Characterization of myosin I in the inner ear

Kelli R. Phillips
West Virginia University

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Characterization of Myosin I in the Inner Ear

Kelli R. Phillips

Dissertation submitted to the School of Medicine
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in
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2007
Abstract

Characterization of Myosin I in the Inner Ear

Kelli R. Phillips

Specialized epithelial cells, called hair cells, detect stimuli perceived by the organs of the inner ear. Such stimuli deflect the sensing organelle of the hair cell, the hair bundle. This force is proposed to create tension in the tip link, an extracellular filamentous structure, and open mechanically-gated ion channels. This process, called mechanotransduction, allows ions to enter the hair cell and results in its depolarization. During prolonged stimuli, the sensitivity of the hair cell is maintained by adaptation. To date, little is known about the molecular mechanisms that regulate either mechanotransduction or adaptation.

Myosin-1c (Myo1c) has been proposed to be the adaptation motor. As such, it must interact with other components of the mechanotransduction complex. Using an in situ binding assay, we previously demonstrated that Myo1c interacts with molecules in the hair bundle, the site of mechanotransduction, through the calmodulin (CaM)-binding IQ domains in its neck region. In the current study, we identify the second CaM-binding IQ domain as a region of Myo1c that mediates CaM-sensitive binding to the hair bundle. As well, we show that the binding of Myo1c in the hair bundle is disrupted by treatments that break tip links.

Recent data indicate that Myo1c interacts in vitro with Cadherin-23 (Cdh23), a component of the tip link, and phosphotidyl-inositol bisphosphate, a phospholipid recently shown to be essential to mechanotransduction. It is not known, however, whether these interactions occur in hair cells. To determine if either of these components contribute to the binding observed in in situ binding assays, we examined Myo1c binding in mice whose hair cells lack Cdh23. In such samples, Myo1c binding was not observed. Additional experiments confirm the correct localization of PIP2 in Cdh23 mutant mice. Collectively, our data suggest that Myo1c and Cdh23 interact in the hair bundle and that this interaction is modulated by CaM.

Myosin-1a (Myo1a), the predominant unconventional myosin expressed in the enterocyte, has also been implicated in hearing disorders. Using antibodies raised against Myo1a we failed to detect Myo1a in the hair cells of murine cochlea. However, alternative, potentially novel, antigens were detected and will be pursued in future work.
Acknowledgments

“The features that, at least to my mind, help make a good or a great scientist really are not some of the ones that our society most often points to. It isn't just enormous intelligence or enormously rapid thought but a couple of other properties are particularly important. One is innovative thought, ideas that run counter to some preconceived notions. Another is simply perseverance. People, in order to work out the details of scientific inquiry, need a lot of time and a lot of patience. And it's very rare that one has a sudden blinding or overwhelming insight that one can promptly follow through. More often one has a hunch or an intuition that one then tries to trace over many months or many years until one finally has the full picture. And it's only in an intuitive sense that you then try to get on paper, in terms of concrete experimentation. Making that transition can be extremely hard but when it's successful it's remarkably rewarding.”

J.A. Hudspeth, Becoming a Scientist, HHMI Lecture Series

Like many of the pivotal events in scientific history, serendipity led me to the laboratory of Dr. Janet Cyr. Retrospectively, I am unsure how I would have completed graduate school without her presence in my life. Together we have spent countless hours at the bench, at the microscope, and in her office. Following her example, I have learned to never settle for mediocrity and to assume responsibility for my decisions. Everyday she teaches me about compassion and fortitude. She has taught me how to lead, to follow, and most importantly, to care in the face of twelve-hour days and negative results.

On an accompanying note, I would like to especially thank Song Tong and Matt Roberts, our laboratory technicians during my tenure. They not only provided me with a steady stream of experimental reagents and protocols, but also filled my hours at the bench with interesting conversations and mischief. As well, I must also recognize my fellow labmates, Anindita Biswas and Jeff Christiansen. If not for simply spreading Janet’s attention, but for keeping the experimental bar high and our chocolate drawer fully stocked.

During the course of my education, I also had the opportunity to work with several scientists at other institutions who significantly contributed to my scientific approach. Specifically, I would like to thank Guy P. Richardson at Sussex University, UK, and Matthew J. Tyska at Vanderbilt University, TN. Both scientists were always available with technical support and rewarding interactions. They each provided me with the diversified perspective that is vital to practicing good science.

The experience I have had with the members of my dissertation committee has been very rewarding. They have acted as my mentors, advocates, and colleagues. Despite busy schedules, each of them has taken time to give me guidance in both my immediate and long-term goals. I would like to thank them for sharing with me both their time and insight.

Finally, I would like to recognize my family. My successes are a direct reflection of the support I have received from them throughout my life. My husband and son relinquish me daily to my science. My parents and grandparents have remained a constant positive presence in my life. They are proud of me when I succeed and when I fail. It is their love and understanding that continuously fuels my fire
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<tr>
<td>3THD</td>
<td>myosin III tail homology domain</td>
</tr>
<tr>
<td>αBB1</td>
<td>antibody recognizing Brushborder I (Myo1a)</td>
</tr>
<tr>
<td>ALA</td>
<td>ankle link antigen</td>
</tr>
<tr>
<td>BB</td>
<td>brushborder</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>CD1</td>
<td>C-like-domain 1</td>
</tr>
<tr>
<td>CdH23</td>
<td>cadherin-23</td>
</tr>
<tr>
<td>CdH23&lt;sup&gt;2J&lt;/sup&gt;</td>
<td>Cadherin-23 mutant mice</td>
</tr>
<tr>
<td>DEG/ENaC</td>
<td>degenerin epithelial sodium channel</td>
</tr>
<tr>
<td>FERM</td>
<td>F for 4.1, E for Ezrin, R for radixin and M for moesin; a unique module involved in the linkage of cytoplasmic proteins to the membrane</td>
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<tr>
<td>IHC</td>
<td>inner hair cell</td>
</tr>
<tr>
<td>MAGUK</td>
<td>membrane associated guanylate kinase</td>
</tr>
<tr>
<td>MET</td>
<td>mechanosensitive transduction</td>
</tr>
<tr>
<td>MV</td>
<td>microvilliar</td>
</tr>
<tr>
<td>Myo15a</td>
<td>myosin-15a</td>
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<tr>
<td>Myo1c</td>
<td>myosin-1c</td>
</tr>
<tr>
<td>Myo1c-N2</td>
<td>Myo1c IQ2 domain recombinant protein</td>
</tr>
<tr>
<td>Myo1c-T701</td>
<td>Myo1c neck and tail recombinant protein</td>
</tr>
<tr>
<td>Myo1c-T701&lt;sub&gt;IQ1RG1&lt;/sub&gt;</td>
<td>Myo1c-T701 with mutations in IQ1</td>
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<tr>
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<td>Myo1c-T701 with mutation in IQ2</td>
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<td>myosin-3a</td>
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<td>myosin-6</td>
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<td>myosin-7a</td>
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<td>myosin-7a mutant mice</td>
</tr>
<tr>
<td>MyTH4</td>
<td>myosin tail homology 4</td>
</tr>
<tr>
<td>NOMPC</td>
<td>no mechanoreceptor potential C</td>
</tr>
<tr>
<td>OHC</td>
<td>outer hair cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PAB</td>
<td>parallel actin bundle</td>
</tr>
<tr>
<td>PBI</td>
<td>PDZ binding interfaces</td>
</tr>
<tr>
<td>Pcdh15</td>
<td>protocadherin-15</td>
</tr>
<tr>
<td>PDZ</td>
<td>PSD-95/SAP90 DISCS-largeZ0-1 homologous</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
</tr>
<tr>
<td>PIP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>phosphatidylinositol bisphosphate</td>
</tr>
<tr>
<td>PLC&lt;sub&gt;δ1&lt;/sub&gt;</td>
<td>phospholipase Cδ1</td>
</tr>
<tr>
<td>PMCA</td>
<td>plasma membrane Ca&lt;sup&gt;2+&lt;/sup&gt; ATPase</td>
</tr>
<tr>
<td>SH3</td>
<td>src homology 3</td>
</tr>
<tr>
<td>TH1</td>
<td>basic tail homology 1</td>
</tr>
<tr>
<td>TLA</td>
<td>tip link antigen</td>
</tr>
<tr>
<td>TM</td>
<td>tectorial membrane</td>
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I. Introduction

A. Sensory mechanoreceptors

Environmental perception is vital to the survival of all organisms. This is achieved largely by the process of mechanosensation; a sensory pathway common to an exhaustive array of biological systems. The breadth of this process ranges from an organism’s detection of pain to a cell’s sensation of fluid flow. Of particular interest are the specialized systems that have evolved to detect minute stimuli; transforming small physical energies into vital neurological messages. Our senses of touch, hearing and balance are three such crucial examples.

To date, very little is known about the details of tactual perception in vertebrates. Because a diverse group of touch receptors are interspersed among the non-sensory cells of the epidermis, the identification of unifying molecules playing a role in somatosensation has been difficult. In the case of the Merkel cell-neurite complex, responsible for vertebrate perception of soft-touch, controversy surrounds the function of each cellular component. It was only recently, following more than a century of debate, that pre-synaptic molecules were identified in the Merkel cell, strongly supporting its role in mechanosensation (Haeberle et al., 2004). In contrast, the identification of the mechanosensory cell of the acousticolateralis system, the hair cell, occurred over 150 years ago and its proposed function has remained undisputed (Corti, 1851). However, as is the case with the somatosensory system, hair cells are sparse in number and traditional biochemical techniques have been only marginally successful in the identification of molecular components.

Common to each of these sensory systems is the mode of signal transduction. In each system, regardless of the specific cellular receptor, a physical force, such as surface pressure, sound waves or gravity, acts to open mechanosensitive transduction (MET) channels. These channels are predicted to work as a part of a complex of proteins that
anchor the MET channel to the cytoskeleton of the receptor cell and to couple it to the cell’s extracellular surface where the stimulation occurs. A large-scale mutagenesis screen in *Caenorhabditis elegans* enabled the initial identification of a collection of proteins that when mutated (*mec* mutants) adversely affected MET-mediated touch (Chalfie and Sulston, 1981; Chalfie and Au, 1989; reviewed in Gillespie and Walker, 2001). From this pool, the degenerin/epithelial sodium channel (DEG/ENaC) family became a popular candidate for the MET channel involved in the perception of touch in *C. elegans* (Huang and Chalfie, 1994). A similar screen was later repeated in *Drosophila* (Kernan et al., 1994; reviewed in Gillespie and Walker, 2001). These results implicated the transient receptor potential (TRP) and the transient receptor potential-vanilloid (TRPV) as strong candidates for the MET channels involved in touch and hearing in flies (Walker et al., 2000; Hamill and Martinac, 2001; Gong et al., 2004; reviewed in Gillespie et al., 2005; reviewed in Nicolson, 2005b; Cheung and Corey, 2006). Later experiments that examined mutations in the TRP protein, NOMPC, which led to the loss of mechanosensation in the fly, confirmed the original hypotheses (Walker et al., 2000). However, investigating these leads in mammals has been challenging. Knockout animals lacking the alpha subunit of the DEG/ENaC channel were shown to exhibit no abnormalities in mechanotransduction (Rusch and Hummler, 1999). In addition, the TRP channels identified in invertebrates to mediate mechanotransduction do not have exact homologues in mammals (Walker et al., 2000; Kim et al., 2003; Gong et al., 2004). As a result, investigators have been screening close mammalian correlates with minimal success (Kwan et al., 2006). To date, the identity of these channels and the complete complement of interacting proteins in mammals are still unknown. It is the long-term goal of our research to elucidate these molecular mechanisms in the auditory and vestibular systems of the inner ear.

**B. Perception of sound and movement**

The acousticolateralis systems of the inner ear are responsible for our senses of hearing and balance. Sensations detected by these systems begin as physical forces that
are detected by specialized organs and then translated to a synaptic signal that is processed by the brain (reviewed in Hudspeth, 1989). In the auditory system, sound transmission begins as sinusoidal waves that are filtered by the outer ear, the pinnae, and directed through the middle ear canal where they make contact with the surface of the tympanic membrane (ear drum; Fig 1A). This physical force is translated to three small bones, the ossicles, and then to the oval window of the cochlea (reviewed in Hudspeth, 1989). In the vestibular system, gravity and directional accelerations act to move the auxiliary structures of the inner ear that stimulate the end organs: the utricle, sacculus and semicircular canals (reviewed in Hudspeth, 1989). Both the auditory and vestibular organs share a common mechanoreceptor, the hair cell.

Mammalian auditory detection occurs within the cochlea. Encased in the temporal bone, this assembly, 150 µm wide by 35 mm long, wraps around the modiolus forming a shape analogous to a snail shell (measurement of the human cochlea; von Bekesy, 1960). The cochlea is separated into three spiral ducts; the tympanic, vestibular, and the cochlear canals (Fig 1A, green, pink and yellow respectively). The tympanic and vestibular canals are filled with perilymph, a fluid analogous to cerebral spinal fluid that has a high concentration of sodium and a low concentration of potassium (reviewed in Dallos, 1992). The cochlear canal is filled with endolymph and, like cellular cytosol, has a high concentration of potassium and a low concentration of sodium (reviewed in Dallos, 1992). These differences in ionic concentrations create an electrochemical gradient that is crucial to auditory signal transduction (Offner et al., 1987; reviewed in Dallos, 1992).

In the cochlear canal, the organ of Corti rests on the basilar membrane and contains four parallel rows of sensory hair cells (Fig 1B). The inner hair cells (IHCs) are poised closest to the modiolus and sit directly upon the basilar membrane. The remaining three rows of sensory cells are referred to as the outer hair cells (OHC). The OHCs rest upon an underlying collection of cells called the Dieter cells (Fig 1B; DC). Each hair cell detects a specific frequency of sound relative to its position along the basilar membrane (reviewed in Hudspeth, 1989). Those hair cells posited at the apex of the cochlea detect
low frequency sound, while those at its base detect high frequency sound (reviewed in Hudspeth, 1989). Additional cochlear supporting cells, pillar cells, separate the IHCs from one another and form a partition between the IHCs and the OHCs (Fig 1B; PC). The remainder of the cochlear epithelium is made up of Henson cells (Fig 1B; HC).

In the vestibular organs, hair cells lie on sensory epithelial sheets in hexagonal arrays separated by supporting cells. In the semicircular canals these sheets occur at the ampule, a bulbaceous region of each canal, and are called crista. In the sacculus and utricle, these sheets are called maculae. Positioned directly above both the crista and maculae is a thin gelatinous layer, referred to as the cupula and otolithic membrane respectively, upon which resides a large mass of carbonate crystals called the otoconia (reviewed in Hudspeth, 1989).

**C. The hair cell**

The hair cell is a polarized epithelial cell. At its apical surface, a sensing organelle, the hair bundle, projects into the endolymph where it is poised to detect stimuli. The OHCs of the cochlea make contact with the tectorial membrane. In the cochlea, the tectorial membrane remains stationary as the basilar membrane oscillates in response to sound waves, creating a shearing movement that acts to deflect the hair bundles of the OHCs (reviewed in Hudspeth, 1989). IHC bundles do not directly contact a structural surface in the cochlea, but instead are deflected solely the fluid displaced by the basilar membrane’s movement through the surrounding endolymph (Dallos et al., 1972; Lim, 1986). The hair bundles of vestibular hair cells make direct contact with the otolithic membrane in the sacculus and the cupula in the semicircular canals. The adjacent otoconia move in response to head movements, linear and angular accelerations, and gravity, shifting the underlying cupula and otolithic membranes, resulting in the deflection of the hair bundle (reviewed in Hudspeth, 1989).
In the cochlea, IHCs are innervated by 95% of the all the afferent nerve fibers extending from the cochlear nuclei, clearly establishing the IHCs as the primary sensory transmitters of the auditory system (Fig 1B; reviewed in Spoendlin, 1985). In contrast, the OHCs have only a few afferent nerve fibers, and instead have efferent synaptic connections from the superior olivary complex (reviewed in Spoendlin, 1985). This asymmetrical pattern of innervation left the role of the OHCs unclear for many years (reviewed in Dallos, 1988). However, it is now known that OHCs serve in a positive feedback loop that amplifies the sensitivity of stimuli detection (reviewed in Dallos, 1992). This cochlear amplification is proposed to occur as the result of two processes: somatic electromotility and stereociliary amplification (reviewed in Fettiplace, 2006). Together these movements are proposed to occur in phase with the shearing motion of the basilar and tectorial membranes and act to increase the flow of fluid that deflects the hair bundles of the IHC. Somatic electromotility describes the expansion and contraction of the OHC body in response to stimuli and subsequent intracellular depolarization (Brownell et al., 1985; Kachar et al., 1986; Ashmore, 1987). Recent data indicate that this phenomenon is mediated by the motor protein prestin (Zheng et al., 2000a; reviewed in Dallos et al., 2006). Immunolocalization experiments indicate that prestin is present at very high concentrations in the somatic membrane of the OHC (Oliver et al., 2001). In response to intracellular depolarization, prestin is postulated to undergo a conformational change that decreases its surface area, thereby causing the OHC to contract (reviewed in Dallos et al., 2006). As evidence of prestin’s role as the OHC motor, the OHCs of prestin knockout mice do not undergo somatic electromotility (Liberman et al., 2002). As well, the frequency selectivity of the cochlea is greatly reduced in prestin KO mice despite normal mechanotransduction (Liberman et al., 2002; Cheatham et al., 2004; reviewed in Dallos et al., 2006).

Acoustic emissions manifesting from acousticolateral amplification have also been measured in non-mammalian invertebrates that do not have OHCs (reviewed in Hudspeth, 1989). This led to investigation and the subsequent theory of stereociliary amplification, which describes the negative movement of the hair bundle in response to calcium (Choe et al., 1998; Chan and Hudspeth, 2005). This process has been shown to
occur in mammals as well as non-mammalian invertebrates and is believed to work in conjunction with the somatic contractility of the OHCs (Chan and Hudspeth, 2005; reviewed in Fettiplace, 2006). Together, both processes clearly provide explanation for the discrepancies noted between the predicted and measured threshold limits of the cochlea (Dallos, 1988). By acting as amplifiers of stimuli, especially at higher frequencies, the OHCs are able to significantly increase the sensitivity of the cochlea. As expected, when OHCs are damaged, hearing persists, but with a limited frequency range (Ryan and Dallos, 1975).

D. The hair bundle

Each hair bundle is composed of a single process called the kinocilium and a collection of many elongated actin-filled processes called stereocilia (Fig 2). The kinocilium is the only true cilium of the hair cell. As such, the core of the kinocilium is composed of microtubules arranged in the 9+2 pattern that is common to all cilia structures (Flock and Duvall, 1965). Despite the localization of numerous hair bundle proteins to the kinocilium, its function is unknown (Hudspeth and Jacobs, 1979; Goodyear et al., 2005; Leibovici et al., 2005; Michel et al., 2005; Shin et al., 2005). Upon its discovery, it was postulated to be the site of mechanotransduction in the hair cell (Hillman, 1969). However, mechanotransduction in the hair cell persists following kinocilium micro-dissection (Hudspeth and Jacobs, 1979). Moreover, the kinocilium degenerates as auditory hair cells mature (reviewed in Eatock, 2000). Current hypotheses predict that the kinocilium acts to tether hair bundles to proximate membranes or, alternatively, contributes to the orientation of the hair bundle in the surrounding sensory epithelium and its resulting axis of sensitivity (Hudspeth and Jacobs, 1979; reviewed in Kelley, 2006).

The mature stereocilium is shaped like a pencil, having a constant diameter along its length and tapering at its point of insertion into the apical surface of the cell. The narrow portion of the stereocilium is referred to as the stereociliary rootlet (Fig 2C). The
rootlet has decreased rigidity, allowing stereocilia to pivot in response to stimuli (Flock et al., 1977). The predominant protein of stereocilia, comprising 50-75% of the total protein, is actin (Flock and Cheung, 1977; Tilney et al., 1980; Shepherd et al., 1989). Stereociliary actin, comprised of both beta and gamma monomers, is organized in parallel hexagonal arrays with the positive, or barbed end, of the actin filament oriented at the tips of the stereocilia (Flock and Cheung, 1977; Tilney et al., 1980).

The development of the hair bundle has been explored extensively in avian models (Tilney and DeRosier, 1986; Tilney et al., 1986; Tilney et al., 1988). During early embryonic chick development, the kinocilium and stereocilia are unable to be distinguished from the short microvillia present on the hair cells apical surface (reviewed in Tilney et al., 1992). However, beginning at approximately embryonic day 10 (E10) in avian models, the kinocilium and stereocilia become thicker and more robust than the surrounding microvilliar processes (Tilney and DeRosier, 1986). Shortly thereafter, the kinocilium migrates to an eccentric position at the abneural edge of the hair cell (Tilney and DeRosier, 1986). During this time, stereocilia continue to elongate sequentially, with those stereocilia closest to the kinocilium growing first (reviewed in Tilney et al., 1992). The rate of growth and length of each stereocilium can be correlated to its positioning relative to the kinocilium. Those nearest the kinocilium are taller and have a faster rate of growth when compared to those furthest away. As a result, the stereocilia of a hair bundle form a staircase pattern (reviewed in Tilney et al., 1992). In the adult cochlea, this staircase pattern forms a crescent, with the kinocilium located at the crescent’s vertex (Fig 2B). In vestibular organs, the bundle is spherical and the staircase pattern gives the bundle a beveled appearance, with the kinocilium at its tallest point (Fig 2A & D). In the cochlea, the kinocilium degrades as the hair bundle matures, while in vestibular organs it remains for the life of the hair bundle.

Stereocilia are often compared to the microvilli that extend from the brush border regions of enterocyte cells in the lumen of the small intestine (Flock and Cheung, 1977). Both processes result from a very similar arrangement of parallel actin bundles (PABs) and consequently the two structures share many of the same proteins (discussed below,
reviewed in Tilney et al., 1980; reviewed in Bartles, 2000). However, stereocilia and microvilli function at different levels within their sub-cellular hierarchies. Microvillar PABs act as a scaffolding for the enterocyte membrane. The increased surface area created by microvillar PABs allow for enhanced nutrient adsorption in the lumen of the small intestine. Mutations in microvillar PAB proteins only moderately affect gut function (Tyska et al., 2005). In contrast, the structure of each stereocilia directly affects the process of signal transduction. Mutations that alter stereocilia structure often lead to complete acousticolateralis dysfunction, making the role of the stereocilia increasingly elaborate when compared to that of the microvilli.

The PABs that make up each stereocilium directly influence the physical properties of the hair bundle. The stereociliary membrane is tightly coupled to the PABs and lies only 100 angstroms away (Tilney et al., 1980). As a result, the length and width of each stereocilium is directly proportional to the hundreds of actin filaments of which it is composed. It is interesting to note that while the height of the hair bundle varies in relation to the hair cells position along the cochlea and the frequency that it is tuned to detect, the total amount of actin utilized in each hair bundle is identical (Tilney and Tilney, 1988). A hair cell with a longer bundle has fewer, thinner stereocilia and a hair cell with a shorter bundle has an increased number of stereocilia with larger girth.

