Developmental Regulation of Fascin and Plastin Actin Crosslinkers in the Chicken Basilar Papilla

By

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Abstract

Actin crosslinkers are essential for the highly specified structure of hair cell stereocilia. Crosslinkers are known to influence the length, diameter and stiffness of actin-based cell protrusions, all qualities that are integral to stereocilia function. Using mass spectrometry, our lab has identified several abundant actin crosslinkers, including fascin-1, fascin-2, plastin-1, plastin-2 and plastin-3. The expression patterns and functions of these crosslinkers during hair cell development have been studied very little. Because of preliminary evidence that one of these crosslinkers, fascin-2, is developmentally regulated, I analyzed fascin-2 during stereocilia development in chicken basilar papilla. Fascin-2 mRNA and protein levels increase at each time point examined, with the largest increases during periods of stereocilia elongation. In mature bundles, fascin-2 is concentrated in the distal portion of the tallest stereocilia. At early stages of stereocilia development, fascin-2 is present in cell bodies and in stereocilia. As bundles mature, fascin-2 is exclusively localized to the distal regions of stereocilia. mRNA levels of the complement of fascin and plastin genes showed a complex developmental expression pattern: fascin-1 decreased, fascin-2 increased, plastin-1 increased, plastin-2 increased then decreased, and plastin-3 stayed constant. These findings suggest that different crosslinkers may be involved in distinct phases of stereocilia elongation and widening, as well as in the long-term structural integrity of the bundle.

Chapter 1. Introduction

Sound and balance are detected in the inner ear by deflections of the stereociliary bundles on mechanosensory hair cells. The actin-based stereocilia have very specific morphology that is partially defined by actin cross-linking proteins. Although there have been some studies of actin crosslinkers in stereocilia since their importance was proposed 30 years ago (Tilney et al., 1980), there has not been a comprehensive developmental analysis of crosslinkers in stereocilia formation and maintenance. The goal of this thesis was to examine actin crosslinker expression during stereocilia development in chicken basilar papilla. Temporal expression patterns along with protein immunolocalization patterns can help in defining distinct functional roles for individual actin crosslinkers. I present mRNA, protein quantitation and protein localization data for the abundant stereocilia crosslinker fascin-2, and survey mRNA expression of other crosslinkers during development.

This introductory chapter reviews hair cell function and morphology and the importance of actin crosslinkers in determining stereocilia shape. Furthermore, I present background on the crosslinkers we have identified in the stereocilia, including the experiments with fascin-2 that prompted these studies. Chapters 2 and 3 detail the methods and results from cloning, quantitative RT-PCR, immunoblots and immunolocalization of fascin-2 in chicken basilar papilla. Quantitative RT-PCR of five crosslinkers over development is also presented. Chapter 4 discusses several possible functions for fascin-2 during development and proposes some mechanisms that would produce the observed localization pattern. Chapter 5 summarizes the conclusions and discusses the implications of the research. The appendix includes the cloning and

qPCR primers, alignment scores for fascin genes, full amino acid sequences of fascin-1 and fascin-2, and peptide sequences for newly synthesized antibodies.

Stereocilia hair bundles of inner ear hair cells are the active element in mechanotransduction

Mechanosensory hair cells detect sound in the cochlea and acceleration due to gravity and head movements in the vestibular system. Hair cells convert displacements of their stereociliary bundles into electrochemical signals that are conveyed to the brain. Deflection of apical stereociliary bundles depolarizes hair cells, transducing a mechanical stimulus into an electrical one. The highly specified morphology of actin-based stereocilia is essential for hearing and vestibular function. Rows of stereocilia within each bundle are arranged in a staircase formation along the axis of maximal sensitivity. When stereocilia are deflected toward the tallest row by movements in the surrounding overlying structures, tip links connecting adjacent stereocilia are tensed, resulting in the opening of mechanically sensitive transduction channels. These channels allow the entry of potassium and calcium, and subsequent depolarization of the cell. The coupling of stimuli to depolarization relies on the precise structure of the stereociliary bundle. Knowledge of the molecular components of the stereocilia and their roles in stereocilia function is at the heart of understanding the basic mechanisms of hearing and mechanotransduction.

