01).

Discussion

All teleost fishes studied here possess two myosin VI genes, while non-teleost fishes and all other vertebrates (including tetrapods) have only one (Hasson and Mooseker 1994; Hasson et al. 1996; Ahituv et al. 2000). Phylogenetic analysis of myosin VI deduced amino acid sequences shows that myo6 paralogs have a sister-group type of relationship, the expected topology for genes that arise via genome duplication (Wolfe 2001; Taylor et al. 2003). Our myosin VI data therefore provides additional support for the fish-specific genome duplication hypothesis. We found two *myo6* genes in *Pantodon*, a member of the basal teleost lineage Osteoglossomorpha. In contrast, only a single *myo6* gene was found in the non-teleost bony fishes (*Acipenser* and *Amia*). Based upon these findings, we suggest that the fish-specific genome duplication occurred after the Amiiformes split from the Actinopterygii, most likely in an ancestral teleost (Fig. 3.6).

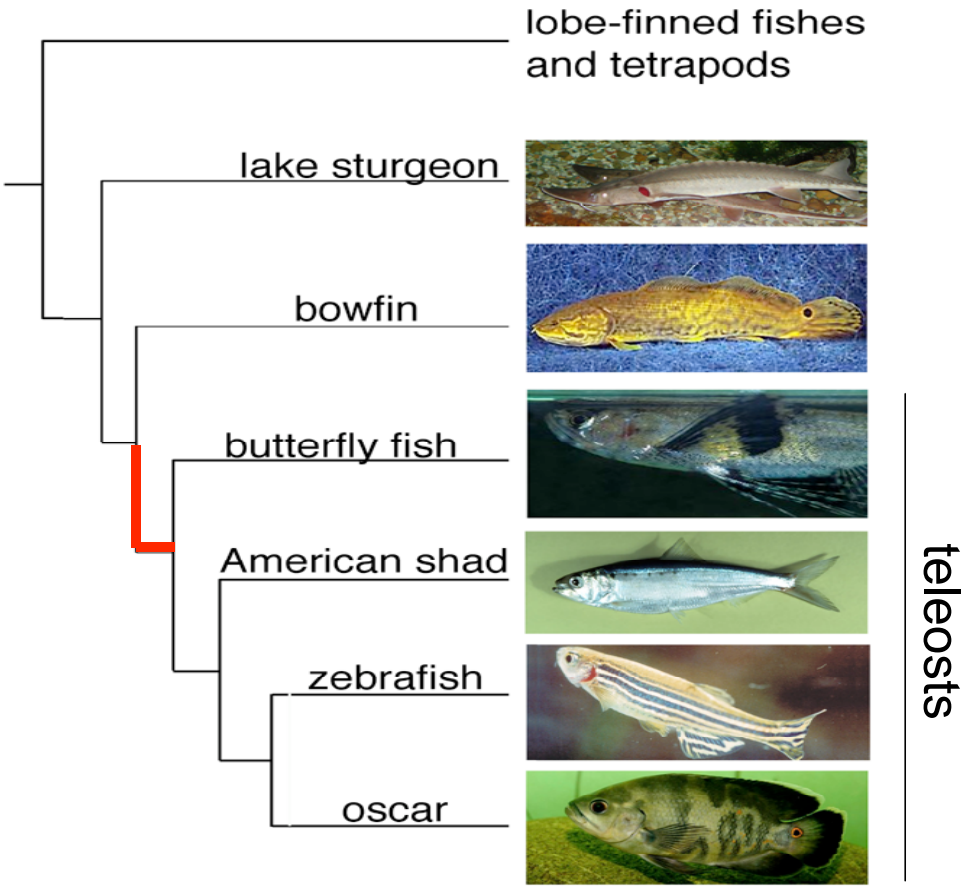


Figure 3.6. Phylogeny redrawn from Figure 3.2 showing the narrowed time window for the fish-specific genome duplication (red line). The present study strongly suggests that this duplication occurred 200-250 million years ago. Pictures are from fishbase.org.