In a stereocilium, approximately 90% of the actin filaments terminate prior to the rootlet (Tilney et al., 1980). The remaining actin filaments are arranged in a donut-like pattern and extend into the cuticular plate, a dense cytoskeletal network that covers most of the apical surface of the hair-cell body (Tilney et al., 1980). This domain has a high concentration of randomly oriented actin filaments that are cross-linked by the actin bundling protein, spectrin (Tilney et al., 1989; Drenckhahn et al., 1991). In addition, the proteins tropomyosin and radixin have been identified in the cuticular plate and are postulated to play a role in stabilizing and anchoring the stereociliary rootlet (Drenckhahn et al., 1991; Pataky et al., 2004). As a result of the cuticular plate’s dense structure, all vesicular traffic appears to be directed around its periphery. This area, referred to as the
pericuticular necklace, consequently maintains a high concentration of vesicles and hair bundle proteins (Kachar et al., 1997).

Stereociliary actin filaments are predicted to undergo a steady-state process of actin renewal called actin treadmilling (Schneider et al., 2002; Rzadzinska et al., 2004; reviewed in Lin et al., 2005). Actin filaments polymerize at the tip of the stereocilia while simultaneously degrading at the base (Schneider et al., 2002). The details of this process were observed in cochlear cultures, subsequent to the over-expression of GFP-actin (Schneider et al., 2002; Rzadzinska et al., 2004). Incorporation of the GFP-actin revealed the identical rates of elongation and degradation of PABs in the stereocilia (Rzadzinska et al., 2004). The rate of actin turnover was shown to adjust according to the height of the stereocilia, allowing the entire hair bundle to renew uniformly; this process takes 48 hours in cochlear hair cells and 72 hours in vestibular hair cells (Schneider et al., 2002; Rzadzinska et al., 2004). Recent evidence also implicates the adjacent stereociliary membrane in the treadmilling process (Grati et al., 2006). One of the most abundant proteins of the hair bundle, plasma membrane Ca\(^{2+}\) ATPase (PMCA), was over-expressed using the same technique used by Schneider et al. (2002). Like actin, PMCA turned over quite rapidly, on the order of 5-7 hours (Grati et al., 2006).

The regulation of actin filament polymerization at the stereociliary tip is only beginning to be elucidated (Mburu et al., 2006). To date, the PSD-95/SAP90 DISCS-largeZ0-1 homologous (PDZ) domain protein whirlin, the membrane associated guanylate kinase (MAGUK) protein p55, and the protein 4.1R have all been implicated in this process (Mburu et al., 2003; Kikkawa et al., 2005; Mburu et al., 2006). These proteins are all localized to the tips of stereocilia in the developing hair bundle, and have been shown to interact with one another in vitro (Mburu et al., 2006). In addition, the interactions of these proteins in the process of actin microfilament assembly is well established in erythrocytes (Marfatia et al., 1995).

Of the identified proteins involved in actin polymerization, the role of whirlin has been explored in the greatest detail (Mburu et al., 2003; Kikkawa et al., 2005). Whirlin is
postulated to be an essential structural protein that acts to organize proteins at the stereociliary tip by virtue of its multiple PDZ domains (Mburu et al., 2003). Null mutations of whirlin, as modeled in the *whirler* mouse, result in dramatically shortened stereocilia in IHCs and malformed OHC bundles (Holme et al., 2002). Two isoforms of whirlin are expressed in the hair cell (Mburu et al., 2003). The long isoform of whirlin contains three PDZ domains (one C-terminal and two N-terminal) and a proline rich domain. Short whirlin lacks the two N-terminal PDZ domains (Mburu et al., 2003). While both isoforms of whirlin are expressed in the hair bundle, experiments using a BAC transgene to express only the short isoform in the *whirler* mouse, show a complete rescue of the mutant phenotype (Mburu et al., 2003).

Antibodies that recognize the short isoform of whirlin have been used to characterize its expression in the developing hair bundles of both IHCs and OHCs in the cochlea (Kikkawa et al., 2005). At murine age P0, whirlin is observed only in the tallest row of stereocilia of all cochlear hair cells (Kikkawa et al., 2005). In OHCs its expression is next detected in each sequentially growing row of stereocilia (Kikkawa et al., 2005). In IHCs, where rows of stereocilia are less defined, a similar pattern of expression is also detected (Kikkawa et al., 2005). In each stereocilium, whirlin’s expression is restricted to the actin-free zone at the tip of the stereocilia where actin polymerization is postulated to occur (Schneider et al., 2002; Kikkawa et al., 2005). The expression of whirlin can no longer be detected in OHCs after murine age P14 or in the IHCs after age P11 (Kikkawa et al., 2005). Conversely, whirlin is expressed throughout adulthood in all stereocilia of vestibular hair cells (Kikkawa et al., 2005).

**E. Actin cross-linkers**

The actin filaments that make up the cytoskeletal core of each stereocilium are extensively crosslinked. The first identified actin crosslinking protein of the hair bundle was fimbrin (Tilney et al., 1989; reviewed in Tilney et al., 1992). Fimbrin is located with regular periodicity along the entire length of the stereocilium and can easily be observed
using light microscopy as transverse stripes crossing the actin filament core (Tilney et al., 1989). As the result of two-tandem actin-binding calponin homology domains, fimbrin acts to densely pack the parallel arrays of actin filaments (Banuelos et al., 1998; Hanein et al., 1998). The crosslinks that fimbrin makes with actin are unique, having a degree of flexibility that allows the stereocilia to bend slightly in response to stimuli (Volkmann et al., 2001).

Fimbrin’s interactions with actin are sensitive to high calcium (Ca\(^{2+}\)). In the absence of the fimbrin-actin interaction, actin bundles lose their reported ordered hexagonal packing, but still remain intact (Tilney et al., 1989). Accordingly, early research focused on the identification of other actin crosslinking proteins in the hair bundle (Tilney et al., 1989). Villin, which works in conjunction with fimbrin in the microvillus of the brush border intestinal cells, was thought to be an obvious candidate. However, early experiments indicated that villin was not expressed in hair cells (Flock et al., 1982; Shepherd et al., 1989). Over a decade later, a second protein that works in unison with fimbrin in the intestine, espin, was found to also be an actin-bundling protein of the hair bundle (Zheng et al., 2000b).

Espin was first identified in the PABs of the Sertoli cells lining the seminiferous epithelium (Bartles et al., 1996; Chen et al., 1999). Since then espin has also been characterized in a variety of sensory cells including taste cells, Merkel cells, hair cells, and the microvilli domains of the enterocyte (Bartles et al., 1998; Sekerkova et al., 2004). Each espin isoforms has like C-terminal actin binding modules and unique N-terminal domains that are postulated to modulate espin’s specific protein-protein interactions (Bartles, 2000). Espin’s affinity for actin is 10 to 100 fold higher than other identified actin-bundling proteins (Chen et al., 1999). As well, in contrast to fimbrin, espin interactions with actin are not modulated by Ca\(^{2+}\) concentrations (Bartles, 2000). In avian cochlear and vestibular hair cells, espin expression is concurrent with stereociliary elongation and is localized along the length of the stereocilium (Li et al., 2004). The concentration of espin in each stereocilium increases with stereociliary height (Rzadzinska et al., 2005), and the over-expression of espin in tissue culture cells results
in microvilliar structures with increased rates of growth (Loomis et al., 2003). In addition, a mutation in the C-terminal actin-binding domain of espin has been identified as the causal mutation of the Jerker mouse (Zheng et al., 2000b). The absence of espin in the stereocilia of these mice results in thin, short stereocilia leading to deafness and vestibular dysfunction (Zheng et al., 2000b; Karolyi et al., 2003). Collectively, data suggest that espin acts to maintain the association of actin filaments, initiate their elongation and regulate the growth required to maintain the hair bundle structure utilizing a concentration-dependent mechanism (Rzadzinska et al., 2005).

**F. Mechanotransduction and the tip link**

**The tip link**

Stereocilia positioned in the tallest row of the hair bundle have oblate tips, while shorter ones have tips described as prolate. This observed tenting that occurs on shorter stereocilia is thought to be the result of mechanical tension on the stereociliary membrane caused by the presence of an extracellular linkage commonly referred to as the tip link (Fig 2E & F: Pickles et al., 1984; Kachar et al., 2000). The tip link, visible in transmission electron micrographs from murine hair cells beginning at E17.5, spans the distance between each stereocilia and its next tallest neighbor (Denman-Johnson and Forge, 1999; Geleoc et al., 2004). The tip-link structure is predicted to be composed of a complex of proteins: up to four helical proteins that span the 150-300 nm between stereocilia and up to three anchoring filamentous proteins at each end that act to secure the structure to the stereociliary membrane (Kachar et al., 2000; Tsuprun et al., 2004).

The integrity of the tip link structure is Ca\(^{2+}\) dependent. When hair cells are exposed to the classical calcium chelating agents, 1,2-bis(\(O\)-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) or Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), the tip-link structures are immediately obliterated (Assad et al., 1991; Zhao et al., 1996; Marquis and Hudspeth, 1997). Following such
treatments tip links reform in approximately 4 hours, imperfectly and independent of protein synthesis (Zhao et al., 1996). Additionally, tip links are unaffected by the protease subtilisin, but they are broken by an excess of the trivalent cation lanthanum (La\textsuperscript{3+}) (Kachar et al., 2000; Goodyear and Richardson, 2003).

**Potential components of the tip link**

Investigators have focused on the identification of the tip-link components since the structure was discovered in 1984 (Pickles et al., 1984). Recently, cadherin-23 (Cdh23) has become a strong candidate for a component of the tip link (Siemens et al., 2004; Sollner et al., 2004; reviewed in Gillespie et al., 2005). Cdh23 is a transmembrane protein that engages in Ca\textsuperscript{2+} dependent extracellular interactions that mediate cell-cell adhesion (Di Palma et al., 2001a). Its extracellular domain is predicted to contain 27 calcium-binding ectodomain (EC) repeats that form homophilic interactions that are common to many transmembrane adhesion glycoproteins (Nollet et al., 2000; Di Palma et al., 2001a). Two alternatively spiced isoforms of Cdh23 (Cdh23\textsuperscript{+exon68} and Cdh23\textsuperscript{-exon68}) are expressed in the inner ear. However, only the expression of Cdh23\textsuperscript{+exon68} is restricted to the hair bundle.

The short intracellular domain of Cdh23 contains two PDZ-binding interfaces (PBIs); one internal and one at the protein’s C-terminus. These domains mediate the interaction of Cdh23 with the structural hair bundle PDZ protein, harmonin-b (Boeda et al., 2002). Harmonin-b contains three PDZ domains (one C-terminal and two N-terminal) and an actin-binding domain at its C-terminus (Bitner-Glindzicz et al., 2000; Verpy et al., 2000). The first PDZ domain of harmonin-b interacts with the internal PBI domain of Cdh23, while its second PDZ domain interacts with the Cdh23 C-terminal PBI (Siemens et al., 2002). The inclusion of exon 68 in Cdh23\textsuperscript{+exon68} isoform disrupts its internal PBI domain and is proposed to specifically direct harmonin-b to interact with only the C-terminal PBI of Cdh23 (Siemens et al., 2002). Mutations in both harmonin-b and Cdh23 have been linked to the human disease Usher’s Syndrome type I, subtypes C and D respectively (Verpy et al., 2000; Di Palma et al., 2001b). This disease is an
autosomal recessive syndromic disorder that results in congenital deafness and concurrent retinitis pigmentosa (reviewed in Petit, 2001). The remaining subtypes of Usher’s syndrome type I are linked to mutations in additional hair bundle proteins and will be discussed below.

The phenotypes of the waltzer mouse and sputnik zebrafish have been linked to mutations that cause the null-expression of Cdh23 (Di Palma et al., 2001b; Sollner et al., 2004). The waltzer mouse is affected by early-onset hearing-loss. This is attributed to the abnormal development of their hair bundles; in most cases the kinocilium is mis-localized, cochlear bundles do not form crescent shapes and mature hair bundles are splayed (Di Palma et al., 2001b). This severe bundle disorganization provides evidence of Cdh23’s crucial role in stereociliary linkages (Bolz et al., 2001; Bork et al., 2001; Di Palma et al., 2001b). A similar dysfunctional morphology is described in the zebrafish Cdh23 mutant sputnik. Moreover, in this model, no discernible tip-link structures are detected. While the absence of tip links could be a secondary effect of the Cdh23 deficiency, the investigators feel that this observation strongly implicates Cdh23 as a tip-link component (Sollner et al., 2004; reviewed in Gillespie et al., 2005).

Immunolocalization data has shown Cdh23 expression in the transient lateral links that traverse the length of the developing hair bundle, in kinociliary links, and in close proximity to the tip-link structure (Siemens et al., 2004; Lagziel et al., 2005; Michel et al., 2005). Investigators reason that if Ch23 is a component of the tip-link complex its localization, detected by fluorescent antibodies, will be identical to that of the tip-link structure observed using electron microscopy (Siemens et al., 2004; Lagziel et al., 2005; Michel et al., 2005). In bullfrog, investigators have found just that; the localization of Cdh23 mirrors that of the tip link. Extracellular exposure to La$^{3+}$ and EGTA results in the apparent disruption of the tip-link structure and eliminates Cdh23 immunoreactivity at stereociliary tips (Siemens et al., 2004; Phillips et al., 2006). However, the correlation between Cdh23 localization and tip-link integrity does not extend to murine cochlear hair cells. In these cells, despite the identical sensitivities of the tip link to BAPTA, EGTA, and La$^{3+}$, the immunolocalization of Cdh23 persists following these treatments (Michel et
al., 2005; Phillips et al., 2006). As well, the expression of Cdh23 in adult (>P30) murine hair cells is controversial (Boeda et al., 2002; Siemens et al., 2004; Sollner et al., 2004; Lagziel et al., 2005; Michel et al., 2005, Rzadzinska et al., 2005). Investigators unable to detect Cdh23 in mature hair bundles discount its predicted role as a principal tip-link component (Lagziel et al., 2005; Michel et al., 2005). However, late onset hearing-loss resulting from Cdh23 mutations has been reported, giving credence to a vital role of Cdh23 in the mature hair cell (Noben-Trauth et al., 2003).

Two models have been proposed assuming Cdh23’s role as the tip link (Corey and Sotomayor, 2004; Tsuprun et al., 2004; reviewed in Gillespie et al., 2005). The first model predicts that Cdh23 forms a dimer of dimers (one dimer originating from each stereocilium to be connected) creating a linkage between stereocilia whose length is dependent upon the extent of dimer overlap (Corey and Sotomayor, 2004). The second model, suggested by Tsuprun et al. (2004), also predicts that Cdh23 molecules form two dimers that extend from each stereocilium, but these molecules only contact at their N-termini. Both models have trouble fitting seamlessly with the high-resolution measurements of the tip link (reviewed in Gillespie et al., 2005). The first predicts a molecular conformation that falls short of the 100-250 nm tip link length (Kachar et al., 2000), and the second predicts a structure that may be too weak to mediate mechanotransduction forces (reviewed in Gillespie et al., 2005).

In addition to Cdh23, the CD1 and 3 isoforms of protocadherin-15 (Pcdh15), referenced for many years as the tip-link antigen (TLA), have been shown to be associated with the tip-link complex (Goodyear and Richardson, 2003; Ahmed et al., 2006). Because the extracellular domains of the Pcdh15 isoforms are much shorter (~47.3 nm) than the estimated length of the central helical tip-link protein, it is predicted to be one of the observed filamentous anchoring proteins (Kachar et al., 2000). Pcdh15 expression was shown to persist in the mature hair bundle at the stereociliary tips and its immunolocalization is sensitive to BAPTA, EGTA, and La³⁺. These data, however, are controversial; other investigators show localization of Pcdh15 at the base of the stereocilia and provide evidence for its interaction with harmonin and Myo7a (Senften et
Mutations in Pcdh15, as modeled in the orbiter zebrafish and Ames waltzer mouse, result in hair cells that do not transduce (Alagramam et al., 2001; Hampton et al., 2003; Seiler et al., 2005; Haywood-Watson et al., 2006). As well, mutations in human Pcdh15 have been shown to be the cause of Usher’s syndrome type IF (Ahmed et al., 2003).

**Mechanotransduction and adaptation in the hair cell**

Tip links connect each stereocilium to its next taller neighbor. They exist in the same 3-dimensional plane as the kinocilium and create the axis of mechanical sensitivity for the hair bundle. When stereocilia deflect, along this axis towards the tall end of the hair bundle as a result of sensory stimuli, the space between congruent points on neighboring stereocilia increases and puts tension on this extracellular filamentous structure. This tension is postulated to physically open a single mechanoelectrical transduction (MET) channel that is located in conjunction with each tip-link complex in the stereociliary membrane (Holton and Hudspeth, 1986; Howard and Hudspeth, 1988). Mechanotransduction can be detected in murine vestibular hair cells at E17, concurrently with the observation of tip links at stereociliary tips, long before the hair bundle is fully mature (Geleoc et al., 2004). As well, when tip links are broken following treatment with BAPTA, EGTA or La³⁺, mechanotransduction is also ablated (Zhao et al., 1996; Marquis and Hudspeth, 1997).

In the absence of stimuli, the open probability of the transduction channel (P₀) is 0.1-0.2 and the resting potential of the hair cell is -60 mV (Hudspeth, 1989; Fettiplace et al., 1992). A stimulus in the positive direction, deflecting the hair bundle toward the kinocilium and increasing tension at the tip link, causes the open probability of the transduction channels to increase (P₀ approaches 1), allowing the positively charged potassium (K⁺) and Ca²⁺ ions from the surrounding endolymph to enter the cell. The subsequent cellular depolarization (with maximum receptor potentials equaling tens of millivolts) causes increased synaptic release and consequent neuronal signaling (Hudspeth and Corey, 1977; Fettiplace and Crawford, 1978). Early kinetic experiments performed to develop transduction models indicated that hair-bundle transduction
kinetics would not fit a traditional Z-state model. Instead, it was proposed that a third, closed state must be added to the traditional two-state model. This added state was referred to as the “spring” element and became the crux of the “gating spring model” (Corey and Hudspeth, 1983). Initially, the tip link was proposed to be this spring element (Assad et al., 1991; Pickles et al., 1991). However, a recent alternative model suggests that the gating spring may not be the tip link, which appears in high-resolution images to be inelastic, but instead an extension of the MET channel (Kachar et al., 2000; Howard and Bechstedt, 2004; Tsuprun et al., 2004).

Electrophysiological recordings from vestibular hair cells have provided substantial data detailing the process of mechanotransduction. An representative electrophysiological trace that depicts the ion flux in a hair cell following a sustained stimulus is shown in Fig 3. The immediate sharp downward spike is indicative of the positive flow of current into the cell. This current then deteriorates as the result of the process of adaptation (Eatock et al., 1987). This process can be separated into two components (Wu et al., 1999; Holt and Corey, 2000). The first, fast adaptation, occurs in a few milliseconds and results from Ca\(^{2+}\) binding to proximal intracellular sites, perhaps located on the channel, causing its rapid closure (Ricci et al., 1998; Wu et al., 1999). The second component, slow adaptation, is believed to occur as the result of the tip link, MET channel, and associated molecules relocating to a lower position on the stereocilia, thereby decreasing tension in the tip link and allowing for the MET channel’s gradual reclosure. Slow adaptation occurs on a timescale of tens of milliseconds following a stimulus (Fig 3: ●; Eatock et al., 1987; Assad and Corey, 1992). Approximately 60-80% of the MET channel sensitivity is regained following slow adaptation (Fig 3: ■; reviewed in Eatock, 2000). When the bundle stimulus is removed or when the bundle is deflected in the negative direction, the tip link becomes lax and transduction channels completely close, (\(P_\circ = 0\); Fig 3: ▲). To reset tip-link tension and maintain maximum bundle sensitivity, the transduction channel complex relocates to a position higher on the stereocilia. This returns the MET channel open probability to \(P_\circ = 0.1\) (reviewed in Eatock, 2000). Both the initial slipping of the MET channel complex in response to
sustained stimuli and the relocation of the complex to a higher point on the stereocilium following the cessation of the stimulus is regulated by the hair cell’s adaptation motor (to be discussed below, Howard and Hudspeth, 1987; Assad et al., 1989).

Many experiments have been done to characterize the physiological properties of the MET channel. It is predicted to be a cation channel that boasts a large, non-selective pore that is sensitive to Ca$^{2+}$ (Corey and Hudspeth, 1979; reviewed in Corey, 2006; reviewed in Ricci et al., 2006). However, to date, the identity of the MET channel is still unknown. Recent advances made using an invertebrate model indicate that TRP family ion channels have a strong role in mechanotransduction. In *Drosophila* both NOMPC (TRPN1) and TRPV have been implicated in mechanosensory transduction (Walker et al., 2000; Kim et al., 2003; Gong et al., 2004). As well, these channels are attractive candidates for hair-cell mechanotransduction in mammals (reviewed in Corey, 2006). They have a conductance amplitude and a low ion selectivity that are similar to those predicted for the MET channel (Owsianik et al., 2006). Furthermore, they possess a series of elastic ankryin repeats (Corey et al., 2004; Howard and Bechstedt, 2004). These repeated domains may have the flexibility needed to act as the gating spring of the MET channel complex (Sotomayor et al., 2005). However, TRPN1 is not expressed in mammals. Alternate research has focused on another gene of the TRP family, TRPA1. This isoform has 17 ankyrin repeats, the largest number reported in mammalian TRP family channels (reviewed in Gillespie et al., 2005). Investigators have examined the characteristics of this channel in the hair bundle extensively (Corey et al., 2004; Nagata et al., 2005). The expression of TRPA1 mRNA levels correlate with the onset of mechanotransduction (Corey et al., 2004). As well, siRNA experiments directed against TRPA1 reduce transduction in murine vestibular hair cells (Corey et al., 2004). However, TRPA1 is localized robustly along the length of the stereocilium, at a much higher concentration than one would expect if TRPA1 was acting as the MET channel, which is postulated to be present only one or two times per stereocilium (Corey et al., 2004; Nagata et al., 2005). In addition, TRPA1 knockout mice maintain auditory function (Kwan et al., 2006). Thus continuing research focuses on identifying the MET channel (reviewed in Corey, 2006).
The phospholipid phosphatidylinositol 4,5-bisphosphate (PIP$_2$) has previously been shown to regulate TRP channels (Hilgemann et al., 2001). Consequently, investigators were interested in the potential role PIP$_2$ may have in the regulation of the MET channel in hair cells. PIP$_2$ was shown to be localized to the upper two-thirds of the hair bundle, and absent from the stereociliary base where phosphatidylinositol lipid phosphatase (Ptprq) is expressed (Hirono et al., 2004). Most interesting, electrophysiological recordings show that the depletion of PIP$_2$ decreases the rates of both fast and slow adaptation (Hirono et al., 2004). As well, PIP$_2$ has been shown to interact with the slow adaptation motor, Myosin-1c (Myo1c), in vitro (Hirono et al., 2004; Hokanson and Ostap, 2006). These data support the hypothesis that the MET channel may be a member of the TRP channel family and illustrate the complexity of MET regulation.