Actin crosslinkers, which bundle actin filaments into parallel fascicles, are an integral part of stereocilia structure and have known roles in determining actin fascicle length, diameter and stiffness. Our lab has recently discovered that several crosslinkers are abundant and highly enriched in stereocilia. It is intriguing that these organelles contain multiple proteins carrying out similar function. The studies presented here begin to address the distinct behavior of the most abundant actin crosslinkers in stereocilia.

Understanding the developmental, regional, and sub-cellular regulation of these proteins in stereocilia will contribute to our understanding of the development and maintenance of stereocilia structure and thus the abnormalities that lead to deafness and balance disorders.

Different types of hair cells require morphologically different stereociliary bundles

Although auditory and vestibular hair bundles in all vertebrates share basic morphological properties such as the directionally oriented stereocilia staircase, other aspects of morphology are specialized to the specific inner ear organ function. Based on its location, each hair bundle is unique in its stereocilia number, diameter and length. Differences have been noted between auditory and vestibular hair cells and between cells of different species. Bundle morphology varies even within organs.

Vestibular hair cells generally have long stereocilia, with cristae ampularis hair bundles reaching 40 μm. In contrast, cochlear hair cell bundles are as short as 1 μm in some species. Not only are bundle heights very different, but bundle shapes also vary widely. Most vestibular cell bundles are nearly conical; in contrast, mammalian inner hair cell bundles are slightly curved, and outer hair cell bundles in the same organ have "v" or "w" shapes (Nayak et al., 2007).

Species differences have also been noted. Mammalian cochlear hair cells differ from other vertebrate cochlear hair cells both in their arrangement and their bundle morphology. Bullfrog saccular hair cells have slightly wider stereocilia that have been useful in studies that require visualization of individual stereocilia. Even the packing of actin filaments within stereocilia differs between species (Tilney et al., 1980).

There can be substantial variation in bundle morphology even in a single hair cell type. Within the bird or mammalian cochlea, hair bundle length varies inversely with frequency, with short bundles responding best to high frequency tones. Thus

stereociliary bundle lengths vary in a regular pattern along the cochlea (Tilney & Saunders, 1983, Tilney & Tilney, 1988). Additionally, in the chicken cochlea (basilar papilla), bundles located near the superior edge have tall stereocilia whereas those near the inferior edge have short stereocilia. Stereocilia width also varies regularly along the basilar papilla, with wider stereocilia at the basal end; stereocilia width varies inversely with the stereocilia height in this organ. The number, diameter and height of stereocilia within each bundle is precisely determined, and this specificity is imperative for proper cell function (Tilney et al., 1992).

Because I wanted to study cells that were developmentally synchronized and make use of the chicken proteomics data in our lab, I did my studies in the chicken basilar papilla. Most of the proteomics data our lab generates is from utricle hair cells because their long bundles are relatively easy to isolate from cell bodies. Although the utricle is a useful model system, the cells turn over every few weeks (Warchol et al. 2001), so there are always cells at all stages of development. In contrast, auditory hair cells in mammals and birds develop together and survive for a lifetime unless damaged.

Stereocilia development in the chicken basilar papilla occurs in several distinct phases from embryonic day 7 to 21

During embryonic development, hair cells in the basilar papilla differentiate from surrounding support cells, sprout and elongate stereociliary bundles, and develop mechanosensory properties. The 10,500 hair cells of the basilar papilla undergo their terminal mitoses by embryonic day 8 (E8) (Katayama & Corwin, 1989) and begin to grow stereociliary bundles. The mature bundles contain from several dozen stereocilia in apical cells to several hundred stereocilia in basal cells. Developing bundles also have a single microtubule-based kinocilium. Orientation of the kinocilium, which is connected to the tallest stereocilia, helps to determine the shape and position of the bundle; the

kinocilium is absent from some mature auditory hair cells, but is important during development (Hirokawa, 1978; Montcouquiol et al., 2003).

Bundle development involves stereocilia elongation and thickening. This development is described in detail by Tilney and colleagues (Tilney et al., 1986). Elongation is achieved by the addition of actin monomers to the distal barbed ends of established actin fascicles. The two primary periods of stereocilia elongation occur from E10 to E12, when the staircase pattern develops, and from E16 to P2, when long apical hair bundles elongate to their mature lengths of up to 5.5 μm. In contrast, increases in stereocilia diameter occur between periods of elongation. Regular cross-bridging of actin filaments within each stereocilium occurs between E8 and E11 and results in stereocilia with predictable diameters (Tilney & DeRosier, 1986). Stereocilia diameter increases between E13 and E16, which coincides with an increase in the number of actin filaments in each stereocilium. Because actin crosslinkers play established roles in determining microvilli length and diameter, different crosslinkers may be important in these distinct phases of bundle development. The proposal that crosslinkers may play developmentally distinct roles is a major focus of this thesis.