Recent work by Hoegg et al. (2004) corroborates our findings. These investigators found duplicates of three nuclear genes (*fzd8*, *sox11*, and *tyrosinase*) in a variety of teleost fishes but not in Siberian sturgeon (*Acipenser baerii*) or Florida gar (*Lepisosteus platyrhynchus*). That study, along with our findings on myosin VI, strengthens the hypothesis that the fish-specific duplication is confined to teleosts and therefore occurred 200-250 million years ago. Our data clearly show that *myo6a* genes from diverse teleosts form a monophyletic group (Fig. 3.3). However, some *myo6b* genes are closely related to the ancestral *myo6* sequences from non-teleosts. Following a gene (or genome) duplication event, selection is often initially relaxed on one or both of the newly duplicated genes and evolutionary rates often increase (Lynch and Conery 2000, 2003; Gu et al. 2005). This is most likely the situation for the cloned region of *myo6a*, while *myo6b* evolved more slowly. Interestingly, there is high bootstrap support for a sister-group relationship between teleost *myo6a* and tetrapod *myo6*. While it is possible that the tetrapod ancestor had two *myo6* genes and lost *myo6b*, it is unlikely that both *Acipenser* and *Amia* independently lost *myo6a*. Comparisons of branch lengths in Figure 3.3 show that the grouping of teleost myo6a with tetrapod myo6 may be due to long branch attraction, a commonly observed phenomenon arising from unequal evolutionary rates (Felsenstein 1978). Long branch attraction was also detected in similar studies of duplicate gene pairs (e.g., Taylor et al. 2001a, 2003). Phylogenetic analysis of the full-length sequences for *myo6* genes in *Morone*, *Danio*, and mouse show the expected sister grouping of monophyletic teleost paralogs (Seiler et al. 2004 and data not shown).

It is possible that *Acipenser* and *Amia* possess two *myo6* genes and that we failed to find the second one. However, we consider this unlikely since our PCR strategy successfully amplified both *myo6* paralogs from diverse teleosts, while the sturgeon and bowfin results are similar to *Petromyzon* and *Raja*, two basal fishes that probably represent the ancestral vertebrate condition. As lake sturgeon are functional tetraploids (Ludwig et al. 2001), it is more likely that we would see more than two myosin VI genes in this species, so the finding of one is telling and suggests that lake sturgeon possess a single *myo6* that is orthologous to *Myo6* in mammals. It is also possible that *myo6a* has reduced expression patterns in basal actinopterygian fishes such that it is not expressed in the ear, eye, or brain tissue sampled here. However, *in situ* hybridization studies show that *myo6a* in zebrafish is expressed broadly, while only *myo6b* has restricted expression (Kappler et al. 2004; Seiler et al. 2004). Therefore, our screen was more likely to find *myo6a* than *myo6b* if both were present. Hoegg et al. (2004) used a degenerate PCR strategy to identify duplicates of three genes in basal and derived bony fishes and found only one copy of each gene in the non-teleosts. This further supports our finding that lake sturgeon and bowfin possess only one copy of *myo6*.

Traditional models of gene duplication suggest that most duplicated genes are lost due to deleterious mutations and that remaining genes evolve novel functions (Ohno 1970). Current estimates suggest that 20-30% of duplicate genes were retained following the fish genome duplication, a much larger percentage than would be expected if duplicates must evolve novel functions in order to be retained (Postlethwait et al. 2000).

The recent duplication-degeneration-complementation (DDC) model provides a different way to view evolution of duplicated genes and offers an explanation for the large number of genes retained following some duplication events (Force et al. 1999). The DDC model proposes that deleterious mutations in regulatory elements will lead to loss of gene expression in a subset of tissues or developmental time points such that the two new paralogs will possess complementary or partially overlapping functions. This hypothesis is supported by gene expression data for the transcription factors *en1* and *sox9* (Force et al. 1999; Cresko et al. 2003).

Myosin VI expression patterns in the zebrafish provide further evidence in favor of the DDC model. Seiler et al. (2004) used whole-mount *in situ* hybridization to show that *myo6a* is highly expressed in zebrafish gut, brain, gill, and kidney, while *myo6b* appears restricted to ear, lateral line, and eye. The single *Myo6* gene in mice is predominately expressed in ear, eye, brain, kidney, and liver, which presumably represents the ancestral distribution pattern (Avraham et al. 1995; Hasson et al. 1997). It is likely that mutations in regulatory regions of the duplicated *myo6* zebrafish genes have led to subfunctionalization of each paralog and therefore retention of both genes. Myosin VI is critical in vertebrate hearing but its precise role in inner ear sensory cells is not fully understood (Avraham et al. 1995; Self et al. 1999). Functional analysis of both zebrafish *myo6* homologs may offer insight into this important protein and its role in human hereditary deafness.