**G. Additional lateral links of the hair bundle**

In addition to the cluster of proteins referred to collectively as the tip link, multiple other lateral links connect adjacent stereocilia at different positions along the length of stereocilia during bundle development. In the avian hair bundle, discrete antibodies label shaft connectors, horizontal top connecting links, ankle links, tectorial membrane (TM) attachment crowns and kinociliary links, providing evidence of each structure’s unique biochemical composition (Goodyear and Richardson, 1992, 1999; Goodyear and Richardson, 2003). As well, each observed link has a unique expression pattern during development and differing sensitivities to Ca$^{2+}$ chelators and proteases (Goodyear et al., 2005). Shaft connectors, which are present along the entire length of the developing hair bundle are first detected at murine age E17.5 and persist until P19 (Goodyear et al., 2005). Shaft connectors are predicted to be composed of Ptprq and are not sensitive to Ca$^{2+}$ chelators or proteases (Goodyear et al., 2003; Goodyear et al., 2005). Horizontal top connectors, first observed at P9, are also insensitive to both BAPTA and subtilisin, and remain present in the adult hair bundle (Goodyear et al., 2005). Ankle
links, present transiently from P2-9, are predicted to be composed of very large G-protein-coupled receptor 1 (Vlgr1) and usherin proteins (Adato et al., 2005; McGee et al., 2006). These have been shown to be sensitive to both Ca\(^{2+}\) chelation and subtilisin treatment and crucial to bundle development (Goodyear et al., 2005; McGee et al., 2006). TM crowns, first observed at P2, are also present in adult hair bundle and degraded by subtilisin, but are unaffected by BAPTA (Goodyear et al., 2005). Kinociliary links, as suggested, connect the kinocilium to the tallest row of stereocilia. These structures are observed in concurrence with the kinocilium, transiently in the cochlea where the kinocilium degrades, and for the lifetime of the hair bundle in vestibular organs where the kinocilium remains (Goodyear et al., 2005). Although the kinocilium is believed to have no direct role in mechanotransduction, antibodies against both Cdh23 and Pcdh15 recognize kinociliary links and investigators postulate that kinociliary links and tip links must be closely related (Siemens et al., 2004; Lagziel et al., 2005; Michel et al., 2005; Ahmed et al., 2006).

**H. Myosins of the hair bundle**

In addition to providing structural support for stereocilia, the high concentrations of actin in the hair cell also enable the directed transport of molecules to the distal tips of the hair bundle. As such, unconventional myosin proteins, a class of actin-based motor molecules, have been determined to be vital for proper hair cell function. Unconventional myosin molecules are a large sub-class of the myosin motor proteins that consist of a single heavy chain, in contrast to the dual self-associating heavy chains of traditional myosins (reviewed in Cheney and Mooseker, 1992). The head domain of the myosin molecule binds to and moves along actin filaments, through an actin-binding region, using the traditional myosin power-stroke movement (Spudich and Watt, 1971). Energy consumed during this movement results from adenosine triphosphate (ATP) hydrolysis by the head domain. The mid-section of the unconventional myosin, the neck domain, is composed of multiple (up to six), tandem calmodulin (CaM)-binding sequences referred to as IQ domains, named as such for the first two amino acids,
isoleucine and glutamine, that make up the CaM-binding consensus sequence: IQX,RGX,R (single amino acid code, where X = any residue; Rhoads and Friedberg, 1997; reviewed in Bahler and Rhoads, 2002). The rate of actin hydrolysis and the resulting step size of the power-stroke is often regulated by the CaM occupancy of this neck region, which in turn, is regulated by Ca^{2+} (reviewed in Bahler and Rhoads, 2002). CaM is able to bind four Ca^{2+} molecules (reviewed in Chin and Means, 2000). When these binding sites are filled, CaM adopts a conformation that is generally unfavorable for its interaction with the IQ domains of myosin molecules (reviewed in Chin and Means, 2000; reviewed in Bahler and Rhoads, 2002). Conversely, when Ca^{2+} concentrations are low, CaM binds the IQ consensus sequences tightly. Each IQ domain has its own specific affinity along this spectrum, allowing for the precise regulation of myosin activity in response to Ca^{2+} flux. A tail domain, the most variable region among myosin molecules, completes the C-terminus of the unconventional myosin (reviewed in Cheney and Mooseker, 1992).

Since the origin of the motor model of adaptation, the hair-cell adaptation motor has been postulated to be a myosin molecule (Howard and Hudspeth, 1987). To test this hypothesis, investigators focused on interrupting general myosin function in the hair bundle by applying saturating concentrations of adenosine diphosphate (ADP) during sustained mechanical stimuli (Gillespie and Hudspeth, 1993). Excess amounts of ADP were predicted to saturate the active site of any myosin molecules present in the bundle and hold them in a state of rigor, thereby preventing both slipping and climbing adaptation (Gillespie and Hudspeth, 1993). Because ADP can be modified to form ATP in vivo, an analog of ADP that can not be phosphorylated, adenosine 5’-β-thiodiphosphate (ADP[βS]), was used (Goody et al., 1972). Following a sustained stimulus given in conjunction with ADP[βS], the transduction current in bullfrog saccular hair cells ceased to adapt, and the resting P_o value increased from 0.1 to 0.8 (Gillespie and Hudspeth, 1993). These data indicated that essentially all MET channels remained open following the stimulus and adaptation was completely blocked (Gillespie and Hudspeth, 1993). It also provided strong evidence that the adaptation motor was in fact a
myosin isozyme, and investigators began to focus on its identification (Gillespie and Hudspeth, 1993).

**Myosin-1c**

Myo1c, 120 kDa, was the first myosin identified in the hair cell bundle and an early candidate for the adaptation motor (Gillespie et al., 1993; Gillespie and Corey, 1997). Myo1c’s neck domain is composed of four tandem IQ motifs and a highly basic tail domain that has been shown to bind to PIP₂ in vitro (Hirono et al., 2004; Hokanson and Ostap, 2006). Myo1c mRNA can first be detected in the murine vestibular hair cell at E17, the same time as the developmental acquisition of mechanotransduction (Geleoc and Holt, 2003). Initial studies in the bullfrog sacculus showed a distinct immunolocalization of Myo1c to the distal two-thirds of the stereociliary shaft, in the pericuticular necklace, and diffusely throughout the hair cell body (Gillespie et al., 1993). Further ultra-structural localization experiments showed the highest density of Myo1c immunoreactivity around the base of the tip-link insertion plaque (Metcalf et al., 1994; Garcia et al., 1998; Steyger et al., 1998).

To directly implicate Myo1c in mechanotransduction, investigators developed a mutant Myo1c by replacing a key tyrosine residue of the ATP-binding pocket with glycine (Y61G; Gillespie et al., 1999). Because glycine is small, Y61G has a larger binding pocket when compared to wild-type Myo1c. This enables larger molecules like N⁶-modified (NMB)-ADP analogs to interact with the Y61G catalytic components. Because NMB-ADP can not be hydrolyzed, its interaction with Y61G results in the complete inhibition of myosin ATPase activity and the arrest of myosin motility (Gillespie et al., 1999). Despite the mutation, the motility and activity of Y61G remains similar to wild-type Myo1c in the absence of NMB-ADP analogs (Gillespie et al., 1999). Y61G, when expressed in transgenic mice, provides an ideal model to assess Myo1c’s contribution to mechanotransduction and adaptation (Holt et al., 2002). Because it is completely functional in the absence of NMB-ADP analogs, investigators were able test for Myo1c function in a targeted manner by perfusing NMB-ADP into vestibular hair bundles during a sustained mechanical stimulus. Under these conditions, the Y61G
mouse showed no adaptative response (Holt et al., 2002). Investigators postulate that the mutant Myo1c molecules, acting as part of a large grouping of Myo1c molecules that function as the adaptation motor, arrest on the actin cytoskeleton and prevent the sliding and climbing of the MET channel complex (Holt et al., 2002; Stauffer et al., 2005).

Myo1c’s role as the slow adaptation motor in vestibular hair cells is a well-established hypothesis. However, the localization of Myo1c in the murine cochlear hair cell is not limited to the stereociliary tips. As a result, the role of Myo1c in auditory hair cells is not as clear, and other myosin molecules have been posited as adaptation motor candidates (Kros et al., 2002). Nevertheless, because of its early identification and interesting localization, Myo1c has been the focus of intense research. Unfortunately, a mouse model for Myo1c mutations, perhaps the most powerful tool in protein characterization, does not yet exist; attempts to develop a Myo1c knockout mouse have been unsuccessful (personal communication, P. Gillespie). Alternately, in situ binding assays using recombinant Myo1c fragments consisting of combinations of its neck and tail domains have been used to characterize Myo1c’s interactions with other constituents of the hair cell (Cyr et al., 2002). Myo1c’s distinct localization at the tip of the stereocilia was shown to be dependent upon only its neck domain and consequently is regulated by CaM occupancy at these domains (Cyr et al., 2002). Excess CaM abolishes all interactions of Myo1c and hair bundle constituents observed using the in situ binding assay. It is proposed that interactions of Myo1c and hair bundle constituents are disrupted as the result of CaM masking an interaction domain present in the neck sequence (Cyr et al., 2002). Of particular focus is the second IQ domain of Myo1c, which shows a level of sequence conservation across species that surpasses that needed for CaM interaction (Cyr et al., 2002).

In addition, Myo1c has recently been shown to interact with Cdh23 when over-expressed together in tissue culture cell lines (Siemens et al., 2004). If Cdh23 is a component of the tip link, as suggested, its interaction with Myo1c might be expected (Siemens et al., 2004). These data, along with its proposed in vivo interaction with PIP2,
may begin to elucidate the molecular components that regulate hair-cell
mechanotransduction (Hirono et al., 2004; Hokanson and Ostap, 2006).

**Myosin-7a**

Myo7a is composed of a head domain, five IQ domains, a coiled-coil domain that enables the formation of homodimers, and a tail domain (Weil et al., 1997). The tail of Myo7a has a myosin tail homology 4 (MyTH4) domain, a SH3 domain, and a FERM domain, which is a region of high sequence homology to that of the 4.1 protein family which associates with integral membrane proteins (Chen et al., 1996). By virtue of its tail, Myo7a interacts directly with the N-terminal PDZ domains of harmonin-b, and this interaction is believed essential to localization of harmonin-b in the hair bundle (Boeda et al., 2002). Mice that do not express Myo7a (*shaker1* mutants) express harmonin-b only in the hair-cell pericuticular necklace, as opposed to the stereociliary tips as seen in wild-type animals. These data, along with evidence of Cdh23 and harmonin-b interactions, has led to the hypothesis that the three proteins interact in a complex that acts to regulate bundle formation and shape (Boeda et al., 2002). However, Cdh23 localization in the *shaker1* mouse is normal, and therefore, the stereociliary transport of Cdh23 is not dependant upon Myo7a. In addition to its interaction with harmonin-b, Myo7a also interacts with vezatin, a protein associated closely with the ankle links of stereocilia and the hair-cell adherens junctions (Kussel-Andermann et al., 2000).

Myosin-7a (Myo7a) mutants have been modeled in seven allelic variations of the *shaker1* mouse. Five of these mutations occur in the head domain of Myo7a, presumably disrupting Myo7a motor function and two occur in the tail domain. All mutations disrupt normal cochlear electrophysiology and only one *shaker1* variant has normal hair-bundle development (Mburu et al., 1997). *Shaker1* mice demonstrate very poor hair-cell mechanotransduction, only transducing following very large mechanical bundle deflections of more than 100 nm (Kros et al., 2002).

In addition to the allelic variations in the *shaker1* mouse family, the mutant mouse, headbanger, also harbors mutations that map to the same locus as Myo7a (Rhodes
et al., 2004). However, the phenotype of these hair cells, an “O” shaped bundle, does not coincide with the shaker1 bundle morphology (Rhodes et al., 2004). As well, headbanger mice are not profoundly deaf and exhibit reduced levels of Myo7a (Rhodes et al., 2004). Myo7a mutations have also been linked to the human disease, Usher’s syndrome type I B (Liu et al., 1997).

Myo7a has been postulated to regulate the function of the MET channel (Kros et al., 2002). However, as is the case for Myo1c, Myo7a is localized along the entire length of the stereocilia of murine cochlea (Hasson et al., 1995). This arrangement suggests that if Myo7a does directly regulate the MET channel at the tips of stereocilia, it must do so by regulating the tension applied to the entire stereociliary membrane, and consequently affect the MET channel function (Kros et al., 2002). Other hypotheses propose that Myo7a may maintain the environment necessary for MET channel function by transporting molecules essential for MET channel regulation to their proper location in the stereocilia (Boeda et al., 2002).

**Myosin-6**

Myosin-6 (Myo6) is the only unconventional myosin protein reported in association with the hair bundle whose actin motility is directed toward the base of the stereocilia (Wells et al., 1999). In addition to its catalytic head domain, Myo6 has only one IQ domain, a coiled-coil domain, and a short tail region with no identifiable binding domains. Myo6, specific to the hair cell, is localized to the rootlet of the stereocilium and is observed most clearly in the cuticular plate (Hasson et al., 1997) (Avraham et al., 1995). It has been postulated to anchor the apical membrane of the stereocilia to the cuticular plate region (Hasson et al., 1997; Kappler et al., 2004).

Mutations in Myo6 cause auditory and vestibular dysfunction that define the Snell’s waltzer mouse phenotype (Melchionda et al., 2001). In early development, normal hair bundle morphology is observed in these mice, but as the hair cell matures, the stereocilia begin to fuse and the hair cell eventually degrades (Seiler et al., 2004). Cochlear hair cells are completely degraded in these mice by 6 weeks of age (Avraham et
al., 1995). In addition to the murine model, mutations to Myo6 or species homologues have been identified in both humans and zebrafish, concurrently with deafness and vestibular dysfunction (Melchionda et al., 2001; Kappler et al., 2004).

**Myosin-15a**

Myosin-15a (Myo15a) is located exclusively at stereociliary tips, concentrated at the space between the end of the actin core (positive-end) and the stereociliary plasma membrane (Belyantseva et al., 2003). It contains two IQ domains in its neck region, followed by a long proline/tyrosine rich region of unknown function, and a tail containing MyTH4, SH3, and FERM domains in a pattern identical to that of Myo7a (Chen et al., 1996; Mburu et al., 1997; Liang et al., 1999). Myo15a transports whirlin to its position at the tips of stereocilia, and without its expression, whirlin remains in a pool in the cuticular plate region, and stereociliary elongation is disrupted (Kikkawa et al., 2005). Mice with mutations in Myo15a (shaker2), much like the whirler mouse, which does not express whirlin, have dramatically shortened stereocilia and are profoundly deaf (Wang et al., 1998; Liang et al., 1999). As hair cells mature (>P9) in mutant animals the links between stereocilia disappear and the hair bundle morphology degrades (Anderson et al., 2000). As well, extended actin filaments can be observed in the basal region of the hair cell indicating an actin organizational deficiency (Anderson et al., 2000). However, prior to bundle degradation, both the shaker2 and whirler mice have normal mechanotransduction following a mechanical stimulus in vitro (Stepanyan et al., 2006). This provides evidence that neither Myo15 or whirlin plays a direct role in MET channel function (Stepanyan et al., 2006).

**Myosin-3a**

In addition to the head and neck domains observed in other unconventional myosins, myosin-3a (Myo3a) has a N-terminal kinase domain that undergoes autophosphorylation (Ng et al., 1996; Komaba et al., 2003), and two unique motifs termed myosin III tail homology domains (3THDI and 3THDII; Dose et al., 2003). Myo3a expression in the stereocilia is localized to the crown of the stereocilia forming a thimble-like compartment at the stereociliary tip (Schneider et al., 2006).
localization occurs even in the absence of the 3THD domains, and the over-expression of Myo3a at stereociliary tips causes abnormal elongation of the stereocilium (Schneider et al., 2006).

**Myosin-1a**

Myosin-1a (Myo1a) is the predominant myosin motor protein found in the enterocyte, the specialized epithelial cell that lines the gut (Fig 5). The MYO1A gene may give rise to multiple Myo1a isoforms; in the gut, full-length Myo1a mRNA transcript is detected, along with a short isoform that is predicted to code for the proteins C-terminus (unpublished data, M. Tyska). Like other unconventional myosins, full length Myo1a is composed of the actin-binding head domain, the neck domain made up of three IQ-binding motifs, and tail domain (reviewed in Mooseker and Cheney, 1995). The tail domain of Myo1a has a highly basic region, the TH1 domain, which binds directly to cellular membranes and has been shown to be involved in Myo1a localization (reviewed in Mooseker and Cheney, 1995). High concentrations of Myo1a exist in the gut as part of the arrays of microvilli that form the brush border domains of the enterocyte (Mooseker, 1985). Here, Myo1a function is unclear, although it is predicted to anchor the enterocyte actin cytoskeleton to extracellular membranes, regulate microvillar organization and packing, or facilitate vesicle release in microvilli. Myo1a function in the brush borders of the gut has been studied using a genetically engineered Myo1a knockout (KO) mouse (Tyska et al., 2005). Extensively characterized, this mouse does not express full-length Myo1a mRNA transcripts or protein in the gut tissue (Tyska et al., 2005). Careful comparison between Myo1a KO mice and wild-type mice reveal that despite the lack of an overt phenotype, Myo1a KO mice have cellular perturbations in the organization and packing of both the brush border domains and microvillar arrays. In addition, the localization of Myo1c at the membrane region of the enterocyte is increased, suggesting potential myosin compensation and functional redundancy (Tyska et al., 2005).
Recently, Myo1a has been linked to auditory dysfunction. Multiple mutations in Myo1a have been identified as a result of broad genetic screening in patients with sensorineural bilateral hearing loss, although the pathogenesis of this hearing loss has not yet been elucidated (Donaudy et al., 2003). As well, Myo1a maps to the newly identified non-syndromic autosomal dominant chromosomal locus DFNA48 (D'Adamo et al., 2003). Despite the detection of Myo1a mRNA transcripts in the cochlea, Myo1a protein expression or localization in the auditory/vestibular systems is unknown, and the Myo1a KO mouse shows no overt auditory or hearing deficits, although careful analysis of auditory function has not been performed (Donaudy et al., 2003, K. Phillips & M. Tyska unpublished data, and unpublished observation, M. Tyska).

I. Hurdles of hair-cell research

Hair-cell biochemistry

Humans have approximately 32,000 hair cells in their cochlea and 134,000 total (reviewed in Hudspeth, 1989). Compared to the 131 million photoreceptors present in one human retina, it is clear that those studying the acousticolateralis systems are at a severe disadvantage with respect to experimental sample size. In addition, hair cells are unable to survive in a cell culture environment for longer than a few days, they do not divide, and there are no hair-cell tissue culture lines. As a result, it becomes very challenging to conduct biochemical research. Therefore, out of necessity, hair-cell research requires innovative laboratory techniques. Initial experiments focused on isolating the cytoskeletal core of the hair bundle and its associated proteins (Tilney et al., 1989). This enabled the early identification of both actin and fimbrin (Flock and Cheung, 1977; Tilney et al., 1989). However, because the membrane was sheared away during these procedures, many low abundant, soluble and membrane-associated proteins were lost.

In an attempt to examine constituents specific to the hair bundle more closely, the “bundle blot” and “twist off” methods were developed in 1989 and 1991, respectively
Each of these methods entailed mechanically separating the bullfrog saccular hair bundle from the hair cell body while it was secured in the macula. In the bundle-blot method, stereocilia were pressed against nitrocellulose sheets, to which they adhered. When the nitrocellulose was pulled away from the macula, the bundles broke at the stereociliary taper. The bundles were then extracted from the nitrocellulose membrane and used as experimental samples. The bundle blot method uses approximately 30 saccular equivalents per silver-stained SDS-PAGE gel lane (Shepherd et al., 1989). Using this method, the high concentrations of actin and fimbrin in the hair bundle were confirmed, as was the absence of villin. In addition, a small number of other unidentifiable proteins were observed. In a similar technique, hair bundles were embedded in warm agarose in the twist-off method (Gillespie and Hudspeth, 1991). After cooling, the hardened agarose was sharply twisted, and the bundles isolated (Gillespie and Hudspeth, 1991). The twist-off method yielded approximately <40 ng of purified protein per saccular equivalent, 50-75% being actin, and as many as 50 saccular equivalents were used per SDS-PAGE gel lane (Gillespie and Hudspeth, 1991). Both experiments were ingenious and technically challenging, but neither provided a complete complement of hair bundle proteins or a way to identify the proteins isolated. Thus, for decades the identification of hair bundle constituents using biochemical techniques was a difficult and unrewarding process.

**Genetic screening and mass spectrometry**

Hair cell research of the 21st century has been increasingly successful as genetically-based research has flourished (Nicolson, 2005a). Many of the recently identified proteins of the hair bundle have been the result of a zebrafish, mouse or human models of deafness. Because zebrafish can be systematically mutated and screened for hearing deficits *en masse*, they have proven to be powerful genetic tools. Myo6 was shown to be essential to mechanotransduction in this manner (Kappler et al., 2004). As well, mice strains that show acousticolateralis dysfunction have been instrumental to recent advances. Currently there are 40 mouse models that have mutations in genes implicated in non-syndromic deafness (hearing-loss not associated with other disorders;
www.jax.org). Of these mouse models, 26 correlate with non-syndromic deafness disorders identified in humans (http://ghr.nlm.nih.gov/ghr/page/BrowseConditions), and 15 directly relate to the hair bundle. From these models, researchers have been able to identify those genes essential to hearing and predict their specific function based on the diseased phenotype.

Supplementing strong genetic tools are the advances in the sensitivities of mass-spectrometry. Proteins isolated from hair cells can now be identified despite their low abundances. As a result, researchers have been able to identify the previously unknown antigens of antibodies created against hair bundle proteins. VLGR1 was identified in such manner (McGee et al., 2006). Known as the ankle link antigen (ALA) for seven years, VLGR1 was immunoprecipitated from avian retina, digested with trypsin, and identified by comparison to proteins catalogued in the National Center for Biotechnology Information human and mouse databases (McGee et al., 2006).

**J. Significance of hair-cell research**

The precise detection abilities of the auditory and vestibular systems occur as a result of hair cell physiology and consequent mechanical signaling pathways. These fragile mechanics are susceptible to both genetic and environmental stresses that can result in partial hearing loss or deafness. Moreover, the hair cells of higher vertebrates are never replaced by mitotic turnover. Thus the only hair cells we have are the ones we begin life with. Insult to hair bundle structure resulting from sudden loud noises or prolonged stimuli can manifest in temporary hearing loss which has been shown to occur on the same time scale as tip link regeneration and actin turnover in the hair bundle (Zhao et al., 1996; Schneider et al., 2002).

Congenital deafness, as a result of hair-cell loss, affects 1 in every 1000 children (Hudspeth, 1997). Additionally, as the United States population’s life expectancy increases, the number of older adults with age-onset hearing loss will also increase.
Hearing loss, related to hair cell damage due to over stimulation, infections, and pharmaceutical agents, is becoming increasingly prevalent. Approximately 33.2% of the adult population over 70yrs of age report deafness or hearing-loss and the resulting problems in daily life (Campbell et al., 1999). Overall, 28 million people in the US are affected by hearing loss, and over 50 billion dollars are spent annually compensating for these deficits (http://ghr.nlm.nih.gov/condition=nonsyndromicdeafness and Hudspeth, 1997).