Actin crosslinkers could influence stereocilia length, number, diameter and stiffness

Actin crosslinkers determine the shape of actin-rich protrusions in many cell types by bundling parallel actin filaments into fascicles. Cells utilize various crosslinkers, often in combination, to create specialized actin protrusions, including intestinal microvilli, *Drosophila* bristles, and hair cell stereocilia.

Our lab has identified hundreds of stereocilia proteins through mass spectrometry analysis of isolated stereocilia from chicken utricles (Shin et al., 2009; Shin et al., 2007). Several actin crosslinking proteins appear among the top twenty proteins

ranked for abundance and enrichment in the stereocilia, when compared to the whole utricular epithelium. Fascin-2 and plastin-1 (I-plastin, fimbrin) are the most abundant of these proteins. Espin, fascin-1, plastin-2 (L-plastin), plastin-3 (T-plastin) and Xin-related protein are also present in stereocilia preps, but in low abundance (**Fig. 1**). In a similar mass spectrometry analysis adapted for rat utricle, plastin-1 was the most abundant crosslinker in postnatal rat hair cells (unpublished). Of the crosslinkers we identified, only espin has been extensively studied in stereocilia, even though its abundance is minor compared to fascin-2 and plastin-1.

Plastin-1 was the first crosslinker localized to stereocilia and was long thought to be the predominant crosslinker because of its ability to bind actin filaments into stiff bundles (Bretscher, 1981; Drenkhahn et al., 1991). Despite its abundance in hair cells, no hearing or vestibular phenotype was reported in a *plastin-1* knockout. However, intestinal microvilli are shortened and lack rootlets in the absence of plastin-1 (Grimm-Gunter et al., 2009). Plastin-2 over-expression has no effect on microvilli in epithelial culture cells (Arpin et al., 1994) and it has not previously been identified in the ear, but like other crosslinkers, it stabilizes actin filaments (Tanoury et al., 2010). Plastin-3, which is expressed transiently in developing rat cochlea (Daudet & Lebart, 2002), increases microvilli length (Loomis et al., 2003) and diameter (Arpin et al., 1994) in epithelial culture cells. Espins are important low-abundance stereocilia crosslinkers that are not investigated in this thesis. They have a known role in microvilli and stereocilia length (Rzadzinska et al., 2005) and width (Loomis et al., 2003).

The fascin proteins bundle actin fascicles into stiff, straight bundles. Fascin-1 and fascin-2 have not previously been identified in hair cells but fascins have established roles in actin-based projections in many systems. Fascin-1 is a 55kD actin bundling protein that crosslinks actin filaments into unipolar, parallel bundles with 8 nm filament

spacing (Kureishy et al., 2002). Its actin binding affinity and bundling activity are greatly reduced by phosphorylation at Ser39 (Ono et al., 1997), which is within one of the two actin-binding domains (Yamakita et al., 1996). Over-expression of fascin-1 causes increased numbers of actin protrusions in CL4 epithelial cells in culture (Loomis et al., 2003). Fascin-2 was previously thought to be restricted to the retina (Lin-Jones & Burnside, 2007). Much of what is known about fascin-2 is inferred from its close homology with the more widely expressed fascin-1. Because of the abundance of fascin-2 and plastin-1, I chose to examine fascin-2 in depth and to evaluate the relative contribution of all of the fascin and plastin genes during stereocilia development.

Fascin-2 expression is developmentally regulated in the mouse and is essential for hair cell maintenance

Our lab, in conjunction with our collaborator Ken Johnson at The Jackson Laboratory, has found that fascin-2, the most abundant crosslinker in chicken utricle at E20, is essential for hair cell maintenance and fascin-2 is upregulated during hair cell development in mouse and chicken (Shin et al., unpublished). A single point mutation that leads to an amino acid substitution causes early onset progressive hearing loss in DBA/2J mice. Our lab has demonstrated that fascin-2 protein is localized to the upper portion of taller stereocilia in mouse cochlea, chicken utricle and frog sacculus. Additionally, *fascin-2* mRNA increases in whole mouse temporal bone between postnatal day 5 and postnatal day 10, but decreases later in development.