In summary, we provide further evidence for the fish-specific genome duplication hypothesis and show that this duplication most likely occurred at the base of the teleost radiation. This timeline supports the idea that genome duplication in

fishes provided the genetic substrate for the diversity of evolutionary events that gave rise to over 23,000 species of teleosts. Large-scale sequencing projects for basal actinopterygian fishes such as *Amia* and *Acipenser* are necessary to validate this timing.

Chapter 4: Summary and Conclusions

Summary

This dissertation examines unconventional myosins in fishes. Chapter two looks at myosin distribution in fish ears, specifically within sensory hair cells. Chapter three uses myosin VI DNA and protein sequences from evolutionarily diverse fishes to identify the timing of genome duplication within actinopterygian (ray-finned) fishes.

The studies in Chapter two use RT-PCR, *in situ* hybridization, and indirect immunofluorescence to look at myosin VI and myosin VIIa gene expression and protein distribution in hair cells of fishes. RT-PCR amplification of *myo7a* from inner ear cDNA confirms that this myosin is expressed in the inner ears of all fishes examined in this study. Myo7a protein is distributed along the length of the stereocilia in all fishes examined, consistent with a role in maintenance of bundle cohesion and associated with stereociliary links such as tip links and ankle links. HEK297 expression of the zebrafish epitope specific for the myo7a antibody shows that the antibody properly binds to fish myo7a.

The myosin VI story is considerably more complex. Recent studies by Kappler et al. (2004) and Seiler et al. (2004) show that zebrafish have two *myo6* genes (*myo6a* and *myo6b*). RT-PCR analysis described in Chapter two of this dissertation suggests that both *myo6* paralogs are expressed in teleost ears, while non-teleost sea lamprey (*Petromyzon marinus*), clearnose skate (*Raja eglanteria*), and lake sturgeon (*Acipenser fulvescens*) ears express a single *myo6* gene.

Myosin VI protein distribution differs between species and end organs. Myo6 is found in all hair bundles of the sea lamprey canal cristae and macula communis, suggesting that this distribution pattern is ancestral for vertebrates. This cosmopolitan distribution is also seen in all end organs of the zebrafish (*Danio rerio*) and in the saccule, lagena, and cristae of the clearnose skate, lake sturgeon, American shad (*Alosa sapidissima*), and oscar (*Astronotus ocellatus*). However, myo6 is not in utricular stereocilia in these last four species. This finding suggests that myo6 may play multiple roles in hair cell stereocilia. Myo6 distribution in utricular hair cells of most fishes is similar to mammalian hair cells, while other epithelia in these fishes and all zebrafish hair bundles differ from the mammalian distribution pattern (Hasson et al. 1997).

Epitope expression studies using NIH3T3 cells show that the myo6 antibody binds to both myo6a and myo6b in zebrafish (and presumably other teleosts). However, *in situ* hybridization with a *myo6a*-specific probe shows that *myo6a* is not expressed in sensory epithelia of the inner ear. Therefore, differences in myo6 distribution within inner ear epithelia of teleosts (e.g., zebrafish, shad) are due to species-specific differences in myo6b distribution and not to differential distribution of the two myo6 paralogs.

Chapter three uses *myo6* sequences to look at genome duplication timing in fishes. Amores et al. (1998) observed that zebrafish had twice as many *hox* gene clusters as mammals and hypothesized that a genome duplication occurred somewhere during fish evolution that caused this *hox* cluster increase. They proposed that a genome-wide duplication event took place in an ancestral teleost, providing the

genetic substrate for the great diversity seen in modern teleost fishes. In Chapter three here, it is shown that all teleost fishes studied, including the derived oscar and the ancestral freshwater butterflyfish (*Pantodon buccholzi*) have two *myo6* genes. Two non-teleost actinopterygian fishes, the lake sturgeon and the bowfin (*Amia calva*), have a single *myo6* gene. The resulting phylogeny is consistent with *myo6* paralogs arising during a genome-wide duplication and provides evidence that the duplication did indeed occur in an ancestral teleost. Recent work by Hoegg et al. (2004) using three other nuclear genes supports the findings in chapter three.

Research Implications

Why are myosins in fish hair cells important?