Despite intensive research, much about the molecular details of auditory and vestibular mechanotransduction is unknown. To date, only a few key proteins have been identified and much about the molecular components comprising the gating spring model is unknown. The long-term goal of our research is to detail the mechanisms and participating proteins of the MET complex in the hair bundle, the site of auditory and vestibular transduction. Identification of these proteins is the first step towards developing genetic therapies and repair.

**K. Summary and research goals**

The hair bundle, an apical protrusion of the hair cell, is the site of mechanotransduction in the acousticolateralis systems. It is composed of numerous stereocilia and a single kinocilium that is lost as the cochlear bundle matures (reviewed in Hudspeth and Jacobs, 1979). The main constituent of the stereocilia is actin. In the hair bundle actin forms unidirectional filamentous arrays that are oriented with the barbed end of the actin pointing towards the tip of the stereocilia (Tilney et al., 1980). These arrays are held together by the crosslinking proteins fimbrin and espin and provide a cytoskeletal structure that shapes the hair bundle (Shepherd et al., 1989; Tilney et al., 1989; Zheng et al., 2000b). The actin filaments are dynamic and undergo constant renewal by a process referred to as treadmilling (Schneider et al., 2002; Rzadzinska et al., 2004). Structural proteins, such as whirlin and harmonin-b, bind to the cytoskeleton and
organize proteins of the hair bundle involved in mechanotransduction (Verpy et al., 2000; Holme et al., 2002).

Mechanotransduction refers to the physical opening of a MET channel, in response to the physical force provided by a stimulus, that results in the transmission of synaptic signal. The tip link, a complex of proteins that connects each stereocilia to its next tallest neighbor, is the sensor for this physical force (Pickles et al., 1984; Assad et al., 1991). While the identity of the tip link is controversial (Michel et al., 2005), much evidence has been published that supports the roles of both Cdh23 and Pcdh15 in the tip-link complex (Siemens et al., 2004; Sollner et al., 2004; Ahmed et al., 2006). Following a stimulus, the components of the MET channel move to a lower position on the stereocilia; this relieves the force applied to the tip link and closes the channel (Eatock et al., 1987). This process, adaptation, allows the hair bundle to be sensitive to new stimuli.

Unconventional myosins, molecular motors that move along filamentous actin, play important roles in proper hair bundle development and function (reviewed in Friedman et al., 1999). Adaptation is believed to be mediated by Myo1c (Holt et al., 2002; Stauffer et al., 2005). In vestibular organs Myo1c localizes in association with tip link insertional plaques and is essential to MET (Garcia et al., 1998; Steyger et al., 1998).

The goal of this research is to describe the interaction of Myo1c with the other unknown components associated with the MET channel. As well, we would like to use the characterization of this interaction to identify these unknown components. In-situ binding assays using recombinant fragments of Myo1c show an identical localization pattern as endogenous Myo1c (Cyr et al., 2002). These interactions are dependent upon only the neck region of Myo1c and are modulated by CaM; excess CaM added to the in situ binding reaction eliminates all Myo1c localization (Cyr et al., 2002). In addition, the sequence conservation across species of the second IQ domain far exceeds that necessary to confer CaM interaction (Cyr et al., 2002). It is our hypothesis that the second IQ domain of Myo1c is important to its interactions with components of the MET channel complex, and when in excess, CaM masks this domain. As well, Myo1c has also been
shown to interact with PIP₂ and Cdh23 in vitro (Hirono et al., 2004) (Siemens et al., 2004). We wish to explore these potential interactions in the hair bundle using the in-situ binding assay.

Finally, there has been recent evidence of Myo1a’s association with hearing disorders in humans (Donaudy et al., 2003). Despite the detection of Myo1a mRNA transcript in the cochlea, the role of Myo1a in hearing is unknown (Donaudy et al., 2003). Moreover, previous studies that have examined the expression of Myo1a protein in vestibular hair cells have been negative (Hasson et al., 1997). To address these questions, we have developed a collaboration with Dr. Matthew Tyska at Vanderbilt University. Using antibodies against Myo1a and Myo1a mutant mice provided by his laboratory, we wish to characterize Myo1a’s expression in the cochlea.
Figure 1. Inner ear morphology  A) Cartoon schematic of the inner ear. A sound-wave is shown in red. *Gray arrow* points to longitudinal cross-section of cochlear labyrinth. The tympanic, vestibular, and cochlear canals are colored *green, pink* and *yellow* respectively. Boxed inset shows the organ of Corti and hair cells. Image, without labels, is used with permission from www.brainconnection.com, copyright 1999, Scientific Learning Corporation  B) Detailed cartoon of the Organ of Corti. Supporting cells are labeled as follows: *PC*, pillar cell; *DC*, Dieter cell; and *HC*, Henson cell.
Figure 2. Hair-bundle morphology  
A) Bright-field image of an isolated bullfrog saccular hair cell. 
B) Electron micrograph (EM) of a mature murine cochlear hair cell 
C) TEM longitudinal section of avian vestibular stereociliary base stained with ruthenium red. Arrows point to shaft connectors. Arrowheads point to ankle links. 
D) SEM of a mature frog saccular hair cell. 
E) TEM longitudinal cross-section of avian vestibular stereocilia tips stained with tannic acid. Arrows point to tip links. Arrowhead point to top connectors.  
F) High-resolution freeze-etch image of the tip link from guinea pig cochlear hair cells. Scale bars: B & F, 100 nm; E, 300 nm. 
**Figure 3. Hair cell transduction and adaptation.** A) Transduction current measured from a bullfrog saccular hair cell following mechanical displacement. Prior to stimulation the open probability of the channel $P(O)=0.1$, indicating that at rest a few channels remain open and current is slightly negative. Following the $1\mu m$ positive displacement the maximum inward rectifying current is reached immediately and $P(O)=1$, ●. The transduction current adapts over time to approximately 60-80 % of baseline, ■. Following a negative displacement, ▲, the current overshoots its resting state briefly, $P(O)=0$, and then returns to baseline. B) Cartoon of adaptation in the hair cell. Immediately following a positive deflection, tension on the tip link increases and the MET channel is mechanically opened (purple channel). As a result of tension the MET channel slips down the length of the stereocilia (see arrow). The space between the tip of the shorter stereocilia and the tip link attachment on the taller stereocilia is thus decreased, tension on the tip link is reduced, and the MET channel closes (blue channel). Adaptation motor is shown in yellow. Stereociliary membrane is green. Mechanotransduction trace and adaptation cartoon modified from Holt & Corey, PNAS, 2000, 97(22):11730-5; copyright 2000 National Academy of Sciences.
Figure 4. Myosins of the cochlear hair bundle. Cartoon of two adjacent stereocilia connected by the tip link, shown in blue (other stereociliary links are not shown for simplicity). MET channel is shown in pink. Myosin key is shown in top right corner. Briefly, Myosin-6 (purple) is located in the stereociliary taper region and in cuticular plate; Myosin-3a (green) caps the stereocilia akin to a thimble; Myosin-15a (orange and yellow) is exclusively at the stereociliary tip; Myosins 7 and 1C (yellow) are distributed along the length of the stereocilia. Stereociliary membrane is shown in pink, the transduction channel in bright pink, the tip-link in blue, and filamentous actin in gray. Figure is not drawn to scale.
Figure 5. Myo1a in enterocyte of the gut  A) Bright-field image of an enterocyte cell. Bracket indicates the enterocyte microvilli; actin-filled structures analogous to hair cell stereocilia. B) SEM of microvilli. C) Magnification of an SEM of microvilli. An example of a Myo1a cross-link is circled. D) Confocal image of gut epithelium of Myo1a+/+ mouse immunolabeled with the Myo1a antibody, αBBI, which recognizes the tail domain of Myo1a (green). Filamentous actin is labeled with phalloidin (red). Yellow labeling indicates the overlap of the two fluorophores. E) Myo1a−/− sample; labeling conditions are the same as in panel D. Scale bars: B, 0.5μm; C, 0.05μm; and E, 10 μm, which applies to D & E. Image in A is courtesy of Matthew Tyska, Vanderbilt University, TN. Images in B & C were adapted from Mooseker and Tilney, Journal of Cell Biology, 1975, 67:725-743; copyright 1975 Rockefeller University Press. Images in D & E were adapted from Tyska et. al., Molecular Biology of the Cell, 2005, 16: 2443-2457, copyright 2005 American Society for Cell Biology.
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II. Stereociliary Myosin-1c Receptors are Sensitive to Calcium Chelation and Absent from Cadherin 23 Mutant Mice

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Abstract

The identities of some of the constituents of the hair-cell transduction apparatus have only recently been elucidated. The molecular motor myosin-1c (Myo1c) functions in adaptation of the hair cell response to sustained mechanical stimuli and is therefore an integral part of the transduction complex. Recent data indicate that Myo1c interacts in vitro with two other molecules proposed to be important for transduction: cadherin 23 (Cdh23), a candidate for the stereociliary tip link, and phosphatidylinositol 4,5-bisphosphate (PIP2), which is abundant in the membranes of hair-cell stereocilia. It is not known, however, whether these interactions occur in hair cells. Using an in situ binding assay on saccular hair cells, we previously demonstrated that Myo1c interacts with molecules at stereociliary tips, the site of transduction, through sequences contained within its calmodulin (CaM)-binding neck domain, which can bind up to four CaM molecules. In the current study, we identify the second CaM-binding IQ domain (IQ2) as a region of Myo1c that mediates CaM-sensitive binding to stereociliary tips and to PIP2 immobilized on a solid support. Binding of Myo1c to stereociliary tips of cochlear and vestibular hair cells is disrupted by treatments that break tip links. In addition, Myo1c does not bind to stereocilia from mice whose hair cells lack Cdh23 protein despite the presence of PIP2 in the stereociliary membranes. Collectively our data suggest that Myo1c and Cdh23 interact at the tips of hair-cell stereocilia and that this interaction is modulated by CaM.
In auditory and vestibular end organs, transduction of physical stimuli into electrical signals occurs in hair cells and is critically dependent upon the hair bundle, which protrudes from the hair-cell apical surface and is comprised of actin-filled stereocilia arranged in rows by ascending height (Hudspeth, 1989). Stereocilia are interconnected by several types of extracellular linkages (Goodyear et al., 2005) including the tip link, which connects the top of a shorter stereocilium to the side of its tallest neighbor (Pickles et al., 1984). Cadherin 23 (Cdh23) has been proposed to be a tip-link component (Siemens et al., 2004; Sollner et al., 2004), however this designation is controversial (Lagziel et al., 2005; Michel et al., 2005; Rzadzinska et al., 2005). In the prevailing model of hair-cell transduction, bundle deflection tenses tip links and opens mechanically-gated, cation-selective transduction channels (Pickles et al., 1984) (Howard and Hudspeth, 1987). During prolonged stimuli, transduction-current magnitude reduces within tens of microseconds (for review; Gillespie and Cyr, 2004). This adaptation occurs at two rates (Wu et al., 1999; Holt et al., 2002; Stauffer et al., 2005) and involves the motor protein myosin-1c (Myo1c; Holt et al., 2002; Stauffer et al., 2005), an unconventional myosin comprised of three domains: a mechanochemical head, a neck containing 3-4 calmodulin (CaM)-binding IQ domains, and a tail (Fig. 1A). CaM binding to Myo1c IQ domains is favored in low Ca\(^{2+}\) and modulates both motor activity and binding to intracellular components (for review; Gillespie and Cyr, 2004).

Slow adaptation is predicted to require, at a minimum, the transduction channel; the tip link, which may, in part, be comprised of (Siemens et al., 2004; Sollner et al., 2004); the adaptation motor, Myo1c (Holt et al., 2002); and phosphatidylinositol 4,5-bisphosphate (PIP2; Hirono et al., 2004). To generate a functional transduction complex, these molecules are expected to interact with one another and perhaps other unidentified transduction-apparatus constituents. To date, Myo1c has been shown to bind, in vitro, to both PIP\(_2\) and Cdh23 (Hirono et al., 2004; Siemens et al., 2004; Hokanson and Ostap, 2006), however, such interactions have not been demonstrated in hair cells.
To examine Myo1c interactions with transduction components, we developed an in situ binding assay to visualize Myo1c binding to hair-cell constituents, which we term “Myo1c receptors” (Cyr et al., 2002). Using this assay, we demonstrated that the Myo1c neck region binds to available receptors at stereociliary tips, the site of transduction; in the pericuticular necklace, a vesicle–rich region encircling the cuticular plate; in the kinocilium, a cilium located at the tall edge of the hair bundle, and also within the hair-cell soma (Cyr et al., 2002). With the exception of the kinocilium, Myo1c binding is modulated by CaM, which implicated the CaM-binding IQ domains in receptor interactions (Cyr et al., 2002). In the current study, we determine that the second IQ domain (IQ2) of Myo1c mediates interactions with stereociliary receptors. In addition, these interactions are sensitive to Ca\(^{2+}\) chelation, a procedure that breaks tip links, and do not occur in hair cells lacking Cdh23.

Materials and Methods

Animals. All animal procedures followed NIH guidelines and were approved by either the Animal Care and Use Committee at West Virginia University School of Medicine or the United Kingdom Home Office and local ethical committee.

Myo1c constructs. To generate Myo1c-T701IQ2Mut (Fig. 1A), a construct with mutations in conserved residues in the second consensus IQ domain of Myo1c, the first two amino acids, Ile728 and Gln729, were each mutated to Ala in the context of the bullfrog Myo1c-T701 construct (Cyr et al., 2002), using the Quick-Change site-directed mutagenesis system (Stratagene, La Jolla, CA) and the oligonucleotides 5'-ATTCCGCGGTAGAGGCCGCGTGGTGGCGTGG-3' and 5'-CCACGCCACCA CGACGCGCCCTCTACCGCGGAAAT-3'. A construct with mutations in the first IQ domain of bullfrog Myo1c (IQ1), Myo1c-T701IQ1RG1Mut, was generated by site-directed mutagenesis using the oligonucleotide primers 5'-GGGTAGCATAGCAACATTCGCCGCGGCTAGGTGGAGAGG-3' and 5'-CCTCTCCACCTAGCCGCGGCGAATGTTGCTATGCTACCC-3' to mutate Leu706 and Gln707 to Ala residues. The residues Arg711 and Gly712 were each also mutated to Ala using the
primers 5'-GCAACATTTCTCCAGGCTAGGTGGGCGGCCTATCATCAA
CGACAG-3' and 5'-CTGTCGTTGATGATAGGCCGCCCACCTAGCCTGGAGGA
ATGTTGC-3' resulting in a construct in which four of the five residues of the IQ
consensus sequence of IQ1 were altered. The mutated Myo1c sequences were ligated
into the pFastbacI expression vector (Invitrogen, Carlsbad, CA) and sequenced. A
construct encoding the second IQ domain of Myo1c, Myo1c-N2 (bullfrog Myo1c amino
acids 721-744), was generated by the polymerase chain reaction (PCR) using pGus-T701
(Cyr et al., 2002) as the template and the oligonucleotides
5'-CATGCCATGGGTCACATGAAACATTCCGCGGTAG-3' and 5'-CCGGAATTC
TCACTACTCATTCTCAGTGCAACGGGG-3'. The amplified product was ligated into the EcoR I and Nco I sites of pBBHis2A-N123 (Cyr et al., 2002) and re-amplified using the
primers 5'-TTGGCGCGCCTATAAATTGCCGCGGGGTTC-3'
and 5'-CCGGAATTCTCACTACTCATTCTCAGTGCAACGGGG-3'. The PCR product was ligated into the BssH I and EcoR I sites of the pFastBacI vector and sequenced.

Recombinant Myo1c protein purification. Recombinant bullfrog Myo1c protein
fragments were co-expressed with CaM in Sf9 insect cells using baculovirus infection as
previously described (Gillespie et al., 1999). Sf9 cells expressing recombinant protein
were sedimented and stored at -80°C. Protein was purified from the frozen pellets using
a Ni²⁺-nitritoltriacetic acid chromatography column (Qiagen, Valencia, CA) as outlined in
Cyr et al., 2002. Eluted fractions exhibiting a high protein concentration were pooled and
stored on ice at 4°C. Eluates were analyzed by SDS-PAGE and gel-filtration
chromatography on a 25-ml Superdex 200 HR 10/30 or Superdex 200 10/300 GL column
(Amersham Biosciences, Piscataway, New Jersey) in 400 mM KCl, 0.1 mM ethylene
glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 15 mM HEPES,
pH 7.5 at room temperature. Protein concentrations were determined using the Bradford
assay with bovine serum albumin (BSA) as a standard (Bradford, 1976).

CaM purification. Bovine CaM was purified from brain tissue using a phenyl-
sepharose affinity column (Gopalakrishna and Anderson, 1982) and was quantified
assuming 330 µM/A₂₇₆nm.
**Myo1c fragment:CaM stoichiometry.** Gel filtration-purified Myo1c-T701, Myo1c-T701IQ2Mut, and Myo1c-T701IQ1RG1Mut protein fragments were each concentrated by trichloroacetic acid (TCA) precipitation (8% v/v) and resuspended in NuPage LDS sample buffer (Invitrogen). Resuspended TCA precipitates were separated by electrophoresis on 12% Bis-Tris NuPage gels in NuPage MOPS SDS running buffer (Invitrogen) and stained with colloidal blue (Invitrogen). Destained gels were scanned using a flatbed scanner and analyzed with Scion Image Beta software (v4.03, Scion Corporation, Frederick, MD). The ratio of mutated Myo1c heavy-chain fragments to CaM was calculated using the wild-type Myo1c-T701 protein complex as a standard. Under our purification conditions, Myo1c-T701 binds 1.8 ± 0.2 CaM molecules per Myo1c polypeptide (Gillespie and Cyr, 2002). Three separate preparations of each purified recombinant protein were analyzed in duplicate on two separate gels. Values are represented as the mean ± standard deviation.

**Cochlear cultures.** Cochlear cultures were prepared from postnatal day 0 (P0)-P2 Cdh23<sup>v2J</sup> and Myo7a<sup>6J</sup> mice as previously described (Russell and Richardson, 1987). Briefly, cochleas were dissected in Hanks’ balanced salt solution buffered with 10 mM HEPES, pH 7.2. (HBHBSS). Cochlear coils were plated onto a hydrated collagen gel on a glass coverslip and maintained in sealed Maximow slide assemblies containing ~50 ml of medium (93% DMEM/F12, 7% fetal calf serum, 10 mg/ml ampicillin). Cultures were grown for 1-2 days in vitro at 37°C after which they were fixed in 3% paraformaldehyde (Agar Scientific, Essex, UK) and stored in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, and 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) at 4°C. To assess the dependence of Myo1c binding on intact tip links, tips links were broken in a subset of the cultures prior to fixation by incubation for 15-30 min at room temperature in either a solution containing 5 mM EGTA or 5 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N’-tetraacetic acid (BAPTA) in HBHBSS supplemented with 0.5 mM MgCl<sub>2</sub> and 0.4 mM MgSO<sub>4</sub> or a solution containing 5 mM LaCl<sub>3</sub> in 155 mM NaCl, 6 mM KCl, 3 mM glucose, 4 mM CaCl<sub>2</sub>, 10 mM HEPES, pH 7.2.
**In situ binding assay.** In situ binding assays were performed as described (Cyr et al., 2002) on bullfrog (*Rana catesbeiana*) sacculi or mouse cochlear cultures. Briefly, the samples were fixed in 3% paraformaldehyde in PBS, washed in PBS, and permeabilized with 0.1% (w/v) Sarkosyl for 1 hour. Following a PBS wash, nonspecific binding sites were blocked with 5 mg/ml BSA (fraction V, Calbiochem, La Jolla, CA) in 25 mM HEPES, pH 7.5, and the samples were incubated overnight at room temperature with purified Myo1c fragments at 20 mg/ml [corresponding to either 230 nM for Myo1c-T701, Myo1c-T701IQ2Mut (n=10), and Myo1c-T701IQ1RG1Mut (n=9) or 1.65 mM for Myo1c-N2 (n=4)] in 5 mg/ml BSA, 25 mM HEPES, pH 7.5, 1 mM EGTA, and 400 mM NaCl. Following washing, bound Myo1c fragments were detected by virtue of the Xpress tag located at the amino terminus of the Myo1c constructs, using the anti-Xpress antibody (5 µg/ml; Invitrogen). Following three washes, bound antibody was detected with Alexa-488 goat-anti-mouse secondary antibody (13 µg/ml; Invitrogen) in 25 mM HEPES, pH 7.5, 1 mM EGTA, and 5 mg/ml BSA. Filamentous actin was labeled with 33 nM Alexa 568-phalloidin (Invitrogen) present during the secondary antibody incubation. All aforementioned washes were performed using 25 mM HEPES, pH 7.5, 1 mM EGTA, and 0.1% Tween-20. Samples were then washed in 25 mM HEPES, pH 7.5, 1 mM EGTA, mounted using Vectashield (Vector Laboratories, Burlingame, CA), and imaged on a LSM-510 Meta confocal microscope (Carl Zeiss Inc., Thornwood, NY). In some experiments, 16 µM bovine brain CaM was added during the recombinant protein incubation step (for co-incubations with Myo1c-T701IQ2Mut, n=9; with Myo1c-T701IQ1RG1Mut, n=4). To break tips links, bullfrog sacculi were treated for 30 min at room temperature with either (n=8) 5 mM EGTA in low-Ca$^{2+}$ saline (110 mM NaCl, 2 mM KCl, 2 mM MgCl$_2$, 0.1 mM CaCl$_2$, 3 mM D-glucose, 10 mM HEPES, pH 7.2) or (n=5) 5 mM LaCl$_3$ in low-Ca$^{2+}$ saline without MgCl$_2$. To assess the efficacy of these treatments, sacculi were subjected in parallel to a 5-sec incubation with 3 mM AM1-43 (Biotium, Inc., Hayward, CA), washed, fixed, mounted, and visualized by confocal microscopy. Images of Myo1c binding to pretreated tissues are gain-matched to control conditions except for Fig. 8C and 8D where the gains for Myo1c-T701 binding are 558 and 565 respectively.
**PIP-strip binding assay.** PIP-strips (Invitrogen and Echelon Biosciences, Salt Lake City, UT) were incubated in blocking solution (1:10 dilution of Liquid Block in PBS; Amersham Biosciences) to reduce non-specific binding. This and subsequent steps were performed at room temperature. Purified recombinant Myo1c protein was added at a concentration of 1 µg/ml (corresponding to either 11.5 nM for Myo1c-T701 and Myo1c-T701IQ2Mut or 82.5 nM Myo1c-N2) with or without 460 nM CaM to blocking solution containing 1 mM EGTA and incubated for 2 hours. Following three 10-min washes with PBS/0.1%Tween-20 (PBS-T), the membranes were incubated with 1 µg/ml anti-Xpress antibody in blocking solution for 1 hour, washed in PBS-T, and incubated with 170 ng/ml goat-anti-mouse alkaline phosphatase antibody (Jackson Immunoresearch, West Grove, PA) in blocking solution for 1 hour. The PIP strips were then washed 3 x 10 min with PBS-T and 2 x 5 min in alkaline phosphatase buffer (100 mM Tris, pH 9.5, 50 mM MgCl₂, 100 mM NaCl), and developed using 1-step NBT/BCIP (Pierce, Rockford, IL) for 5-15 min.