The discovery of fascin-2 as an important crosslinker in stereocilia and our preliminary results showing its developmental regulation prompted my studies of its mRNA and protein expression and localization over the period of stereocilia development. I found that fascin-2 transcription and translation are upregulated during hair cell development, and that fascin-2 is exclusive to the stereocilia in mature hair

cells. The complement of fascin and plastin actin crosslinkers had a complex pattern of upregulation and downregulation over development, indicating distinct developmental roles for these proteins. The differential localization of fascin-2 and plastin-1, the two must abundant crosslinkers at E20, indicates that they have distinct functional roles in mature hair bundles.

Chapter 2. Methods

Animals

Fertilized *Gallus gallus domesticus* (White Leghorn chicken) eggs from Featherland Farms (Coburn, OR) were kept at 100°F in humidified rocking incubators.

Fascin-1 and fascin-2 sequencing using degenerate primers and EST fragments

Over two thirds of the chicken *FSCN2* sequence was unknown. To clone chicken *FSCN2*, I amplified the coding region using 3’ primers complementary to the chicken *FSCN2* sequence (accessed from www.ensembl.org) and degenerate primers at the 5' end based on alignments of mouse, human, rat, bovine, and *Xenopus FSCN2*. A degenerate base was substituted wherever nucleotides differed between species. See the appendix for primer sequences. I cloned the cDNA into Invitrogen pENTR-SD-D, which contains M13 primer sites for sequencing and is Gateway-enabled for easy transfer into destination vectors. I also designed internal sequencing primers once I had successfully cloned *FSCN2* with the degenerate primer. A search for newly added chicken ESTs using the mouse sequence as a probe revealed ESTs that exactly matched my nearly complete sequence. I re-cloned the entire gene into pENTR with primers based on the 5' sequence of the EST and confirmed the sequence from the M13 primer sites. The majority of the *FSCN1* sequence was also absent from genomic databases. I pieced together a complete *fascin-1* sequence using chicken ESTs and I verified its sequence by cloning it from chicken temporal bone cDNA by the same methods.

Quantitative PCR of fascin-1, fascin-2, plastin-1, plastin-2 and plastin-3

Tissue preparation and RNA isolation: I dissected whole basilar papillae from chickens at embryonic day 7, 9, 12, 14, 16, and 21 in cold, oxygenated chicken saline (100 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 2 mM MgCl2*6H_2O, 3 mM D-glucose, 10 mM HEPES), spun down the tissue, added 500 μl Trizol and stored the samples at -20°C. For each age, triplicates were performed. Because the size of the basilar papilla increases about 10-fold over development, each triplicate contained between 6 and 30 basilar papillae, based on age. I homogenized the tissue in Trizol and proceeded with the Life Technologies total RNA isolation protocol. I resuspended the RNA in 10 μl DEPC water and verified RNA concentration and quality. With the iScript kit and protocol, I used 0.5 μg of RNA for each sample in a 20 μl reaction. I diluted the cDNA 1:100.

Primer design and qPCR reaction: I designed primers using the Primer3 online program at http://frodo.wi.mit.edu/primer3/. Most primers were designed to span multiple exons so that contaminating genomic DNA would not be amplified. Two sets of primers were designed for each gene to account for primer specific effects. The qPCR primers are listed in the appendix. For the SYBR Green qPCR reactions, I used 200nM of each primer and 2 μl of diluted cDNA in 20 μl reactions. qPCR reactions were performed in triplicate and only directly compared within each plate. I ran 46 qPCR cycles and selected fluorescence thresholds within the linear range of the amplification curves.

qPCR data analysis: I determined the relative abundance of each gene compared to GAPDH by calculating 2^[Ct(GAPDH)-Ct(gene of interest)]. I first averaged the triplicates for each sample, then averaged the results from the two primer pairs for

each gene, and finally averaged these results for the three separate cDNA preps. I presented the averages and standard errors of the means of the three separate runs.

Peptide antibody production

Peptides from the chicken fascin-2 and plastin-1 sequences were based on predictions at http://bio.dfci.harvard.edu/Tools/antigenic.pl, minimizing hydrophobicity and minimizing sequence similarity with other related proteins. A cysteine residue was added on either the N or C terminal and no internal cysteines were included. Peptides were produced and used to inoculate rabbits by Genemed Synthesis. I tested antibodies for immunohistochemistry and immunoblot on chicken utricle and selected the antibodies that had the most consistent staining and least background.