This dissertation offers important insight into conservation of unconventional myosins in vertebrate hair cells. Myo7a expression and distribution is conserved between fishes and mammals, suggesting a highly conserved role for this myosin in hair cells. Hasson et al. (1997) found differences in myo7a distribution between bullfrogs and rodents and suggested that myo7a associates with stereocilia lateral linkages. Immunofluorescence studies in Chapter two of this dissertation show that myo7a distribution in *Xenopus* hair cells is similar to that seen in both fishes (this dissertation) and mammals (Hasson et al. 1997) and suggest that bullfrog saccular hair cells may be unique in terms of myo7a distribution.

While the precise molecular function of myo7a in hair cells remains unknown, several studies in the past five years have uncovered important interactions between myo7a and other proteins in mammalian hair cells. Myoa7a binds to harmonin b and

vezatin, both hair bundle proteins, which then mediates interactions with cadherin23 and protocadherin15 to form a functional unit that is probably important for hair bundle formation (Boëda et al. 2002; Belyantseva et al. 2003a). In addition to a developmental role in hair bundles, mammalian myo7a is also necessary for maintaining proper bundle tension, thereby allowing transduction to occur within the physiological operating range (Kros et al. 2002). As there are no observable differences in myo7a distribution between fishes and mammals, these roles in bundle development and maintenance of resting tension are probably conserved in fishes.

Myo7a defects result in syndromic hereditary deafness in humans (Usher syndrome type 1) as well as dominant and recessive non-syndromic deafness (Weil et al. 1995; Liu et al. 1997; Weil et al. 1997; Bolz et al. 2004). The *shaker1* and *headbanger* mouse mutants are models for recessive and dominant non-syndromic deafness, respectively, but no mouse model exists for syndromic myo7a-induced deafness, possibly because the mouse lifespan is too short for progressive retinal degeneration to manifest (Gibson et al. 1995; Weil et al. 1995; Rhodes et al. 2004). As myo7a distribution is conserved across most vertebrate taxa, fishes are good models for myo7a-induced deafness and probably other myo7a-related disorders. The zebrafish *mariner* mutant is a myo7a mutant similar to the *shaker1* mouse, with hair cell pathology and no retinal phenotype (Ernest et al. 2000). A longer-lived fish such as the oscar may provide a better model for myo7a-associated syndromic deafness and blindness.

Differential distribution of myosin VI in hair cell epithelia of fishes raises some interesting questions regarding the role of myo6 in hair bundles. In mammals,

myo6 localizes to the base of stereocilia and is hypothesized to maintain bundle integrity by actively anchoring the plasma membrane in between individual stereocilia (Hasson et al. 1997; Self et al. 1999). Fish hair cells are also enriched for myosin in this region and it may play a similar role. Myo6 is also implicated in clathrin-mediated endocytosis and localizes to the cuticular plate, a site of known endocytic activity in mammals (Hasson et al. 1997; Kachar et al. 1997; Hasson 2003). However, distribution along the length of all hair bundles in the zebrafish and in the saccule, utricle, and canal cristae of other fishes suggests an additional role, one within the stereocilia themselves.

Myo6 is a known vesicle transport motor, moving along actin filaments to transport clathrin-coated vesicles away from the plasma membrane and into the cytoplasm (reviewed in Hasson 2003). Fish hair bundles may exhibit receptor-mediated endocytic activity, where myo6 transports membrane-bound receptors down stereocilia and into the cell body for endocytosis at the cuticular plate. Myosin VI is implicated in a similar role in microvillar brush border cells of the kidney proximal tubule (Biemesderfer et al. 2002).

As zebrafish and mammalian hair cells differ in myo6 distribution, the zebrafish *satellite* mutant (Kappler et al. 2004; Seiler et al. 2004) may not be the best fish model for human myo6-induced hereditary deafness. Studies in a fish such as the oscar or American shad offer better opportunities to examine myo6 function as utricular myo6 distribution is similar to that of mammals. Furthermore, these fishes provide a unique opportunity for comparative studies between hair cells that differ in myo6 distribution within the same organism.