**Myo1c immunolabeling.** Cochlear cultures from Cdh23<sup>−/−</sup> or Myo7a<sup>−/−</sup> mice were fixed with cold 3% paraformaldehyde in PBS for 25 min. Samples were washed and stored in PBS at 4°C until they were processed for immunocytochemistry at which time the tissue was permeabilized with 0.2% saponin in PBS for 1 hour. This and subsequent steps were performed at room temperature unless otherwise indicated. Non-specific binding sites were blocked using 5% goat serum, 1% BSA, and 0.1% saponin in PBS for 1 hour. The tissue was incubated overnight at 4°C with 3.4 µg/ml anti-Myo1c antibody x (R2652; gift from P. Gillespie, Oregon Health & Science University; Dumont et al., 2002) in blocking solution. Samples were washed 3 x 10 min in PBS-T and incubated with 13 µg/ml Alexa 488 goat-anti-rabbit antibody and 33 nM Alexa-568 phalloidin in PBS containing 1% BSA and 0.1% saponin for 1.5 hrs. Samples were washed 3 x 10 min in PBS-T followed by 1 x 10 min in PBS. Samples were mounted in Vectashield and imaged by confocal microscopy. Immunolabeled images acquired for treated samples are gain-matched to control conditions. Ten apical and 10 basal cochlear coils from 5
heterozygous Cdh23v2J mice were examined, and 16 apical and 16 basal cochlear coils from 8 homozygous Cdh23v2J animals were examined. Likewise, 4 apical and 4 basal cochlear coils from 2 heterozygous Myo7a6J mice and 9 apical coils and 10 basal cochlear coils from 5 homozygous Myo7a6J mice were analyzed.

**Cdh23 immunolabeling.** Cdh23 immunolabeling was performed on cochlear cultures from wild-type mice, acutely dissected vestibular organs (P3 and P6) from wild-type mice, or acutely dissected bullfrog sacculi with anti-Cdh23 antiserum (T8, gift from T. Friedman, NIDCD; Lagziel et al., 2005) as outlined for Myo1c immunolabeling except the tissue was permeabilized for 30 min with 0.5% Triton X-100 in PBS and the blocking solution was 2% BSA, 5% goat serum in PBS. To break tip links, samples were subjected to the designated treatments prior to fixation as indicated above for cochlear cultures or as outlined in the *in situ* binding assay for bullfrog sacculi. Four apical and 4 basal mouse cochlear turns, 5 mouse utricules, and at least 3 frog sacculi were examined for each treatment. Immunolabeled images acquired for treated samples are gain-matched to control conditions.

**PIP2 immunolabeling.** Mouse cochlear cultures (P0 + 1 day *in vitro*) from Cdh23v2J animals were fixed in 4% paraformaldehyde (diluted from ampules of a 16% stock solution) in 0.1 M sodium phosphate pH 7.2, washed in PBS, and stored at 4°C overnight. The tissue was permeabilized with 1% (w/v) Sarkosyl in PBS for 1 hour, blocked for 1 hour with 5 mg/ml BSA in Tris-buffered saline (TBS; 10 mM Tris-HCl pH 7.4, 150 mM NaCl), and incubated with 10 µg/ml anti-PIP2 monoclonal antibody (clone 2C11; Invitrogen) in blocking solution overnight at 4°C. The tissue was washed with 5 mg/ml BSA in TBS, incubated with FITC-conjugated goat anti-mouse IgM secondary antibody (1:200 dilution; Sigma Aldrich) and Texas Red-conjugated phalloidin (1U/ml, Invitrogen) in blocking solution for 2-3 hours, washed, mounted, and visualized by confocal microscopy. All steps were performed at room temperature unless otherwise noted. The gain setting for the heterozygous image presented in Fig. 9C is 590 and for the homozygous image in Fig. 9D is 540. A total of 10 apical and 10 basal cochlear turns
were examined from 5 homozygous Cdh23v2J mice; 8 apical and 8 basal cochlear coils from 4 heterozygous Cdh23v2J mice were examined.

**Biolistic transfection of hair cells with the EGFP-tagged pleckstrin homology domain of PLCδ1.** A mammalian expression vector containing the N-terminal pleckstrin homology (PH) domain of phospholipase Cδ1 (PLCδ1) ligated in frame with the enhanced green fluorescent protein (PLCδ1PH-EGFP; Watt et al., 2002) was a kind gift from Peter Downes (University of Dundee, UK). Gold microcarrier particles (1 mm diameter) were coated with plasmid DNA and used to transfect cochlear cultures prepared from 1-2 day old Cdh23v2J mouse pups using a Helios Gene Gun (Bio-Rad Laboratories, Hercules, CA; Schneider et al., 2002; Rzadzinska et al., 2004). After a further 20-24 hours in vitro, cultures were fixed with 3.7% formaldehyde in 0.1 M sodium phosphate buffer pH 7.4 for 1 hour, preblocked and permeabilized with 10% horse serum in TBS containing 0.1% Triton X-100, and stained with rabbit anti-GFP antibody (4 mg/ml; Invitrogen) followed by FITC-conjugated swine anti-rabbit Ig (4 mg/ml; Dako Ltd, Ely, UK) and Texas Red conjugated phalloidin (1 U/ml; Invitrogen). Samples were mounted and imaged by scanning confocal microscopy. A total of seventeen transfected hair cells from five heterozygous Cdh23v2J cultures, and thirty-seven hair cells from nine homozygous Cdh23v2J cultures were analyzed.

**Results**

**The second IQ domain of Myo1c is necessary, but not sufficient, for interaction with stereociliary receptors**

To evaluate the role of the Myo1c IQ domains in binding to stereociliary receptors we exploited two previous observations: (1) CaM blocks the binding of Myo1c-T701, a fragment of Myo1c containing the neck and tail regions (Fig. 1A), to the tips of stereocilia (Fig. 2A,B and Cyr et al., 2002) and (2) amino-acid sequences in the second Myo1c IQ domain (IQ2) are highly conserved across species suggesting that this domain
may be involved in receptor interactions (Cyr et al., 2002). We hypothesized that if IQ2 is the receptor-binding site of Myo1c, then mutations that prevent CaM binding to IQ2 would abolish the ability of CaM to block interactions between Myo1c and its receptors. Because CaM binding to IQ domains critically depends upon the IQ consensus sequence IQX3RGX3R (single amino acid code where X is any amino acid; Rhoads and Friedberg, 1997), we sought to mutate the Myo1c IQ2 consensus sequence to prevent CaM interaction, but retain the ability of the domain to bind to stereociliary receptors. Accordingly, we changed the first two amino acids of the bullfrog Myo1c IQ2 consensus sequence, isoleucine 728 and glutamine 729, to alanine residues. These mutations, I728A and Q729A, were made in the context of Myo1c-T701, resulting in a recombinant Myo1c fragment referred to as Myo1c-T701IQ2Mut (Fig. 1A).

Using baculovirus infection, Myo1c-T701IQ2Mut was co-expressed with CaM and purified by virtue of an N-terminal His6 tag (Fig. 1C). As with the purification of wild-type Myo1c-T701, CaM co-purifies with the Myo1c-T701IQ2Mut protein indicating that CaM is bound to one or more of the Myo1c IQ domains (Fig. 1B,C). To verify that the mutations made in IQ2 disrupt CaM binding, the complex of Myo1c-T701IQ2Mut and CaM was further purified by gel-filtration chromatography and the amount of CaM co-purifying with the mutated Myo1c fragment was compared to that which co-purifies with the wild-type Myo1c-T701 fragment using densitometry. Gel-filtration chromatography and sucrose-density centrifugation indicate that an average of 1.8 ± 0.2 CaM molecules are bound per wild-type Myo1c-T701 under identical purification conditions (Gillespie and Cyr, 2002). However, it is not known which IQ domains are bound by CaM in the wild-type protein. Analysis of Myo1c-T701IQ2Mut reveals that one CaM (1.0 ± 0.3, n = 3) is bound per Myo1c-T701IQ2Mut molecule, confirming that the mutations in IQ2 were successful in disrupting CaM interactions.

The ability of Myo1c-T701IQ2Mut to bind to Myo1c receptors in hair cells was confirmed using our in situ binding assay. Like the wild-type Myo1c-T701, Myo1c-T701IQ2Mut bound to receptors located at the tips of stereocilia (Fig. 2A,C), in the vesicle-rich pericuticular necklace, and also in the hair-cell soma (data not shown). This
interaction profile is indistinguishable from that of the wild-type protein (Fig. 2A), demonstrating that the IQ2 mutations did not adversely affect the ability of the molecule to bind to stereociliary receptors. To ascertain whether IQ2 is the site of CaM-sensitive interaction between Myo1c and its receptors, we tested the ability of excess CaM to block the observed interactions. As previously reported, wild-type Myo1c-T701 binding to stereociliary tips is blocked in the presence of excess CaM (Fig. 2B and Cyr et al., 2002). Conversely, Myo1c-T701IQ2Mut binding is not altered despite the presence of excess CaM (Fig. 2D). These data confirm that the second IQ domain comprises a vital component of the interaction site between Myo1c and its receptors and that when this domain is occupied by CaM, binding is prevented.

To determine whether the Myo1c IQ2 domain is sufficient for receptor interaction, a protein fragment containing only the second IQ domain of Myo1c, termed Myo1c-N2, was generated (Fig. 1A). This Myo1c fragment was co-expressed with CaM, purified (Fig. 1D), and tested for its ability to bind stereociliary receptors. If the receptor-interaction domain of Myo1c is composed solely of IQ2, Myo1c-N2 would be expected to bind to stereociliary receptors in a manner similar to Myo1c-T701. However, purified Myo1c-N2 protein did not bind to stereocilia (Fig. 2E) or the pericuticular necklace region (data not shown), suggesting that IQ2 is insufficient for binding. These data support previous results in which the addition of excess IQ2 peptide did not block the interaction of Myo1c-T701 and Myo1c receptors (Cyr et al., 2002).

The presence of one CaM bound to Myo1c-T701IQ2Mut indicated that CaM is bound to one of the other three Myo1c IQ domains. The weak IQ consensus sequence and low apparent affinity of CaM for IQ4 (Gillespie and Cyr, 2002) suggested that the IQ domain occupied by CaM was either IQ1 or IQ3. To determine whether CaM is bound at IQ1 and if so, whether this CaM is important for receptor interactions, we mutated the IQ1 consensus sequence to prevent CaM binding. The Myo1c-T701IQ1RG1Mut protein was expressed, purified, and tested for receptor binding in our in situ binding assay (Fig. 1E and supplemental Fig. 1). Despite the simultaneous alteration of four of the five residues
of the IQ1 consensus sequence (L706A, Q707A, R711A, and G712A), no loss of CaM was detected from the purified protein (1.5 ± 0.4 CaMs bound per T701IQ1RG1Mut, n=3) and binding to stereociliary receptors was only slightly reduced compared to the control (supplemental Fig. 1A,B). In addition, as seen with the wild-type Myo1c-T701, incubation with excess CaM blocked the interaction of Myo1c-T701IQ1RG1Mut with stereociliary receptors (supplemental Fig. 1C). These data suggest that CaM is not bound to IQ1 in the Myo1c-T701 construct and therefore is likely bound at IQ3.

**The second IQ domain of Myo1c mediates CaM-sensitive binding to PIP2 in vitro**

Recent data demonstrate that PIP2, which is present in stereociliary membranes and plays a role in hair-cell transduction, binds to Myo1c in vitro (Hirono et al., 2004; Hokanson and Ostap, 2006). The region of Myo1c that binds to PIP2, however, is not clear; both the Myo1c neck and tail regions have been identified as sites of interaction (Hirono et al., 2004; Hokanson and Ostap, 2006). Using a nitrocellulose membrane-based assay and PIP2-containing vesicles, Hirono *et al.* (2004) determined that the neck region of Myo1c interacts with PIP2. In addition, Myo1c interactions with PIP2 vesicles were blocked by CaM in the presence of EGTA or Ca²⁺ (Hirono et al., 2004). We sought to further characterize Myo1c and PIP2 interactions and to explore the possibility that PIP2 may represent the Myo1c receptors observed in our *in situ* binding assay. To do so we examined whether Myo1c-T701, Myo1c-T701IQ2Mut and Myo1c-N2 bound to PIP2 in a membrane-based assay in a manner similar to their binding to stereociliary receptors. In 1 mM EGTA, Myo1c-T701 bound to PIP2 and this binding was blocked by the presence of excess CaM (Fig. 3A,B). Similar to the results seen in our *in situ* binding assay, Myo1c-T701IQ2Mut bound to PIP2 and this interaction was not blocked by the addition of excess CaM (Fig. 3C,D). In addition, Myo1c-N2, which did not bind to stereociliary receptors in the *in situ* binding assay (Fig. 2E), did not bind to PIP2 in the membrane-based assay (Fig. 3E). Finally, co-incubation of Myo1c-T701 with an excess amount of a synthetic peptide encoding IQ2 did not block binding to PIP2 (Fig. 3F). These data demonstrate that, in an *in vitro* assay, Myo1c interacts with PIP2 and the
stereociliary receptors in a similar manner. Although PIP$_2$ binds to Myo1c in a membrane-based assay, further evidence (detailed below) suggests that PIP$_2$ is not the receptor detected in our in situ binding assay.

**Tip-link loss abolishes Myo1c binding to stereociliary receptors**

Myo1c has been shown to interact with Cdh23, a proposed component of the tip link, when the proteins are co-expressed in tissue culture cells (Siemens et al., 2004). To examine the requirement for intact tip links on Myo1c interactions with its receptors, we exposed bullfrog saccular hair cells to either lanthanide ions or calcium chelators, agents which break tip links and eradicate mechanotransduction (Baumann and Roth, 1986; Assad et al., 1991; Michel et al., 2005), prior to fixation and Myo1c-T701 binding. Treatment with 5 mM La$^{3+}$ or 5 mM EGTA successfully broke tip links as assayed by either a complete, or near-complete, loss of AM1-43 loading into hair cells through transduction channels (Fig. 4A,B,E,F). Moreover, pretreatment with either La$^{3+}$ or EGTA eliminated virtually all Myo1c-T701 binding at both the hair-cell stereociliary tips and pericuticular necklace (Fig. 4D,H and data not shown) in the bullfrog sacculus.

To assess whether intact tip links are also needed for Myo1c binding in the mouse cochlea, we broke tip links with either 5 mM EGTA, BAPTA or La$^{3+}$ and determined whether Myo1c bound to stereociliary tips following these treatments. In untreated cochlear hair cells, Myo1c-T701 bound at the tips of stereocilia as seen in the bullfrog saccus (Fig. 5A). Pretreatment of cochleas with BAPTA eliminated all binding of Myo1c to stereocilia (Fig. 5B), while La$^{3+}$ pretreatment reduced, but did not eliminate, Myo1c binding (Fig. 5D). EGTA pretreatment gave variable results ranging from a slight reduction in binding (data not shown) to a loss of binding (Fig. 5C); this variability could be found within a single cochlea and did not correspond to location of the hair cells along the length of the organ.
Effects of tip-link loss on Cdh23 immunolocalization in bullfrog sacculi and mouse cochlea

The elimination of Myo1c binding to stereociliary tips following tip-link loss led us to revisit the question of Cdh23 localization in stereocilia following such treatments in both bullfrogs and mice. To date, seemingly contradictory results have been reported regarding the effects of tip-link loss on Cdh23 localization in hair cells (Siemens et al., 2004; Michel et al., 2005). Siemens et al. (2004) demonstrate that EGTA treatment results in a loss of Cdh23 immunolocalization in bullfrog saccular hair bundles and an appearance of immunoreactivity observed at the pericuticular necklace, a vesicle-rich region surrounding the cuticular plate. However, in the mouse cochlea, similar treatments result neither in a loss of Cdh23 immunoreactivity in stereocilia nor the appearance of immunoreactivity in the pericuticular necklace (Michel et al., 2005). These ostensibly disparate results were performed using different antibodies against Cdh23 and may reflect differences in the antibodies used, species, or type of hair cell. To distinguish between these possibilities, we examined the immunolocalization of Cdh23 in the hair cells of the bullfrog sacculus, mouse utricle, and mouse cochlea following tip-link loss using an antiserum raised against the cytoplasmic domain of Cdh23 (T8 antiserum; Lagziel et al., 2005). In the bullfrog sacculus, this antibody detects Cdh23 at the tall edge of the hair bundle, presumably between the tallest stereocilia and the kinocilium as previously reported (Fig. 6A and Lagziel et al., 2005). Similar to the results of Siemens et al. (2004) treatment of bullfrog saccular hair cells with La$^{3+}$ did not abolish immunolabeling (Fig. 6B), however, treatment with BAPTA (Fig. 6C) or EGTA (Fig. 6D) eliminated Cdh23 immunolabeling. The loss of labeling was observed throughout the hair cells of the sacculus including the immature cells located at the periphery of the sensory epithelium (data not shown). Unlike the results of Siemens et al. (2004), we did not detect Cdh23 immunoreactivity in the pericuticular necklace following EGTA treatment (data not shown). Moreover, as reported by Michel et al. (2005) using two different antibodies to the cytoplasmic domain of cadherin 23, we observed that treatment of mouse cochlear hair cells with La$^{3+}$, BAPTA, or EGTA or had no detectable
effect on Cdh23 immunolocalization (Fig. 6E-H). These data suggest that reported differences in Cdh23 immunolocalization following tip-link loss in bullfrog saccular (Siemens et al., 2004) and mouse cochlea (Michel et al., 2005) are not due to the use of different antibodies.

To determine whether the effects of tip-link loss on Cdh23 immunolocalization differs in auditory and vestibular hair cells within a single species, we examined Cdh23 immunolabeling in mouse vestibular hair cells following treatment with EGTA (Fig. 6I,J). In untreated samples, Cdh23 was present in hair bundles (Fig. 6I) and occasionally could be seen at stereociliary tips (Fig. 6I, arrowhead). Smaller utricular hair bundles were much more intensely labeled than larger hair bundles (Fig. 6I, asterisk). In contrast to that observed in bullfrog saccular hair cells, tip-link loss in mouse utricular hair cells did not coincide with a loss of Cdh23 immunoreactivity (Fig. 6J). Hair bundles remained immunolabeled for Cdh23 to varying degrees. Notably, smaller hair bundles remained intensely labeled following EGTA treatment and often the immunolabeling was observed along the length of stereocilia rather than concentrated at stereociliary tips (Fig. 6J and inset). Such a Cdh23 distribution following Ca\(^{2+}\) chelation was observed when the EGTA incubation was done at both room temperature and 4°C (Fig. 6J, inset and data not shown). These data suggest that the differences seen in Cdh23 immunolocalization following tip-link loss are species dependent and do not reflect a distinction between auditory and vestibular hair cells. We did, however, observe a slight difference in immunolabeling in the mouse utricle in comparison to the saccular following EGTA treatment. Saccular hair cells exhibited reduced labeling following treatment, although labeling was still apparent (data not shown).

The molecular basis for the observed differences in Cdh23 immunoreactivity following Ca\(^{2+}\) chelation in the mouse and frog inner ear is unknown, but suggest that the properties of Cdh23 differ between species or within a given species. It is possible that within a given species, there exist subpopulations of Cdh23 that differ in their clearance from the stereociliary membrane, their conformational state, or their interaction with
other molecules following Ca\(^{2+}\) chelation. Indeed, multiple isoforms of Cdh23 have been reported in the mouse inner ear, which may reflect such Cdh23 subpopulations (Lagziel et al., 2005).

**Interaction of Myo1c-T701 and stereociliary receptors does not occur in Cdh23\(^{v2J}\) mice**

The loss of Myo1c binding to the tips stereocilia following tip-link loss implicates Cdh23 as a stereociliary interaction partner for Myo1c. Although mouse cochlear hair cells retain Cdh23 immunoreactivity following tip-link loss, we are unable to determine whether a small fraction of the Cdh23 immunoreactivity, perhaps that located at very the tips of stereocilia which binds to Myo1c, is eliminated by treatment with EGTA, BAPTA or La\(^{3+}\) (Fig. 6E-H). We, therefore, further explored whether the Myo1c receptors detected in our *in situ* binding assay are Cdh23. To do so, we examined the binding of Myo1c-T701 to stereocilia of mice lacking the Cdh23 protein. Immunolocalization studies using antibodies directed against the N-terminal and C-terminal domains of Cdh23 show that *Cdh23\(^{v2J}\)* homozygous mice have no Cdh23 protein present in stereocilia (Michel et al., 2005; A. Lagziel and T. Friedman, personal communication). Moreover, other antibodies against the C-terminus do not detect Cdh23 protein in cochleas of *Cdh23\(^{v2J}\)* homozygous mice by immunoblotting (Rzadzinska et al., 2005). To further characterize the hair bundles of the *Cdh23\(^{v2J}\)*, we first examined the localization of endogenous Myo1c in cochleas of both *Cdh23\(^{v2J}\)* heterozygous and homozygous mice. In heterozygous mice, which have normal hair-bundle morphology, Myo1c was located along the length of stereocilia in both inner and outer hair cells, however, immunolabeling was more prevalent in the outer hair cells (Fig. 7A and inset). Although the hair bundles of *Cdh23\(^{v2J}\)* homozygous mice are drastically malformed, Myo1c is also present in the mutant hair bundles of both inner and outer hair cells (Fig. 7B and inset). As seen with bullfrog saccular hair cells (Cyr et al., 2002), Myo1c-T701 binds to the tips of stereocilia and to the kinocilium in cochlear hair cells from heterozygous *Cdh23\(^{v2J}\)* mice (Fig. 7C and inset). In contrast, no binding was detected in hair cells of homozygous *Cdh23\(^{v2J}\)* animals (Fig. 7D and inset).
Interaction of Myo1c-T701 and stereociliary receptors occurs in Myo7a<sup>6J</sup> mice

The loss of tip links due to exogenous treatments is expected to result in transduction-channel closure, which could alter the available binding sites for our recombinant Myo1c probes. To examine whether transduction-channel closure prevents Myo1c binding in our in situ binding assay, we examined the ability of Myo1c-T701 to bind to the stereocilia in cochleas from Myo7a<sup>6J</sup> mice which harbor a mutation in myosin VIIa resulting in bundle abnormalities and transduction channels that are closed at rest (Richardson et al., 1997; Self et al., 1998; Gale et al., 2001; Kros et al., 2002). Cochlear hair cells from heterozygous Myo7a<sup>6J</sup> animals exhibit normal bundle morphology, however those from homozygous Myo7a<sup>6J</sup> mice have severely disorganized hair bundles that are fragmented into several smaller units each comprised of variable numbers of individual stereocilia (Fig. 8B,D). As demonstrated by immunolabeling, endogenous Myo1c is present in the stereocilia of both heterozygous and homozygous animals (Fig. 8A,B and insets). Unlike the Cdh23<sup>v2J</sup> homozygotes (Fig. 7D), however, Myo1c-T701 binds to receptors in Myo7a<sup>6J</sup> homozygotes as well as the heterozygotes (Fig. 8C,D). Binding to homozygous hair bundles was often less obvious than that seen in the heterozygous samples, but was above background levels. Collectively, these data suggest that neither aberrant bundle morphology nor channel closure affect the availability of Myo1c binding sites in our assay.