Immunoblot

I performed the dissections as described above and then froze the tissue at -20°C. After a storage period of up to one week, I homogenized the tissue in BCA sample buffer (50 mM Tris pH 6.7, 1% SDS) and assayed the protein concentration using the Pierce BCA (bisinchoninic acid) assay. I diluted each sample into sample buffer for final concentrations of 10% glycerol, 10 mM EDTA, 0.008% Bromophenol Blue, 100 mM DTT, 62.5 mM Tris pH 6.7, 2% SDS. I ran 20 mg of each age and a dilution series of the E21 sample as a standard in a 10% SDS PAGE gel; I also loaded SeeBlue Plus2 pre-stained standard (Invitrogen) in MOPS buffer.

I transferred the proteins to an Immobilon P-PDVF membrane in cold transfer buffer (10 mM CAPS, 5% MeOH) at 100 V for 90 min and then let it incubate overnight. I rinsed the membrane in water twice to remove any residual SDS and stained the total proteins with India Ink in PBSt (PBS plus 0.3% tween) for 1 hr. The membrane was blocked in 2% block in PBSt for 1 hr and incubated with the primary antibody [rabbit anti-

ggFSCN2 peptide #36 (0.6 mg/ml) or goat anti-hmGAPDH peptide (Chemicon)] diluted 1:1000 in blocking solution for 2-3 hrs. For some experiments, the blot was simultaneously labeled with the GAPDH and FSCN2 primary antibodies. I then washed the membrane 3 X 5 min in PBSt. I incubated the membrane with the secondary antibody (either goat-anti-rabbit-HRP or donkey-anti-goat-HRP) for 1 hr in blocking solution and then washed 7 X 5 min in PBSt. I developed the blot in SuperSignal West Pico solution.

Before re-probing with additional antibodies, I stripped the blot for 30 min in blot stripping solution (50mM Tris pH 7.5, 150 mM NaCl, 2%SDS, 100mM 2-ME) at 50°C, rinsed it three times in PBSt, rewet it with methanol, rinsed it three times in PBS and proceeded with blocking as before.

Immunohistochemistry

I dissected basilar papillae and utricles, exposing the sensory epithelium, in chicken saline and fixed the tissue in 4% formaldehyde for 20 min at room temperature. I incubated the tissue in blocking solution (3% normal goat serum, 2% bovine serum albumin, 0.2% saponin in PBS) for one hour at room temperature. I incubated the tissue with primary antibodies diluted in blocking solution with no saponin overnight at 4°C. I used rabbit anti-ggFSCN2 peptide antibody #36 (0.6 mg/ml) at 1:100 for the developmental basilar papilla series. I used rabbit anti-ggFSCN2 peptide antibody #38 and anti-PLS1 mouse monoclonal 3G10 (1 mg/ml, Abnova) both at 1:100 for utricle triple labeling experiments. Phalloidin Alexa-488 (6.6 M) and secondary antibodies Alexa-568 (1 mg/ml) and Alexa-633 (6.6 M) were used at 1:200 for one hour. I washed the tissue 3 X 5 min in PBS between primary and secondary antibody incubation and 6 X 5 minutes after secondary antibody incubation. I mounted the utricles and basilar papillae in

Vectashield (Vector) on glass slides with 0.12 mm Secure-Seal Imaging Spacers (Schleicher & Schuell) and #1.5 cover slips.

During mounting, I folded utricles to allow for imaging of the stereociliary bundles in profile. Using forceps, I folded each utricle in half so that the hair cells faced out and I pinched the outer edges of the utricle together. I positioned coverslips on top of the folded utricles so that weight of the coverslip held the folded utricle in place. With this procedure, I could image a few rows of hair cells that extended from the folded edge at the center of the utricle.

Fascin-2/plastin-1 immunofluorescence analysis

My analysis included 14 bundles from three E21 utricles. I excluded any bundle that was not fully captured within the stack, one that had no plastin-1, one tiny bundle with no PLS1 or FSCN2, and one that lacked a clear cuticular plate to align. To measure the intensity, I first did maximum projections in ImageJ. Then, using ImageJ, I plotted the PLS1, FSCN2 and actin profiles along a region of interest that included about half the width of the cell and spanned the cell body and bundle. I aligned the actin peaks associated with the cuticular plate and used data from that point to the tip of the bundle. Because the actin profiles aligned very well for all the bundles, I did not interpolate to make all the bundles the same length. To find relative fluorescence, I divided the PLS1 or FSCN2 intensity by the actin intensity. I then normalized the actin to its peak fluorescence for each bundle, and did the same for PLS1/actin and FSCN2/actin. I calculated the averages and standard errors along the bundle for actin, PLS1/actin and FSCN2/actin. I plotted the averages +/- SEM.