Genome duplication and fish evolution

Chapter three provides evidence that genome duplication occurred in an ancestral teleost rather than early in actinopterygian evolution. This timing concurs with similar findings by Hoegg et al. (2004) and supports the hypothesis that genome duplication played an important role in teleost evolution (Amores et al. 1998; Meyer and Schartl 1999; Taylor et al. 2001a). Duplication in the teleost ancestor would provide twice as many genes on which natural selection could act, leading to greater adaptive ability and therefore species radiation as seen in modern teleosts. Gene (or genome) duplication events are often followed by a period of relaxed selection and increase evolutionary rates (Lynch and Conery 2000, 2003; Gu et al. 2005). Therefore, genome duplication would allow for rapid expansion of the teleost lineage. Ohno (1970) argued that gene duplication events provided genetic material for evolutionary change. While most tandemly duplicated genes are silenced by deleterious mutations within a few million years, duplicates that arise from polyploidy may initially be preserved due to dosage effects (Lynch and Conery 2000; Papp et al. 2003). This "grace period" allows for evolution of new functions (neofunctionalization) or for partitioning of ancestral functions (subfunctionalization) (Force et al. 1999; Postlethwait et al. 2004).

Evidence for neofunctionalization of duplicate genes in teleosts lends support to these ideas. Rainbow trout (*Oncorhynchus mykiss*) have duplicate glucocorticoid receptors (rtGR1 and rtGR2) that differ in sensitivity to cortisol levels (Bury et al. 2003). Multiple rtGR receptors therefore provide more complex endocrine responses

by expanding the range of cortisol-activated transcription. Similarly, a duplicated pepsin A gene in the notothenioid rock cod (*Trematomus bernacchii*) is cold-adapted, allowing the fish to metabolize food in cold Antarctic waters while preserving the normal operating range of the paralog (Carginale et al. 2004). Visual opsins in zebrafish show evidence of duplication in both red and green opsin genes (Chinen et al. 2003). Duplication of these short wavelength opsins is adaptive for color vision in shallow-water habitats and therefore may contribute to the zebrafish's success in this environment.

Multiple examples of complementary expression patterns in teleost paralogs support the notion that subfunctionalization of duplicate genes also occurs in fishes. *Sox11* is a developmental gene expressed in mammalian CNS and somites during embryogenesis. Zebrafish have two *sox11* paralogs with distinct somite expression patterns that together mimic mammalian *Sox11* expression (de Martino et al. 2000). Similar scenarios are seen for *sox9*, *en1*, and *myo6* (Force et al. 1999; Cresko et al. 2003; Seiler et al. 2004).

Just as retention and adaptation of duplicate genes can promote evolution, so too can differential loss of genes in allopatric populations. Divergent resolution occurs when a population is separated shortly after a genome duplication event (Lynch and Conery 2000; Lynch and Force 2000; Taylor et al. 2001b). As the fate of most duplicates is nonfunctionalization, genes will be lost independently in each population. When the two populations are later reunited, F1 hybrids will have one functional and one null allele of many genes. Twenty-five percent of F2 individuals will be homozygous null and probably non-viable. In this way, genome duplication

provides a passive mechanism for increased rates of speciation (Lynch and Conery 2000). Therefore, genome duplication in an ancestral teleost probably allowed for both active and passive evolutionary mechanisms that led to the teleost radiation.

Future studies

This dissertation provides new and important information on unconventional myosins in fishes. Like most research, however, the findings shown here prompt new research ideas, which are discussed briefly below.

Understanding myosins in fish hair cells

Hasson et al. (1997) found differences in myo7a distribution between hair cells of rodents and bullfrogs and proposed that myo7a associates with lateral links between stereocilia. As fish myo7a distribution is similar to that in mammals, fishes offer an opportunity to test this hypothesis. Electron microscopy is needed to localize lateral links in fish hair bundles.

The finding of inter-epithelial differences in myo6 distribution in some fishes (Chapter two) raises questions regarding the role(s) of this protein in hair cells. Hasson et al. (1997) reported that Myo6 is excluded from mammalian hair cell stereocilia while it is present in stereocilia of bullfrogs (*Rana catesbeiana*). Chapter two shows that in many fishes such as American shad (*Alosa sapidissima*) and lake sturgeon (*Acipenser fulvescens*) myo6 is present in stereocilia of the saccule and lagena but absent in utricular stereocilia. This finding presents a unique opportunity

to perform a "comparative study" of myo6 function in a single animal that shows both mammalian and amphibian-like myo6 distribution in a single sensory system. Particular attention should focus on apical receptor-mediated endocytosis in fish hair bundles that contain myo6. High resolution TEM studies of vesicle distribution in fish hair bundles may provide morphological evidence for myo6-associated endocytosis in some fish hair bundles. Additionally, hair cell immunolocalization studies should be performed for proteins such as megalin, a scavenger receptor that probably interacts with microvillar-bound myosin VI in the kidney (McDonough and Biemesderfer 2003). As myosin VI mutations lead to deafness in humans and in animal models (Avraham et al. 1995; Melchionda et al. 2001; Ahmed et al. 2003; Seiler et al. 2004), a thorough understanding of the multiple roles of myosin VI in hair cells may lead to therapies for this form of hereditary deafness.