**PIP<sub>2</sub> is present in Cdh23<sup>v2J</sup> hair bundles**

In bullfrog saccular hair bundles, PIP<sub>2</sub> is present in stereociliary membranes and is important for transduction (Hirono et al., 2004). The binding of Myo1c fragments to PIP<sub>2</sub> in vitro (Fig. 3 and Hirono et al., 2004) and the loss of Myo1c-T701 binding to hair cells in Cdh23<sup>v2J</sup> homozygous animals (Fig. 7) led us to examine whether Cdh23<sup>v2J</sup> homozygous animals have PIP<sub>2</sub> within their stereociliary membranes. To examine PIP<sub>2</sub> localization, an expression construct comprised of the PLCδ1 pleckstrin homology domain coupled with EGFP was transiently transfected into cochlear hair cells of...
homozygous and heterozygous Cdh23\textsuperscript{v2J} animals. PLCδ1PH-EGFP binds to PIP\textsubscript{2} providing a fluorescent reporter of PIP\textsubscript{2} localization in the membranes of living cells (Downes et al., 2005). Between 20-24 hours after transfection, inner and outer hair cells of both Cdh23\textsuperscript{v2J} heterozygous and homozygous mice show robust fluorescence of their stereociliary membranes verifying the presence of PIP\textsubscript{2} in the hair bundles of living cells (Fig. 9A,B). We then examined whether PIP\textsubscript{2} remains in the stereociliary membrane following fixation and extraction with sarkosyl, the detergent used to permeabilize the tissue in our in situ binding assay. Using a monoclonal antibody against PIP\textsubscript{2}, we found PIP\textsubscript{2} in the stereociliary membrane and concentrated at stereociliary tips in both Cdh23\textsuperscript{v2J} heterozygous and homozygous cochlear hair cells even after extraction of the tissue with 1\% Sarkosyl for one hour (Fig. 9C,D). Similar results were obtained in the wild-type bullfrog sacculus (data not shown). In addition, PIP\textsubscript{2} immunolabeling was detected in hair cell microvilli, which are very abundant at the cell’s apical surface (Fig. 9C, arrow). Thus, the lack of Myo1c-T701 binding to stereociliary receptors in homozygous Cdh23\textsuperscript{v2J} hair cells is not due to the absence of stereociliary PIP\textsubscript{2}.

**Discussion**

*The second IQ domain of Myo1c mediates binding to intracellular receptors*

We have previously established the importance of the Myo1c neck domain in interactions of the adaptation motor with its receptors stereociliary tips (Cyr et al., 2002). This interaction is blocked by excess CaM, indicating that binding is modulated by the CaM occupancy of the Myo1c IQ domains, which in turn is modulated by Ca\textsuperscript{2+}. In the current study we demonstrate that mutations in IQ2 that are predicted to prevent CaM binding to this IQ domain also prevent the CaM modulation of Myo1c binding to its receptors. The second IQ domain of Myo1c, which is highly conserved across species (Cyr et al., 2002), therefore, mediates CaM-sensitive interactions of the adaptation motor at the site of transduction.
Although a CaM-free IQ2 domain is necessary for receptor interactions, it may not be sufficient for binding. Myo1c-N2, which contains only the Myo1c IQ2 domain and detection tags, does not bind to stereociliary components. These data are consistent with previous results showing an excess of the IQ2 peptide does not block Myo1c-T701 binding at stereociliary tips (Cyr et al., 2002) and support our earlier observations that micromolar Ca\(^{2+}\) concentrations, which are expected to remove CaM from neighboring IQ domains, can prevent Myo1c-T701 binding to stereociliary receptors (Cyr et al., 2002). Collectively these results suggest that additional portions of the Myo1c protein, perhaps CaM molecules bound at neighboring IQ domains, are also required for binding.

Sucrose-density centrifugation and gel-filtration chromatography demonstrates that \(\sim 2\) CaMs are bound to Myo1c-T701 following purification (Gillespie and Cyr, 2002). The weak affinity of IQ4 for CaM (Gillespie and Cyr, 2002) suggests that these CaMs are bound at two of the first three IQ domains. Moreover, the loss of a CaM from Myo1c-T701 following mutations in IQ2 suggests that one of the two CaMs that co-purify with Myo1c-T701 is bound at IQ2. In order for Myo1c-T701 to bind to receptors, therefore, CaM must be displaced from IQ2 during our assay, perhaps due to competition, thereby unmasking IQ2 and allowing receptor interactions. Simultaneous mutation of four of the five residues comprising the CaM-binding consensus sequence of IQ1, however, did not result in a loss of a CaM molecule from Myo1c-T701. These results suggest that the second CaM that co-purifies with Myo1c-T701 is not bound at IQ1 and therefore is likely bound at IQ3.

**Myo1c binding to PIP\(_2\)**

PIP\(_2\), which is abundant in stereociliary membranes, is important for hair-cell transduction and binds to the Myo1c neck in vitro (Hirono et al., 2004). Using a nitrocellulose-based assay we determined that CaM occupancy of the second IQ domain of Myo1c also modulates the interaction of the motor protein with PIP\(_2\) in vitro. However, as evidenced in cochleas from homozygous Cdh23\(^{\text{v2J}}\) mice, which have
stereociliary PIP2 but no Myo1c binding, our *in situ* binding assay does not detect Myo1c interactions with PIP2, suggesting that the conditions of the assay are not favorable for detecting this interaction. Nevertheless, the *in situ* binding assay enables the examination and characterization of Myo1c interactions with other stereociliary components.

**Which molecules are the observed Myo1c receptors?**

Our results allow us to eliminate several candidate molecules as the receptors visualized in our *in situ* binding assay. As mentioned above, while Myo1c may interact with immobilized PIP2 *in vitro*, we do not detect Myo1c interactions with PIP2 in our *in situ* binding assay. Similarly, because stereocilia of homozygous Cdh23*^v2J* mice contain endogenous Myo1c but do not bind recombinant Myo1c probes, the observed Myo1c receptors are not endogenous Myo1c molecules. Another candidate Myo1c receptor is PHR1, which binds to a Myo1c fragment containing IQ4 and the tail. Because a Myo1c fragment containing only IQs 1-3 is sufficient for binding to stereociliary tips (Cyr et al., 2002), it is unlikely the PHR1 molecules are the receptors observed in our assay unless Myo1c has two independent PHR1 interaction domains. Our data do not preclude the possibility that PHR1 and Myo1c interact in hair bundles. Indeed, molecular motors often interact with more than one molecule.

Treatment of bullfrog vestibular or mouse cochlear hair cells with agents that break tip links including EGTA, BAPTA, and La^{3+} eliminates or greatly reduces Myo1c binding. The effects of tip-link loss on Myo1c binding implicate a component of the tip-link complex as the binding partner for Myo1c in our assay. As a candidate tip-link molecule, Cdh23, therefore, became an obvious contender for the Myo1c receptor. To examine the role of Cdh23 in the interactions of Myo1c with its receptors in hair cells, we made use of the homozygous Cdh23*^v2J* mouse, which does not express Cdh23 protein in hair cells, has gross hair-bundle abnormalities, and exhibits both auditory and vestibular defects (Di Palma et al., 2001; Lagziel et al., 2005; Michel et al., 2005; Rzadzinska et al., 2005). Although Myo1c binds to the hair bundles from heterozygous Cdh23*^v2J* cochleas, no binding is detected in the cochleas from homozygous animals. This lack of binding is
unlikely to be simply due to hair-bundle disarray or transduction-channel closure because Myo1c binds to stereociliary tips of homozygous Myo7a6J cochlear hair cells.

Our data suggest that the lack of Myo1c binding to hair bundles with broken tip links and in homozygous Cdhl23v2J cochleas reflects a perturbation of molecular interactions that involve both Cdhl23 and Myo1c. It is formally possible that these interactions are not direct and that the loss of tip links and Cdhl23 from hair bundles results in the loss of another molecule, which directly interacts with Myo1c. However, the interaction of Cdhl23 and Myo1c in tissue culture cells (Siemens et al., 2004) lends support to the conclusion that these molecules also interact in hair cells.

The immunolocalization of Cdhl23 in stereocilia of mouse cochlear hair cells following treatment with agents that break tip links is not incongruous with our hypothesis. We propose that the structural alterations of the Cdhl23 ectodomain due to Ca\(^{2+}\) chelation results in a conformational change in the protein’s intracellular domain, thereby altering its interaction with other molecules. In support of this hypothesis, in bullfrog saccular hair cells, distinct immunolabeling patterns for Cdhl23 following EGTA treatment have been obtained using different antisera raised against the Cdhl23 cytoplasmic domain (this study and Siemens et al., 2004). Specifically, Seimens et al. (2004) report an appearance of Cdhl23 immunoreactivity in the pericuticular necklace following EGTA treatment. However, using a different antibody against the Cdhl23 cytoplasmic domain, we do not detect Cdhl23 in this subcellular domain. These differences in labeling patterns suggest that the cytoplasmic domain of the protein undergoes conformational changes, perhaps by binding to or dissociating from other molecules, which render the epitopes recognized by certain antibodies inaccessible. Similar types of intra- or intermolecular changes in Cdhl23 might also alter the ability of Myo1c probes to interact with this domain in our in situ binding assay. Thus following Ca\(^{2+}\) chelation, Cdhl23 may be present, but unable to bind to exogenously applied Myo1c fragments.
**Myo1c interactions with transduction components**

Our data demonstrate that the second IQ domain of Myo1c is important for Myo1c interactions with PIP2 in vitro and at stereociliary tips in situ. In hair cells, the later interaction requires intact tip links and is absent in hair cells lacking Cdh23, a proposed component of the tip link (Siemens et al., 2004; Sollner et al., 2004). The ability of IQ2 to bind CaM, which blocks the interaction, suggests that the local Ca\(^{2+}\) concentration plays a critical role in determining which molecule is bound to the Myo1c neck at a given point in time (supplemental Fig. 2). Due to the high degree of identity of IQ2 across species, the ability of a Myo1c fragment containing only IQs 1-3 to bind to stereociliary tips (Cyr et al., 2002), and the effects of mutations in IQ2 on the ability of CaM to block stereociliary interactions, we propose that the domain of Myo1c that modulates interactions with stereociliary receptors, which are likely to be Cdh23, is IQ2. We predict, therefore, that interactions of Myo1c and Cdh23 will depend both upon the CaM-occupancy of the Myo1c IQ domains and the conformation of the Cdh23 cytoplasmic domain (supplemental Fig. 2).

Accordingly, large increases in the Ca\(^{2+}\) concentration, perhaps immediately after channel opening and before fast adaptation has occurred to reduce channel open probability, should favor the loss of CaM from the Myo1c IQ domains and reduce the interaction of Myo1c with Cdh23. As the stereociliary plasma membrane Ca\(^{2+}\)-ATPase begins to reduce the Ca\(^{2+}\) concentration, CaM would re-associate with the high-affinity IQ domains (IQ3 and perhaps IQ1) and Cdh23 binding would be favored. It would be predicted that interactions with Cdh23 would be maintained unless both the local Ca\(^{2+}\) concentration and the relative affinity for CaM favors CaM re-association at IQ2 in vivo or hair cells are exposed to Ca\(^{2+}\) chelators in vitro (supplemental Fig. 2).
Figure 1. Recombinant Myo1c proteins. A) Construct maps. Wild-type Myo1c includes a catalytic head domain, a neck composed of four tandem IQ domains that bind CaM, and a tail region. The recombinant protein Myo1c-T701 encompasses the Myo1c neck and tail domains. Myo1c-T701IQ2Mut is identical to Myo1c-T701, with the exception of mutations made to the first two amino acids of the IQ consensus sequence of the second IQ domain: I728A and Q729A. Myo1c-N2 consists of only IQ2. Purification and detection tags (H, His6-tag; S, S-tag; X, Xpress-tag) have been appended to the N-terminus of all recombinant constructs and are indicated by the light gray box. Myo1c-T701IQ1RG1Mut is identical to Myo1c-T701, with the exception of mutations that made four amino acids of the IQ consensus sequence of the first IQ domain: L706A, Q707A, R711A, and G712A. The diagram is not drawn to scale. B & C) Coomassie blue-stained SDS-PAGE gels of recombinant Myo1c protein fragments Myo1c-T701 and Myo1c-T701IQ2Mut purification. HSS, High-speed supernatant; FT, flow through from Ni²⁺-NTA column; Wash, Ni²⁺-NTA column wash; Eluate, protein eluted from Ni²⁺-NTA column. Myo1c-T701 and Myo1c-T701IQ2Mut migrate at 34 kDa (*). CaM (#) copurifies with each Myo1c fragment and migrates at 16 kDa. D) Purification of recombinant Myo1c protein fragment N2. Lanes are labeled as in B. In addition, Gel filtration shows the Myo1c-N2 protein further purified by size-exclusion chromatography. CaM is purified bovine CaM. Note that although CaM is co-expressed with Myo1c-N2, CaM does not co-purify with the Myo1c-N2 protein. E) Coomassie blue-stained SDS-PAGE gel of Myo1c-T701IQ1RG1Mut protein purification. Lanes are labeled as indicated in B. Molecular weight markers (kDa) are indicated to the left of each gel.
Figure 2. Binding of Myo1c fragments to stereociliary receptors. Each panel shows a single confocal section containing 10 hair bundles of a whole-mount bullfrog sacculus. Each group of three panels depicts the following: left, fluorescently conjugated phalloidin labeling of filamentous actin; middle, bound recombinant Myo1c fragments; right, overlay of actin (red) and Myo1c binding (green). A) Binding of Myo1c-T701 to receptors at the tips of stereocilia. B) Excess CaM abolishes Myo1c-T701 binding to stereociliary receptors. C) Myo1c-T701Q2Mut binding to stereociliary receptors. D) Excess CaM does not block interactions of Myo1c-T701Q2Mut with stereociliary receptors. E) Myo1c-N2 does not bind to stereociliary receptors. Scale bar: (in E) 5 µm.
Figure 3. Binding of Myo1c fragments to anionic phospholipids. A–F) Each panel shows one PIP-strip, a nitrocellulose membrane, which has been spotted with anionic lipids, that has been incubated with the indicated Myo1c fragment. Bound Myo1c fragments were detected by virtue of the N-terminal Xpress epitope tag. The diagram to the right indicates the location and identity of the anionic lipids. A) T701-Myo1c binds weakly to several of the anionic lipids and strongly to PI(4,5)P₂ (PIP₂). B) The addition of excess CaM abolishes Myo1c-T701 interactions with anionic lipids. C) Myo1c-T701\textsubscript{IQ2Mut} binds to the same subset of anionic lipids as Myo1c-T701. D) Excess CaM does not block the interactions of Myo1c-T701\textsubscript{IQ2Mut} with anionic lipids. E) Myo1c-N2 does not bind anionic lipids. F) Excess IQ2 peptide, when incubated with Myo1c-T701, does not block the binding of Myo1c-T701. G) Antibody only control.
Figure 4. **Myo1c binding to stereociliary receptors in the bullfrog sacculus requires intact tip links.** A) Sensory epithelium of a bullfrog sacculus showing loading of hair cells with AM1-43, which enters through open transduction channels. B) Treatment of a sacculus with 5 mM La$^{+3}$ breaks tip links as assayed by the loss of AM1-43 entry into hair cells. C & D) Treatment with 5mM La$^{+3}$ results in the loss of Myo1c-T701 binding to stereociliary tips. E) Bullfrog sacculus showing AM1-43 loading of hair cells. F) Treatment with 5 mM EGTA breaks tip links as assayed by the marked reduction of AM1-43 entry into hair cells. G & H) Treatment with 5 mM EGTA results in the loss of Myo1c-T701 binding to stereociliary tips. All images are single confocal sections. Scale bars: (in B) A, B, E, F, 20 µm; (in D) C, D, G, H, 5 µm. AM1-43 uptake is *white*, phalloidin labeling of filamentous actin is *red*, and Myo1c-T701 binding is *green*. 
Figure 5. Myo1c binding to stereociliary receptors in the mouse organ of Corti requires intact tip links. Each panel shows a single confocal section containing inner and outer hair cells from a whole-mount mouse organ of Corti. Left column, Fluorescently conjugated phalloidin labeling of filamentous actin; middle column, bound recombinant Myo1c fragments; right column, overlay of actin (red) and Myo1c binding (green). A) Binding of Myo1c-T701 to mouse cochlear hair cells. B–D) Treatment of the organ of Corti with 5 mM BAPTA, EGTA, or La³⁺ results in the loss or marked reduction of Myo1c-T701 binding to stereociliary tips. Scale bar: (in D) 10 µm.
Figure 6. **Cdh23 immunolocalization in hair cells after loss of tip links.** Each panel depicts a single confocal section of whole-mounted bullfrog saccule, mouse cochlea, or mouse utricle. The left panel for each group shows the merged image of actin (*red*) and Cdh23 immunolocalization (*green*). The right panel depicts the Cdh23 immunolabeling channel alone. A) Cdh23 immunolocalization in the bundles of bullfrog saccular hair cells. B–D) Cdh23 immunolocalization in the bundles of bullfrog saccular hair cells after treatment with 5 mM La$^{3+}$, BAPTA, or EGTA, respectively. E) Cdh23 immunolocalization in the bundles of mouse cochlear hair cells (P1 plus 1 d *in vitro*). F–H) Cdh23 immunolocalization in the bundles of mouse cochlear hair cells after treatment with 5 mM La$^{3+}$, BAPTA, or EGTA, respectively. I) Cdh23 immunolocalization in the bundles of mouse utricular hair cells. Asterisks demark representative small hair bundles, which are intensely labeled. An arrowhead denotes an example of stereociliary tip labeling. J) Cdh23 immunolocalization in the bundles of mouse utricular hair cells (P6) after treatment with 5 mM EGTA. Inset, A small hair bundle with labeling along the length of the stereocilium. This example is from a P3 mouse utricle treated with EGTA at 4°C. Similar results were seen with P6 utricles treated at room temperature. Scale bars: A, E, I, (for A–D, E–H, I–J), 10 µm; J, inset, 2 µm.
Figure 7. Myo1c and Myo1c receptor localization in cochlear hair cells of Cdh23<sup>v2J</sup> mice. Each panel shows three rows of outer and one row of inner hair cells of heterozygous (+/-) or homozygous (-/-) Cdh23<sup>v2J</sup> mutant mice (P2–P4). The left column of each panel shows phalloidin labeling alone, and the middle column of each panel shows the bound Myo1c antibody or Myo1c-T701 binding alone. The right column of each panel shows the overlay of phalloidin-labeled filamentous actin (red) and the binding of either a Myo1c antibody (green; A, B) or the Myo1c-T701 fragment (green; C, D). A–D) Cochlear cultures from Cdh23<sup>v2J</sup> mice. A & B, Endogenous Myo1c is present in stereocilia of both the Cdh23<sup>v2J</sup> heterozygous and homozygous mice. C) The Myo1c receptor is detected in the stereocilia of the Cdh23<sup>v2J</sup> heterozygous mice. D) Myo1c receptor is undetectable in the stereocilia of Cdh23<sup>v2J</sup> homozygous mice. Insets depict the hair bundles of outer hair cells. Scale bar: (in D) 5 µm; (in C, inset), A, B, C, insets, 2 µm; D, inset, 2 µm.
Figure 8. Myo1c and Myo1c receptor localization in cochlear hair cells of Myo7a<sup>6J</sup> mice. Cochlear cultures from Myo7a<sup>6J</sup> heterozygous (+/-) or homozygous (-/-) mice (P2–P4) are shown in each panel. The left column of each panel shows phallloidin labeling of filamentous actin, and the middle column of each panel shows Myo1c antibody or Myo1c-T701 binding alone. The right column of each panel shows the overlay of phallloidin labeling (red) and the binding of either a Myo1c antibody (green; A, B) or the Myo1c-T701 fragment (green; C, D). A & B) Endogenous Myo1c is present in the stereocilia of both Myo7a<sup>6J</sup> heterozygous and homozygous mice. C & D) The Myo1c receptor is detected in the hair cells of Myo7a<sup>6J</sup> heterozygous and homozygous mice. All insets are the hair bundles of outer hair cells except for that in A, which is an inner hair cell. Scale bars: (in D) 5 µm; B, inset, 2 µm; (in D, inset) A, C, D, insets, 2 µm.
Figure 9. PIP2 is present in the stereocilia of Cdh23v2J mice. A & B) Localization of PIP2 in a cochlear apical coil from a heterozygous, A, and a homozygous, B, Cdh23v2J mouse using the PH domain of PLC 1 conjugated to EGFP as a fluorescent indicator of PIP2 localization. C & D) Immunolocalization of PIP2 in a cochlear basal coil from a heterozygous, C, and a homozygous, D, Cdh23v2 mouse. All images are single confocal sections. In the merged image to the left, phalloidin-labeled filamentous actin is depicted in red, and PLC 1PH-EGFP or an anti-PIP2 antibody is green. In the image on the right, PLC 1PH-EGFP or the antibody labeling alone is shown. The arrowhead in B denotes a fluorescent mutant hair bundle. The arrows in D denote microvilli labeling. Scale bars: (in B) A, B, 5 μm; (in D) C, D, 5 μm.
Supplemental Figure 1. Myo1c-T701IQ1RG1Mut binds to stereociliary receptors and binding is blocked by CaM. A-C) Each panel shows a single confocal microscopy section containing ~40 hair bundles of a whole-mount bullfrog sacculus. Left panel shows the overlay of the phallodin labeling of filamentous actin (red) and the bound Myo1c protein (green). Right panel shows Myo1c fragment binding only. A) Wild-type Myo1c-T701 binding to stereociliary receptors. B) Myo1c-T701IQ1RG1Mut binding to stereociliary receptors. C) Excess CaM blocks Myo1c-T701IQ1RG1Mut binding to stereociliary receptors. Scale bar in A is 10 µm and applies to all panels.
**Supplemental Figure 2. Proposed model for Myo1c and receptor interactions.**

Predicted influence of transduction-channel open probability and thus intracellular Ca$^{2+}$ concentration on Myo1c interactions with stereociliary receptors, presumably Cdh23, depicted at the top. Myo1c head, IQ domains (numerals 1-4), and tail are indicated. The stereociliary membrane (gray), transduction channel (yellow), Cdh23 (blue), Myo1c (green), CaM (purple) and an actin filament (red) are shown. The predicted effect of Ca$^{2+}$ concentration on the CaM occupancy of the Myo1c IQ domains for each condition is depicted at the bottom. High intracellular Ca$^{2+}$ concentrations (High [Ca$^{2+}$]) would be expected to strip all CaMs from Myo1c and prevent Myo1c interactions with Cdh23 (left panel). Myo1c interactions with Cdh23 would be predicted to occur at Ca$^{2+}$ concentrations that favor disassociation of CaM from IQ2 (second and third panels). In vitro, when the extracellular Ca$^{2+}$ concentration is very low due to chelation (Low [Ca$^{2+}$]$^{\text{ext}}$), the tip link breaks which closes the transduction channel and the cytoplasmic domain of Cdh23 is predicted to undergo a conformational change rendering it unable to bind to Myo1c (right panel). Conditions that favor CaM occupancy of all Myo1c IQ domains would also be predicted to block Myo1c interactions with Cdh23. The ability of IQ4, which has a weak IQ consensus sequence, to bind CaM is uncertain, but if it occurs would only be predicted to occur in low Ca$^{2+}$ and high CaM concentrations. Based upon its proposed role as a tip-link component, Cdh23 is drawn as an integral membrane protein that interacts with the transduction channel. Both its designation as the tip link and its interaction with the transduction channel await further verification.
References


III. Two novel antigens in the hair bundle detected using myosin-1a directed antibodies

Introduction

Hair-cell stereocilia have been compared to the brush border (BB) microvilli of intestinal enterocytes for decades (Flock and Cheung, 1977). Groups of both processes extend away from the apical surface of their respective cell and are structurally dependent upon parallel actin bundle (PAB) arrays that compose their core (see chapter 1, Fig 5B &C). In each case, the PABs share uniform polarity and are oriented with the positive end of the actin filaments extending away from the cell body (Flock and Cheung, 1977). As well, in both stereocilia and microvilli, the rootlet of each PAB tapers and inserts into a dense cytoskeletal network, referred to as the cuticular plate and the terminal web, respectively (see chapter 1, Fig 2C & 5C; DeRosier and Tilney, 1989). Despite structural similarities, stereocilia and microvilli have unique roles. Minute movements of stereocilia in relation to one another dictate an organism’s perception of hearing and balance, while microvilli provide structural expansion to the dense surface area of the small intestine, allowing for the maximum adsorption of nutrients. Despite these functional differences, the core components of the stereocilia and microvilli remain the same and consequently the two structures share many of the same proteins. The actin bundling proteins, fimbrin and espin, have been identified in both the stereocilia and microvilli (reviewed in Mooseker, 1985; Tilney et al., 1989; Bartles et al., 1998; Zheng et al., 2000). As well, both projections utilize unconventional myosins. These motors are a subclass of the myosin superfamily, which is the only family of molecular motors identified to move on actin filaments.

The most abundant unconventional myosin of the enterocyte BB domain is Myosin-1a (Myo1a; Heintzelman et al., 1994). Myo1a, initially referred to as 110K-CM in the early literature, was first identified as a 110 kDa protein that closely associates with calmodulin (CaM; reviewed in Mooseker, 1985). It was classified as an unconventional myosin isoform in 1989 and given the name brush border myosin I, which was later changed to myosin-1a (Mooseker and Coleman, 1989; Gillespie et al., 2001). Myo1a,
like other members of the unconventional myosin class, has three domains: a single catalytic head domain, a neck domain composed of three tandem IQ motifs capable of binding CaM, and a C-terminal tail consisting of a TH1 domain that binds directly to acidic phospholipids (Mooseker and Cheney, 1995). Myo1a is located along the length of the microvilli, and appears to crosslink the space between the PAB and the microvillar membrane (see chapter 1, Fig 5C; Mooseker and Coleman, 1989).

Despite the high concentration of Myo1a in the microvilli of the enterocyte, its specific role remains unclear. Since its identification in 1989, investigators have hypothesized that Myo1a may act to tether the PABs to the microvillar membrane, buffer intracellular Ca\(^{2+}\) levels by virtue of its associated CaM molecules, or power vesicle trafficking to the BB membrane (Fath et al., 1994; reviewed in Tyska and Mooseker, 2002). To address these hypotheses and elucidate the contributions of Myo1a to the organization and function of the enterocyte brush border domains, a Myo1a knockout (KO) mouse was developed (Tyska et al., 2005). In these mice, the first three exons of the Myo1a gene, including its start site for protein synthesis, were replaced with a neomycin resistance cassette. This mutated allele was expressed in 129X1/SvJ mice and was bred to homogeneity. Both immunoblot and immunolocalization experiments assaying intestinal tissue indicate that mice homozygous for this mutation, denoted Myo1a\(^{-/-}\), do not express Myo1a protein (see chapter 1, Fig 5D & E; Tyska et al., 2005). As well, evidence of the full-length Myo1a mRNA transcript was not detected in Myo1a\(^{-/-}\) mice (Tyska et al., 2005). Despite the null expression of Myo1a, Myo1a\(^{-/-}\) mice exhibit no overt phenotype. Animal size, intestinal function, and reproductive rates of Myo1a\(^{-/-}\) mice were not significantly different than wild-type mice of the same background (Tyska et al., 2005).

However, a detailed analysis of the epithelium of the gut did uncover subtle abnormalities in the organization of the enterocyte BB domains. In the Myo1a\(^{-/-}\) mouse, microvillar packing was less organized and some microvilli exhibited herniations of the membrane (Tyska et al., 2005). In addition, the intestinal epithelium appeared to be in a prolonged state of stress and had an increased number of apoptotic cells as reported by TUNEL staining (Tyska et al., 2005). As expected, no cross-bridges between the PAB and the microvillar membrane were observed in the Myo1a\(^{-/-}\) mice and the
membrane often was collapsed onto the PAB core (Tyska et al., 2005). Most interesting, the addition of ATP to BB preps of wild-type mice activated terminal web contractility, attributed to the conventional myosin, myosin 2a, and initiated the shedding of the microvillar apical membrane. However, in BB preps from Myo1a<sup>−/−</sup> mice only terminal web contractility was observed (Tyska et al., 2005). This suggests that Myo1a is required for the ATP-dependant microvillar membrane shedding observed in these <i>in vitro</i> assays.

Investigators also assayed the expression of other proteins in the Myo1a<sup>−/−</sup> mouse intestinal epithelium relative to their expression in wild-type mice. While the expression of many proteins was unaltered, a decrease in myosin-1e and CaM was noted, as well as an increase in cytokeratins 8 and 18, and myosin-1c (Myo1c; Tyska et al., 2005). The increase in Myo1c expression was confirmed using immunofluorescence experiments that showed its redistribution from the enterocyte cell body to the microvilli at the cell periphery (Tyska et al., 2005). Investigators hypothesized that the subtle phenotypical differences detected in Myo1a<sup>−/−</sup> mouse may be the result of Myo1c’s compensation for the absence of Myo1a (Tyska et al., 2005). This is the first recorded observation supporting the potential compensation between unconventional myosin molecules.

Myosin isoforms expressed in the hair bundle were identified during investigations aimed at identifying candidate molecules for the hair-cell adaptation motor. In the first of these screens, Myo1a mRNA was detected in the sensory cells of bullfrog sacculus (Solc et al., 1994). Subsequently, these results were repeated using mouse utricle (Dumont et al., 2002). However, in each study the amount of Myo1a mRNA detected was very low, and investigators were skeptical as to whether Myo1a protein was expressed in the hair cell (Solc et al., 1994; Dumont et al., 2002). Despite these results, Myo1a has recently been implicated in hearing loss (Donaudy et al., 2003). Because MYO1A was mapped to DFNA48, a DNA locus attributed to nonsyndromic autosomal-dominant hearing-loss defects, a large pool of hearing impaired patients were screened for mutations in MYO1A (D'Adamo et al., 2003; Donaudy et al., 2003). These studies indicate that a MYO1A nonsense mutation and six missense mutations occur concurrently with hearing loss (Donaudy et al., 2003). This study also repeats the
detection of Myo1a transcript in acousticolateralis end organs (Solc et al., 1994; Donaudy et al., 2003).

Based on these recent findings, our lab in collaboration with Matthew Tyska, Ph.D. (Vanderbilt, TN) sought to characterize the potential expression and localization of Myo1a protein in the hair cell. At our disposal were both the Myo1a−/− mice used in Dr. Tyska’s previous studies and polyclonal antibodies raised against the Myo1a head and tail domains (Tyska et al., 2005). To our dismay, these antibodies proved to be non-specific in the cochlea, despite previous characterization showing their specificity in gut epithelial tissue. As such, we were unable to identify any labeling in hair cells that could be attributed specifically to Myo1a. However, both antibodies tested did detect specific antigens that are expressed in the cochlear hair cells of both Myo1a++ and Myo1a−/− mice. These antigens occur in distinct patterns and may represent two novel hair-bundle proteins.

**Methods**

**Myo1a KO mice** Myo1a KO mutant mice were supplied by Matthew Tyska (Vanderbilt, TN) as part of the collaborative project (referenced in Tyska et al., 2005).

**Genotyping** Tail samples were taken from all mice immediately following sacrifice and stored at −20°C. DNA was extracted using Wizard Genomic DNA Purification Kit (Promega, Madison, WI). To assess the incorporation of the neomycin cassette into the Myo1a locus, resulting in a nonfunctional Myo1a allele, we used the following primers; Myo1aF1584: 5’-TCAGGTGGGCAGACAGAGGTAGT-3’, Myo1aR3330: 5’-CCCTCCCATCAGGTTAACTATCAGA-3’, and NeoF1a 5’-GGATTGCACGCAGGTTCCTCC-3’ in a PCR cocktail with the puReTaq Ready-To-Go PCR beads (GE Healthcare, Piscataway, NJ) and purified DNA. PCR gave products of 1800 bp for wild-type samples, 1400 bp for homozygous Myo1a KO samples and both 1800 bp and 1400 bp for heterozygotes.
Cochlear samples for RT-PCR Six to ten cochleas were acutely dissected in minimal essential medium (MEM) with Glutamax supplemented with 10 mM HEPES (Invitrogen, Carlsbad, CA) and stored inRNAlater buffer (Qiagen, Valencia, CA). RNA was extracted using RNAeasy RNA isolation kit (Qiagen). To digest any contaminating genomic DNA, DNase I was incubated with the RNA sample while on the RNA isolation column for 15 minutes at RT. Isolated RNA was used as a template for the reverse-transcription reactions using the Thermoscript RT kit (Invitrogen). As an additional control for genomic contamination, one sample did not receive reverse transcriptase (-RT). The synthesized cDNA and the -RT control were used in PCR reactions with primers complementary to the head and tail domains of Myo1a (completed in the Tyska laboratory).

Scanning Electron Microscopy Cochleas were harvested as described above and fixed overnight at 4°C using 3% gluteraldehyde in SEM Buffer (0.1 M Na-phosphate buffer and 0.1 M sucrose). Samples were washed 2 X 5 minutes using SEM buffer and subjected to an additional fixation using 1% osmium tetroxide on ice for 1 hour. Samples were again washed 2 X 5 minutes using SEM buffer and then dehydrated using a series of ethanol washes (35%, 50%, 70%, 95% and 100%; 15 minutes each). Samples were either stored at this step or the ethanol was replaced with hexamethyldisilazane (HMDS). The HMDS was then evaporated in the hood and sample was mounted and coated with gold. Samples were imaged on a Hitachi X-650 scanning electron microscope.

Primary Antibodies Primary polyclonal antibodies were supplied by Matthew Tyska (Vanderbilt, TN) as part of the collaborative project. Anti-brush border I (αBBI) was raised against a portion of the Myo1a tail domain and characterized extensively in the gut epithelium tissue (see chapter 1, Fig 5 D & E; Tyska et al., 2005). The antibodies 3p1 and 4p1 were raised against multiple peptides of the head domain of Myo1a. The denotations 3p1 and 4p1 correspond to the rabbit identification number followed by the specific peptide used for antibody purification on an affinity column. Peptide 1, consisting of amino acids 1-14 of the Myo1a head domain; MPLLEGSVGVEDLV, was
used for the purification of both antibodies. The monoclonal antibody, 6a, also recognizes Myo1a, and was supplied by Dr. Tyska (Carboni et al., 1988).

**Immunocytochemistry** Following sacrifice at P7, cochleas were acutely dissected in MEM with Glutamax supplemented with 10 mM HEPES (Invitrogen). Some samples were incubated with 5 mM 1,2-bis(O-aminophenoxy)ethane-N,N,N9,N9-tetraacetic acid (BAPTA) for 20 minutes to break tip links. All samples were fixed using 3% paraformaldehyde diluted from a 16% stock (EM Sciences, Hatfield, PA) in cold phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, and 1.5 mM KH2PO4, pH 7.4). Following a brief PBS wash, samples were permealibilized using 0.5% Triton X-100 in PBS for 30 minutes. Samples were washed 2 X 10 minutes in PBS and blocked for 1 hour in 2% BSA (fraction V; Calbiochem, La Jolla, CA) and 5% goat serum (Jackson Laboratories, West Grove, PA) in PBS at room temperature (RT). Primary antibodies were incubated at 1:200 with samples overnight at 4°C in PBS with 2% BSA and 5% goat serum. Samples were washed 3 X 10 minutes in PBS-0.1% Tween-20 and incubated with Alexa 488 goat anti-rabbit secondary antibody (13 µg/ml; Invitrogen) and 33 nM Alexa 568 phalloidin (Invitrogen) for 2 hours at RT. Samples were washed 3 X 10 minutes in PBS-0.1% Tween-20 and 1 X 10 minutes in PBS, mounted with Vectashield (Vector laboratories, Burlingame, CA) and imaged on a LSM-510 Meta confocal microscope (Zeiss, Thornwood, NY).

**Immunoblot Tissue Preparation** Following sacrifice at age P7, the entire murine gut was immediately extracted and submerged in 1 mL of cold sucrose dissociation buffer (SDB; 200mM sucrose, 0.02% sodium azide, 12 mM EDTA-K, 19 mM KH2PO4 monobasic, 78 mM Na2HPO4 anhydrous, dibasic) and incubated on ice up to 30 minutes. The cochleas of each animal were dissected in 10 mM HEPES-buffered MEM with Glutamax (Invitrogen, Carlsbad, CA) and submerged in 1 mL of cold SDB and incubated on ice up to 30 minutes. All samples were agitated in 1.5 mL microcentrifuge tubes using micro stir-bars for 30 minutes at 4°C. Solubilized tissue was removed and centrifuged at 14000 rpm for an additional 30 minutes at 4°C. Cell pellets were resuspended in cold homogenization buffer (10 mM imidazole, pH 7.2, 4 mM EDTA-K, 1 mM EGTA-K,
0.02% sodium azide), supplemented with 1 mM DTT and 1 mM Pefablock® SC (Pentapharm, Norwalk, CT). Samples were homogenized using a motorized hand homogenizer for 15 sec. An equal volume of 10X sample buffer (8% SDS, 2.4% 10X stacking gel buffer [1.4 M Trizma base, 1.2 N HCl, pH 6.8], 580 mM sucrose, 9.5% 2-β Mercaptoethanol, 3 mM bromphenol blue, 10 mM EGTA-K) was added. Samples were boiled for 5 minutes and either frozen at –20°C or immediately used for immunoblots.

**Immunoblotting** Samples were separated on a 4-12% gradient NuPage Bis-Tris gel in MES buffer (Invitrogen). Gel was transferred overnight at 4°C onto HyBond ECL nitrocellulose membrane (GE Healthcare) and blocked with 5% nonfat dry milk in tris-buffered saline-Tween-20 (TBS-T; 1.5 M NaCl, 0.5 M Trizma base, 0.1% Tween-20, pH 8.0) for 1.5 hours at RT. Primary antibodies (αBBI, 3p1, 4p1, or 6a) were added at 1:1000 in TBS-T for 2 hours at RT. Membranes were washed 3X10 minutes in TBS-T and incubated with goat anti-rabbit or goat anti-mouse conjugated to horseradish peroxidase secondary antibody (1:1000; Cell Signaling, Danvers, MA) for 1 hour at RT. ECL Plus Western Blotting Detection System (GE Healthcare) was used to detect bound secondary antibody.

**Results**

**Myo1a mRNA expression**

The past characterization of Myo1a expression in the Myo1a°/° mouse was based upon null RT-PCR screens directed at exons 2-8 (head°/°) of the Myo1a head domain (Tyska et al., 2005). Because this sequence occurs early in the Myo1a head domain, it was felt to be an accurate indicator of Myo1a expression in the Myo1a°/° mouse. However, a recent entry into Genbank, based upon analysis of the MYO1A gene’s open reading frame, predicts the expression of a short isoform of Myo1a that results from a novel start site in exon 24 of the Myo1a tail domain (XP_904541; www.ncbi.nlm.nih.gov/Genbank,
Based on these predictions, we felt it imperative to any future experiments to complete a thorough characterization of Myo1a transcript expression in both the Myo1a<sup>+/+</sup> and in vivo in the small intestinal epithelium of either the Myo1a<sup>+/+</sup> or Myo1a<sup>-/-</sup> mouse. To determine whether this novel tail isoform, denoted Myo1a<sup>TAIL</sup>, is transcribed in mouse, primers were designed to amplify the previously screened exons, head<sup>2-8</sup>, as well as exons 13-16 of the head domain (head<sup>13-16</sup>), exons 24-27 of the Myo1a tail domain (tail<sup>24-27</sup>), and the unique sequence specific to Myo1a<sup>TAIL</sup>. Results of these experiments replicate past data that detect mRNA transcripts that code for head<sup>2-8</sup> of Myo1a in the gut of the Myo1a<sup>+/+</sup> but not the Myo1a<sup>-/-</sup> mouse. However, mRNA that codes for head<sup>13-16</sup>, as well as Myo1a<sup>TAIL</sup>, is detected in gut epithelial tissue of the Myo1a<sup>+/+</sup> mouse, as well as the Myo1a<sup>-/-</sup> mouse (unpublished data, M. Tyska; Fig 1B). This suggests that Myo1a<sup>TAIL</sup>, as well as an additional isoform containing the head<sup>13-16</sup> sequence, may be transcribed in the gut epithelium of both the Myo1a<sup>+/+</sup> and Myo1a<sup>-/-</sup> mouse.

Previous experiments have identified Myo1a mRNA transcripts in the vestibular organs of the bullfrog as well as in mouse utricle and cochlea (Sole et al., 1994; Dumont et al., 2002; Donaudy et al., 2003). However, the published experimental methods provided little technical detail. Therefore, we wished to replicate these experiments using the 129X1/SvJ mouse strain, as well as explore the potential expression of Myo1a<sup>TAIL</sup> in the cochleas of both the Myo1a<sup>+/+</sup> and Myo1a<sup>-/-</sup> mouse. Cochleas from Myo1a<sup>+/+</sup> and Myo1a<sup>-/-</sup> littermates, age P7, were acutely dissected. A cDNA pool was made from the RNA extracted from these samples and used as a template in PCR reactions designed to amplify the head<sup>2-8</sup> of Myo1a. Like similar reactions done in gut, DNA sequence complementary to mRNA transcript coding for head<sup>2-8</sup> of Myo1a was detected in the Myo1a<sup>+/+</sup> cochlear sample and was absent in the Myo1a<sup>-/-</sup> cochlear sample (Fig 1C). In contrast to results using gut tissues, a product of approximately 750 bps, in addition to the expected 600 bp product that corresponds to head<sup>2-8</sup>, was detected in Myo1a<sup>+/+</sup> cochleas. Sequencing of the novel 750 bp product reveals two distinct mRNAs; one with a 99 bp intronic insertion, denoted 750a, and one with 198 bp intronic insertion, denoted 750b (Fig 1D). When translated to amino acid sequence, the 750b transcript codes for multiple
in-frame stop codons, suggesting that it is likely to be an incompletely spliced mRNA artifact (Fig 1D & E; red sequence). However, the 750a transcript codes for no stop codons in its open reading frame. As this transcript is never detected in gut samples, it may represent a cochlear specific Myo1a isoform (Fig 1D & E; green sequence). DNA sequence complementary to mRNA coding for Myo1aTAIL and tail24-27 was also amplified in Myo1a+/+ and Myo1a−/− cochlear samples, similar to what was observed using gut epithelium (Fig 1C).

Cochlear morphology of Myo1a−/− mice

A first step in the assessment of a potential role of Myo1a in the hair cell is the examination of the cochlear morphology of the Myo1a−/− mouse. If Myo1a plays a significant role in hearing, then its absence may lead to the physical perturbation of the cochlear hair cells, as is the common result of many mutations that lead to hearing loss. Cochlear samples, postnatal day 7 (P7), of Myo1a+/+ and Myo1a−/− litter-mates were acutely dissected and processed for scanning electron microscopy (SEM). Surprisingly, no significant cochlear morphological differences between the Myo1a+/+ and the Myo1a−/− mouse were noted, indicating that the absence of Myo1a in the Myo1a−/− mouse did not grossly effect either the organ of Corti or the hair cell structural integrity at this age (Fig 2, A&B). In some samples, regardless of genotype, large irregular gaps between stereocilia were observed (Fig 2C). As well, the bundles of some samples appeared to be relaxed, making the characteristic “V” shape of the cochlear bundle indiscernible (Fig 2C). As a control, an alternate strain of mouse, FVB/NJ, was processed. The cochlear bundles of this mouse strain were uniformly packed and the “V” shape of the hair bundle was easily identified (Fig 2D).

Immunoflourescent localization of the anti-brush border I (αBBI) antibody

Next we sought to determine whether Myo1a is expressed in the hair cell and if so, its location. The antibody used in these experiments, αBBI, was raised against a portion of the tail domain of Myo1a. It had been previously used to characterize Myo1a
expression in the gut where it recognized only Myo1a in Myo1a\textsuperscript{+/+} mice and had no immunoreactivity in Myo1a\textsuperscript{+/−} mice, as indicated by immunoblots and immunocytochemistry (see chapter 1, Fig 5D & E; Tyska et al., 2005). For all immunolabeling experiments, P7 cochleas from FVB/NJ, Myo1a\textsuperscript{+/+}, or Myo1a\textsuperscript{−/−} mice were used. In FVB/NJ and Myo1a\textsuperscript{+/+} mice, αBBI localized weakly to the bottom two-thirds of the stereocilia (data not shown), and was strongly concentrated at the tips of the stereocilia (Fig 3A-D). In inner hair cells the tips of the tallest stereocilia were only weakly labeled relative to the tips of shorter stereocilia (Fig 3C). In outer hair cells the tips of the middle and shorter rows of stereocilia were brightly labeled while the tallest row of stereocilia were only occasionally labeled (Fig 3D). The αBBI antigen was also highly expressed in surrounding pillar and Dieter cells (Fig 3A). Immunolabeling experiments were repeated on Myo1a\textsuperscript{−/−} mice and an identical labeling was observed (data not shown). No discrepancies between Myo1a\textsuperscript{+/+} and Myo1a\textsuperscript{−/−} mice could be detected, either in the hair cells or in surrounding supporting cells in the organ of Corti.

Because αBBI recognizes an antigen at the tip of stereocilia, concurrent with the location of the mechanoelectrical transduction (MET) channel complex, we wished to further characterize antigen localization following treatment with the Ca\textsuperscript{2+} chelator, BAPTA, which disrupts the tip links which are part of the MET channels. Following such treatment, αBBI immunoreactivity could not be detected at either the tips of the stereocilia or in supporting cells (data not shown, J. Cyr).