The finding of myo6 in the stereocilia of most fishes presents an obvious question-how does it get there? Mammalian myosin VI is a backwards motor that moves toward the barbed end of actin filaments (Wells et al. 1999). As hair bundle actin is oriented with the barbed end toward the cell body, myo6 should not be able to transport itself up the hair bundle shaft. Two competing hypotheses are proposed. Fish myosin VI may move toward the plus end of actin filaments, up to the tips of stereocilia. Myosin directionality is determined by an unknown region of the core motor domain (Homma et al. 2001) so directionality studies of the zebrafish myosin VI motor domain would test this first hypothesis. An initial test of zebrafish myo6 directionality would be to express GFP-tagged myo6 motor domains in cultured COS7 cells. Similar studies using mouse myosins show that mammalian myo6

localizes to the cell body, while plus-ended myosins such as myo7a localize to filapodia tips (Belyantseva et al. 2005). If zebrafish myo6 moved to filapodia tips in cultured cells, in vitro motility assays using fluorescently labeled actin could then be used to confirm the direction of movement (Wells et al. 1999).

Opposite direction of fish myo6 is unlikely, however, as most fish utricular stereocilia do not have myo6. Also, immunolocalization studies in mammalia kidney show that mouse myo6, a proven minus-ended motor, is found in microvillar structures (Biemesderfer et al. 2002). This suggests transport of myo6 up the stereociliary (or microvillar) shaft by a plus-ended motor such as myo7a. This interaction could occur by direct binding of the two myosins or via binding with an adaptor protein. Several myo6 binding partners such as Dab2 and GIPC have been identified in yeast two-hybrid studies (Inoue et al. 2002b; Hasson 2003). Immunolocalization of myo6 binding partners in fish stereocilia is a first step in better understanding myo6 distribution and function within fish ears.

The discovery of two *myo6* paralogs in fishes also presents a unique opportunity to study myo6 function (Kappler et al. 2004; Seiler et al. 2004). The duplication-degeneration-complementation (DDC) model of Force et al. (1999) states that duplicate genes often partition ancestral functions, such that each paralog takes on a portion of the ancestral function in a complementary fashion. Gene expression studies by Seiler et al. (2004) suggest that the two *myo6* paralogs in zebrafish have complementary expression patterns. Therefore, comparative studies of myo6 function between a teleost (with two *myo6* genes) and a non-teleost such as the bowfin (with one *myo6* gene) will also provide new insights into myo6 functions as

well as knowledge of how gene subfunctions are partitioned following duplication events.

Validity of genome duplication timing in fishes

Finally, the results in Chapter three, while highly suggestive of a genome-wide duplication in the teleost ancestor, are not conclusive. The complete sequencing and annotation of the pufferfish genome (*Tetraodon nigroviridis*) confirms that a complete duplication event did take place in at least the common ancestor of zebrafish and pufferfish (Jaillon et al. 2004). Large-scale sequencing of a non-teleost actinopterygian and a basal teleost is necessary to validate the timing proposed in Chapter three. As the probable sister group to teleosts, bowfin are a good candidate for the non-teleost fish, while an osteoglossiform fish such as the freshwater butterfly fish or arapaima (*Arapaima gigas*) are good choices for the basal teleost (Nelson 1994; Inoue et al. 2003).

Final Conclusions

In summary, teleost fishes have two *myo6* genes that probably arose during a whole genome duplication in the teleost ancestor. Both of these *myo6* paralogs are expressed in ear tissue as shown by RT-PCR but only *myo6b* (and ancestral *myo6*) is expressed in hair cells. Most fishes examined here have differential distribution of myo6 protein within hair cells of different end organs, providing novel model organisms for further study of this important hair cell protein. The studies presented here demonstrate that fishes are an excellent group for the study of a wide-range of biological questions, from basic evolutionary questions to biomedical pursuits. As there are more fishes than all other vertebrates combined, comparative cell and molecular studies of diverse fishes offer a wealth of opportunities for biological discovery.

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