We also wished to characterize αBBI immunoreactivity in vestibular hair cells. In the bullfrog sacculus, αBBI bound to antigens expressed in the supporting cells of the sensory epithelium, but did not recognize any antigens in the hair cell (Fig 3E&F). As well, no αBBI immunoreactivity was observed in mouse vestibular organs (data not shown).
**Immunofluorescent localization of the 3p1/4p1 antibody**

In response to the identification of the Myo1aTAIL transcript that may code for a headless Myo1a isoform in the Myo1a^/- mouse and the apparent non-specificity of αBBI in the cochlea, two new affinity purified antibodies, 3p1 and 4p1, were generated by Dr. Tyska’s laboratory. These antibodies were affinity-purified against a peptide identical to the first 14 amino acids of the Myo1a head domain, and were predicted to detect only the full-length isoform of Myo1a. In intestinal epithelial tissue, 3p1 and 4p1 showed identical patterns of reactivity, specifically recognizing Myo1a in immunocytochemistry experiments and on immunoblots (M. Tyska, personal communication). In the cochlea, the immunoreactivity of 3p1 and 4p1 was also identical, and showed a localization pattern distinct from the αBBI antibody. In outer hair cells 3p1/4p1 localized to the bottom two-thirds of the stereociliary shaft, extending well into the cuticular plate of the hair cell (Fig 4A, sections 2-5). In inner hair cells immunoreactivity was observed only at the base of the stereocilia, extending into the cuticular plate (Fig 4B, sections 3 & 4). Supporting cells in the organ of Corti were unlabeled (Fig 4A & B). The reactivity of 3p1/4p1 was identical in Myo1a^/- mice, indicating that despite the specificity of the 3p1/4p1 antibody in gut tissue, the immunoreactivity observed using the 3p1/4p1 antibody in the cochlea may not be attributed to Myo1a localization (Fig 5). As well, no immunoreactivity was observed using the 3p1/4p1 antibody on wild-type mouse vestibular hair cells (Fig 6).

**Cochlear immunoblots using 3p1/4p1 antibody**

In an effort to use an alternative method to assess Myo1a protein expression, and perhaps identify the unknown antigens of the 3p1/4p1 antibody, we used cochlear samples for immunoblot experiments. In all immunoblotting experiments we used purified gut microvillar samples and crude gut extract samples as positive controls. The immunoreactivity of 3p1/4p1 with purified microvillar samples was highly specific; usually only 1-2 bands were observed (Fig 7; MV). The most intense band ran at approximately 120 kDa, the size of Myo1a. A smaller band was sometimes observed at 90 kDa and is believed to be a degradation product of Myo1a. Crude gut extract,
prepared in our lab, was also run as a positive control (Fig 7; Myo1a\(^{+/+}\) gut: WTg, and Myo1a\(^{+/−}\) gut: HETg). In these samples a 120 kDa band was clearly visible along with a laddering of proteins from approximately 80-50 kDa. Because crude gut extract samples taken from Myo1a\(^{−/−}\) mice show no immunoreactivity (Fig 7; KOg), this laddering is also thought to be degradation products of Myo1a, accelerated due to the method of sample preparation. The absence of a 120 kDa band in the Myo1a\(^{−/−}\) crude gut extract sample indicates that, as previously published, full-length Myo1a is not expressed in the gut of these animals (Fig 7 B&C; Tyska et al., 2005). Cochlear samples had very low immunoreactivity (Fig 7; Myo1a\(^{+/+}\) cochlea: WTe, and Myo1a\(^{+/−}\) cochlea: HETe, and Myo1a\(^{−/−}\) cochlea: KOe). In cochlear samples from wild-type, heterozygous and homozygous mice, no protein band at the expected size of 120 kDa could be detected (Fig 7 B&C). This was true for 3p1 and 4p1 antibodies in addition to a monoclonal Myo1a antibody (6A; data not shown). A few proteins of various molecular weights are observed inconsistently between samples, but even at long exposures no protein was ever observed at 120 kDa. Five attempts to immunoprecipitate Myo1a from cochlea using the 4p1 antibody were unsuccessful.

**Discussion**

The Myo1a\(^{−/−}\) mouse may express a novel Myo1a isoform, Myo1a\(^{TAIL}\)

Prior to this investigation, Myo1a\(^{−/−}\) mice had not been screened for the presence of alternative mRNA transcripts (Tyska et al., 2005). Because a neomyocin cassette was inserted in the place of the first three exons of Myo1a, including the start site, it had been assumed that all potential for Myo1a protein production had been eliminated. Accordingly, the absence of Myo1a protein was confirmed using the αBBI antibody in gut epithelial tissue of Myo1a\(^{−/−}\) mice (see chapter 1, Fig 5E; Tyska et al., 2005). However, the identification of the novel start site in exon 24 of the Myo1a gene spurred the reexamination of mRNA expression in Myo1a\(^{−/−}\) mice. Unexpectedly, in Myo1a\(^{+/+}\) and Myo1a\(^{+/−}\) gut samples, mRNA coding for head\(^{13−16}\), tail\(^{24−27}\), and the novel tail isoform, Myo1a\(^{TAIL}\) were detected (Fig 1D and data not shown). These transcripts were also detected in the cochleas of both Myo1a\(^{+/+}\) and Myo1a\(^{−/−}\) mice. It is unknown, however,
whether any of these transcripts are ever translated *in vivo* in either tissue. In the gut, immunofluorescence data using αBBI, an antibody directed at the Myo1a tail, suggests that despite the existence of the head\textsuperscript{13-16}, tail\textsuperscript{24-27}, and Myo1a\textsuperscript{TAIL} transcripts in the Myo1a\textsuperscript{−/−} mouse, the corresponding protein remains untranslated (see chapter 1, Fig 5E; Tyska et al., 2005). In cochlear tissue, where immunoreactivity with αBBI was observed in both the Myo1a\textsuperscript{+/+} and Myo1a\textsuperscript{−/−} samples, the head\textsuperscript{13-16}, tail\textsuperscript{24-27}, or Myo1a\textsuperscript{TAIL} transcripts may be the antigenic source, as discussed below.

**Detection of cochlear-specific Myo1a transcripts**

In the cochlea, in addition to the expected 600 bp product that correlates to head\textsuperscript{2-8}, a larger product of approximately 750 bp was also detected by RT-PCR. Sequencing of this product showed two distinct Myo1a transcripts, containing segments of intron 6 sequence, that we refer to as 750a and 750b (Fig 1A, *pink* box). The smaller insertion, 750a, maintains the open reading frame of the Myo1a protein, while 750b sequence codes for multiple stop codons (Fig 1E, *underlined codons*). Analysis of the 750a sequence reveals that the additional 99 bps code for 33 amino acids that, if translated, would lie in the globular head domain of Myo1a. The potential function of the 750a insertion is unknown. Protein database searches indicate that no conserved protein domains exist in this sequence (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). As well, the 750a sequence is composed of 18 percent proline residues, making it unlikely that the alpha-helical tertiary structure of the Myo1a head domain could be maintained consequent to the expression of this sequence (Fig 1F). Because this disruption would occur very close to the Myo1a ATP-binding pocket, the 750a sequence may alter the motor activity of Myo1a, possibly rendering it inactive. Therefore, the expression of the 750a transcript *in vivo* could act as a dominant negative isoform, regulating Myo1a function in a cochlear specific manner. However, it seems more likely that both the 750a and 750b transcripts are the products of incompletely spliced mRNAs.

While the various expression patterns of Myo1a mRNA in the gut and cochlea are very intriguing, such experiments do not reflect protein expression. From these data we
can only conclude that full-length Myo1a is not expressed in the gut epithelium or the cochlea of the Myo1a<sup>−/−</sup> mouse. Alternate mRNA transcripts that are detected in Myo1a<sup>+/+</sup> and Myo1a<sup>−/−</sup> mice, as well as the full-length Myo1a transcript detected in the cochlea, may or may not lead to protein translation. To unequivocally determine protein expression, antibodies specific to the amino acid sequence coded for by the novel mRNA transcripts must be generated and tested on tissue using either immunofluorescent or immunoblotting techniques.

**Cochlear morphology of Myo1a<sup>−/−</sup> mice**

Many mutations linked to hearing-loss manifest in a disruption of the structural organization and morphology of the hair bundle. In the microvilli, which share many structural features with the hair bundle, Myo1a expression is quite robust. As such, we felt that the characterization of the cochlear morphology of Myo1a<sup>−/−</sup> mice, with specific focus on bundle integrity, might provide substantial insight into the pathogenesis of the hearing loss recently linked to Myo1a mutations (Donaudy et al., 2003). We were dismayed however, to find that the structural integrity of the wild-type 129X1/SvJ hair bundles was compromised in some samples. Consequently, any subtle changes that may exist between Myo1a<sup>+/+</sup> and Myo1a<sup>−/−</sup> hair bundle morphology are undetectable. Any minor perturbation that may occur as a result of the absence of Myo1a may be masked by the overall nonconformity of the cochlear sample. To determine whether the abnormalities could be attributed to experimental technique, wild-type mice from an alternate strain, FVB/NJ, were also processed. Hair bundles from these animals looked as expected, providing evidence of a 129X1/SvJ strain-specific problem. As a result, breeding is underway to move the Myo1a<sup>−/−</sup> mutation to the FVB/NJ background for future experiments.

**Immunofluorescent localization of the αBBI and the 3p1/4p1 antibodies**

Using the Myo1a antibodies, directed to Myo1a head and tail domain, 3p1/4p1 and αBBI respectively, we were unable to detect any immunolocalization that could be
attributed to full-length Myo1a protein expression in the cochlea. The immunolabeling observed using these antibodies was identical in both the Myo1a<sup>+/−</sup> and Myo1a<sup>−/−</sup> mice. Because no subset of immunolabeling observed in the Myo1a<sup>+/−</sup> mice was absent in Myo1a<sup>−/−</sup> mice, we must conclude that if full-length Myo1a protein is expressed in cochlear hair cells, it exists at a concentration too low to detect with our reagents. However, both antibodies displayed extraordinary patterns of non-specific reactivity suggesting that each recognizes potentially novel proteins in the cochlear hair bundle.

The αBBI antibody recognizes antigens present at the tips of hair bundles, sparsely along the length of the stereocilium and in the supporting cells. Of particular interest was the immunoreactivity at the tip of the stereocilia. Any protein localized to this area has the potential to interact with the mechanoelectrical transduction (MET) channel complex. To pursue this hypothesis, we repeated immunolocalization experiments with αBBI following hair cell exposure to BAPTA, a reagent known to disrupt tip links, vital components of the MET channel complex. In these experiments, immunoreactivity at the tip of the hair bundle and in the pillar and Dieter cells was absent, suggesting that the conformation of the αBBI antigen, and its reactivity with the αBBI antibody, may be linked to MET complex integrity.

The immunoreactivity of the αBBI antibody was identical in the Myo1a<sup>+/−</sup> and Myo1a<sup>−/−</sup> cochlear tissues. Because the mRNA message for full-length Myo1a can not be detected in Myo1a<sup>−/−</sup> cochlea, we must conclude that the αBBI antibody does not detect full-length Myo1a in these tissues. However, mRNA representing the novel tail isoform of Myo1a, Myo1a<sup>TAIL</sup>, is expressed in the Myo1a<sup>−/−</sup> cochlea. Therefore, the unique localization of the αBBI antigen may be attributed to the expression of the Myo1a<sup>TAIL</sup> transcript. The localization of this headless Myo1a isoform at the tips of stereocilia would position it for association with the intricate scaffolding network that occurs at the stereociliary tip. Because calcium chelation experiments also link the localization of the αBBI antigen to tip-link integrity, and its concentration is increased in shorter stereocilia, one might predict that the Myo1a<sup>TAIL</sup> isoform may interact directly with tip-link complex at the prolate tip of the shorter stereocilia. Because no protein has been identified that
contributes to tip-link asymmetry observed using high-resolution microscopy (Kachar 2001), this would be a novel and interesting avenue to pursue.

The αBBI antibody did not react with any antigen in the saccular cells of bullfrog or mouse vestibular organs, but it was visible at the apical surface of supporting cells. This was as expected; Myo1a−/− mice show no overt vestibular dysfunction (i.e. circling) as is often observed in mutant mice with vestibular defects (Tyska et al., 2005).

The antibodies 3p1/4p1 recognize antigens expressed along the bottom two-thirds of the stereocilia in outer hair cells and at the stereociliary base in inner hair cells. In both types of hair cells, the immunoreactivity extended several microns into the cuticular plate. As well, no supporting cells in the organ of Corti were labeled with 3p1/4p1. Like the αBBI antibody, the immunoreactivity observed in the Myo1a+/− and Myo1a−/− was identical. These data, coupled with the absence of Myo1a mRNA transcripts complementary to the first twelve exons of Myo1a in the Myo1a−/− mouse, allowed us to eliminate Myo1a as the 3p1/4p1 antigen. Concurrently, we hypothesized that the 3p1/4p1 antibody may be recognizing an alternate myosin, such as myosin-6 (Myo6). Myo6 is localized to the rootlet of the stereocilia and robustly in the cuticular plate (Hasson et al., 1997). Comparison of the expression patterns of the 3p1/4p1 antigen and Myo6 clearly precludes this possibility. In the OHC, the immunoreactivity of the 3p1/4p1 antibody is associated with a large portion of the stereocilia. In both cell types, this labeling pattern continues along the stereocilia taper in the cuticular plate; no extraneous labeling in the actin meshwork of the cuticular plate is observed. Therefore, we can safely eliminate Myo6 as a candidate for the 3p1/4p1 antigen. However, because their localizations overlap, once the 3p1/4p1 epitope is identified, further experiments to test for its interaction with Myo6 may be intriguing. To our knowledge, the reported 3p1/4p1 antigen expression pattern has never before been described in the hair bundle. Such a localization pattern would lend itself to stereociliary maintenance at the negative end of the PAB or stereociliary anchorage to the cuticular plate. Little is known about either of these processes.
Cochlear immunoblots using 3p1/4p1 antibody

Despite our inability to detect Myo1α on immunoblots using the 3p1/4p1 antibodies in cochlear samples, we can not eliminate the possibility of Myo1α protein expression. Many factors may contribute to Myo1α’s absence in immunoblotting experiments. Perhaps the number of cochleas used per gel sample was too low. If Myo1α is expressed at a very low concentration in the cochlea, an increased number of cochleas may have to be used for each gel sample. As well, the preparation of the sample, while optimized for Myo1α preservation in gut samples, may not be appropriate for the cochlea. A nuance in sample preparation could result in Myo1α degradation and its inability to be detected by immunoblot.

In addition to the detection of Myo1α, we also hoped to identify other proteins that may act as the epitope responsible for the non-specific labeling in 3p1/4p1 immunocytochemistry experiments. In addition to Myo1α, we also routinely detected a smaller protein, migrating at 40 kDa (Fig 7B&C, blue asterisk). This protein was present in Myo1α+/+, Myo1α+/− and Myo1α−/− samples. However, because it is detected in gut as well as the cochlea, and we do not observe non-specific binding in the Myo1α−/− gut samples, this protein is not likely to be the 3p1/4p1 antigen observed in the cochlea. As well, an additional protein, which migrated slightly higher than Myo1α was detected in in Myo1α+/+, Myo1α+/− and Myo1α−/− cochlear samples (Fig 7B&C, black asterisk). This protein is the strongest candidate for the 3p1/4p1 antigen, however its detection varied across experiments. We did not observe any further candidates, although the same technical problems listed above may also apply. As well, because our sample preparation was biased for myosins, we may have eliminated the non-specific proteins from the sample preparation.

The future direction of this project will depend upon the optimization of immunoblot experiments. We will pursue this by increasing the number of cochleas per gel sample, as well as modifying the protein extraction method based on both myosin and non-myosin protocols. The detection of Myo1α protein in cochlear samples would prompt us to return to immunocytochemistry experiments to examine non-sensory cells.
of the cochlea for Myo1a expression. If we are able to reliably detect a candidate for the observed binding observed in hair cells with the immunocytochemistry experiments, we will continue our research with 3p1/4p1 immunoprecipitation experiments in an attempt to identify these unique hair-cell antigens.
A. Motor domain

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B. WT KO

C. HEX RT HEX RT

D. HEX OLIGO t-RT HEX OLIGO t-RT HEX OLIGO t-RT

E. KO cochlea

F. Amino Acid translation; Wild type Myo1a amino acids #175-185

GGVTN--------------------------YLLEK

predicted Amino Acid translation; Myo1a insertion, 750a, detected using RT-PCR

GGVTNCMPPLISLTNLHFCPFMPICSRWMHPNSLPDLEK
Figure 1. Myo1a mRNA expression in Myo1a+/+ and Myo1a−/− tissues. A) Blue diagram depicts basic Myo1a protein structure. The Myo1a introns (green line) and exons (green boxes) are shown. The novel start site for the predicted tail isoform of Myo1a, Myo1aTAIL, is shown in red. Intron 6, which is alternatively spliced into the mRNA transcripts, 750a and 750b, is shown in pink. PCR primers were designed to amplify regions denoted in black as well as the novel start site shown in red. B) The expression of Myo1a mRNA in Myo1a+/+ (WT) and Myo1a−/− (KO) gut tissue was assayed using primers that amplified exons 2-8 and 13-16 of the head domain as well as the novel start site of Myo1aTAIL transcript diagrammed in A. C) The expression of Myo1a mRNA in Myo1a+/+ (WT) cochlear tissue was assayed using the head and tail primer sets diagrammed in A. HEX: cDNA template was amplified using random hexamer primers. -RT: negative control, no reverse transcriptase was added to cDNA reaction. Oligo dT: cDNA template was amplified using oligo dT primers. The red asterisk denotes the cochlear variants described in D & E. D) The expression of Myo1a mRNA in Myo1a−/− cochlear samples. The expected products are marked with a red asterisk (*). The product amplified in Myo1aTAIL -RT lane is believed to be non-specific, and not the result of genomic DNA contamination. E) DNA sequence of products shown in C, lane: WT cochlea, head primers, HEX template. The lower band in C corresponds to WT Myo1a sequence, black. The top band in C corresponds to two variant sequences: 750a and 750b, green and red respectively. Underlined DNA bases in red sequence code for translational stops. Intron 6 sequence lies within the gray box. F) Amino-acid translation of the wild-type Myo1a sequence and the 750a sequence, black and green respectively.
Figure 2. Scanning electron micrograph of Myo1a mutant cochlea. Acutely dissected cochlea from Myo1a mutant mice, P7, were fixed, dehydrated, and imaged using a scanning electron microscope. A) Three rows of outer hair cells from a Myo1a++ mouse. B) Two rows of outer hair cells from Myo1a−− mouse. C) Outer hair cell from sample with irregularities in stereocilliar packing in a Myo1a−− mouse. Spaces are indicated with red arrows. D) Outer hair cell from FVB/NJ mouse strain. Scale bar in B also applies to A and is equal to 4.29 μm, in C is equal to 1.50 μm, and in D is equal to 1.20 μm.
Figure 3. The αBBI antibody interaction with cochlear hair cells. A) The αBBI antibody interacts with antigens at the tips of stereocilia and in the supporting cells of the cochlea. In all panels the αBBI antibody is labeled green and actin is labeled red. The inner hair cell row is marked with an asterisk (*). The three rows of hair cells to the right are outer hair cells. The row of supporting cells between the inner and outer hair cells are pillar cells, P, and the rows of supporting cells between outer hair cells are Dieter cells, D. B) The same image as shown in A, but αBBI (green) channel only. C) Magnified view of an inner hair cell. Stereociliary-tip labeling is less intense in the tallest row of stereocilia. D) Magnified view of an outer hair cell. Stereociliary-tip labeling is most intense in middle and short rows of stereocilia. Arrowheads point to stereocilia of the tallest row. E) Bullfrog saccular hair cells. No αBBI labeling is observed in the stereociliary tip (white arrow), but is present in surrounding supporting cells (yellow arrow). Scale bars in A also applies to B, in C also applies to D, and in E also applies to F. Scale bars in A and E are equal to 5 µm. Scale bar in C is equal to 2.5 µm.
Figure 4. The 4p1/3p1 antibody interaction with cochlear hair cells. In all data panels, the 4p1/3p1 antibody is labeled in green and actin is labeled in red. Numbers in bottom left corners are in µm and denote the distance from slice 1. A: 1-6) serial confocal sections of outer hair cell bundles labeled with the 4p1/3p1 antibody. No immunoreactivity is observed at the tips of stereocilia (1). Approximately 1/3rd down the length of the stereocilia, 4p1/3p1 labeling can be detected in all three rows of stereocilia (2). Labeling begins to fade in the shortest row of stereocilia at the cuticular plate (3). Labeling of the middle row of stereocilia also fades (4). Labeling remains in the rootlet of the tallest row of stereocilia several microns into the apical surface of the hair cell (5). Labeling fades and is undetectable approximately 2µm below the apical surface of the cell (6). B) Serial confocal sections of inner hair cell bundles. No 4p1/3p1 immunoreactivity is detected at the tips or in the shaft of stereocilia (1 & 2). The 4p1/3p1 antibody interacts with epitopes located in each inner hair cell stereocilia rootlet, extending into the cuticular plate (3 & 4). Scale bars in A6 and B4 are equal to 5 µm. C) Cartoon of the cochlear hair cells, depicting the focal plane for the first and third slices shown in both the three rows of OHC, depicted in panels A1-6 and the IHC, depicted in panels B1-4.
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**Figure 5.** 4p1 immunolabeling of Myo1a mutant mice in cochlear hair cells. Cochleas were acutely dissected from Myo1a mutant mouse littermates at P7. In all overlayed images (A & B, left and middle panels) actin-labeled phalloidin is shown in red and the 4p1 antibody is shown in green. A) Myo1a+/+ mouse. B) Myo1a−/− mouse. In the left panels of A & B the focal plane is at the tip of the hair bundle. In the middle and right panels of A & B, the focal plane is at the base of the hair bundle. Scale bars in A and B is 5 µm and applies to each panel of the respective figure.
Figure 6. 3p1 immunolabeling of murine vestibular hair cells. Acutely dissected wild-type mouse vestibular organs. A) Confocal image of the sagital plane of vestibular hair cells. Actin is labeled with phalloidin, shown in red, and 3p1 antibody is shown in green. B) Confocal image of the horizontal plane of vestibular hair cells. Yellow arrowhead points to the cuticular plate at the base of hair cells, white arrowhead points to the tip of a hair bundle. Scale bars shown in the right panels are equal to 5 µm.
Figure 7. 3p1/4p1 antibody immunoblots. A) DNA agarose gel with products of Myo1a mutant genotyping PCR reaction. Each reaction contains a forward primer, complimentary to the DNA sequence upstream of exon 1, and 2 reverse primers, one complementary to the DNA sequence in intron 3 and one complementary to the neomyosin cassestte, indicative of the knockout allele. WT, Myo1a+/- DNA, HET, Myo1a+/- DNA and KO, Myo1a-/- DNA. B & C) Gut and cochlear samples were ran on SDS page gels and immunoblotted using antibodies 3p1 and 4p1. Ladders, shown to left are in KD. Each lane as labeled: MV, microvilar positive control. WTg, wild-type gut sample. WTe, wild-type cochlear sample. HETg, Myo1a KO+/- mouse gut sample. HETe, Myo1a KO+/- mouse cochlea sample. KOg, Myo1a KO-/- mouse gut sample. KOe, Myo1a KO-/- mouse cochlea sample. Myo1a is denoted with a red asterisk. The 40 kDa antigen is denoted with a blue asterisk. The protein marked with the black asterisk is a potential candidate for the 3p1/4p1 antigen.
References