Chapter 3. Results

Chicken fascin-1 and fascin-2 are highly homologous to other fascin genes

When our lab identified fascin-1 and fascin-2 in a proteomics study of isolated hair bundles, only about 30% of each sequence was available in the Ensembl database. In order to improve the quality of the mass spectrometry analysis by searching with complete protein sequences and to facilitate further study of these genes, I cloned and sequenced chicken *fascin-1* and *fascin-2* genes from chicken temporal bone cDNA using a combination of degenerate primers and EST database searches. I completed the sequencing of the 5' portions and submitted the complete cDNA sequences to NCBI BankIt (*fascin-1* GU952756; *fascin-2* GU907099).

Chicken fascin genes share substantial sequence similarity with homologous fascin-1, 2, and 3 genes in human, mouse, zebrafish, and *Xenopus* (**Fig. 2**). Chicken fascin-2 shares 70% amino acid sequence identity with human fascin-2 and mouse fascin-2. Chicken fascin-1 is 82% identical to human and mouse fascin-1. Additional alignment scores are listed in the Appendix. Fascin-3 genes are the most evolutionarily divergent, but all of the fascin genes in these species are highly conserved throughout the length of the gene, indicating that much of their function is conserved. Notably, the regulatory serine at position 39 (S43 in fascin-3) is conserved in all fascin sequences examined. Phosphorylation at this site reduces actin binding-affinity for human fascin-1 and *Xenopus* fascin-2 (Lin-Jones & Burnside, 2007). Additionally, arginine 109, which is mutated to a histidine in DBA/2J mice, is conserved in all sequences.

Fascin-2 mRNA and protein levels in the basilar papilla increase during development

To determine the transcriptional and translational components of fascin-2 regulation, I isolated fascin-2 mRNA and protein from whole basilar papilla during the period of stereocilia elongation (**Fig. 3a-b**). Quantitative RT-PCR of basilar papilla cDNA revealed that fascin 2 mRNA increased in whole basilar papilla at each time point between E7 and E21 (**Fig. 3c**). The total fascin 2 mRNA level increased about six fold over the course of stereocilia formation and elongation, when normalized to GAPDH mRNA.

Using the full-length fascin-2 protein sequence, we designed chicken fascin-2 peptide antibodies to allow us to probe for fascin-2 in immunoblots and by immunohistochemistry. The antibodies bound to 55kDa proteins in western blot as expected, and we observed the same unique subcellular localization pattern with four polyclonal antibodies produced against different peptides. The developmental increase of fascin-2 mRNA and protein levels and the concurrent decrease in fascin-1 mRNA indicates that the fascin-2 antibodies do not detect fascin-1.

By immunoblot of basilar papilla total protein, protein levels increased about 10 fold from E7 to E21 when normalized to a dilution series of E21 basilar papilla total protein (**Fig. 3d-e**). Fascin-2 increased most between E12 and E14 and between E16 and E21. During the two days between E12 and E14, the fascin-2 protein level nearly doubled, and it more than doubled again between E16 and E21. These ages correspond to periods of stereocilia elongation.

Fascin-2 is transiently expressed in cell bodies and subsequently only toward the tips of stereociliary bundles

From E12, the earliest age examined by immunohistochemistry, fascin-2 is expressed specifically in hair cells and becomes concentrated in the stereocilia tips of taller rows (**Fig. 4**). Fascin-2 is excluded from the base of the stereocilia (**Fig. 4, Fig. 5**). Specific staining is absent from supporting cells and cells outside the sensory epithelium, indicating that hair cells account for the vast majority of fascin-2 in whole basilar papilla preparations. Although the brightest staining is in the stereocilia tips even at E12, at younger ages there is also substantial fascin-2 staining in the hair cell soma. This pattern is especially pronounced in cells near the basal end of the basilar papilla. Hair cells at the basal end have shorter bundles than do apical cells. Cell body staining is still obvious in cells near the base through E21, whereas it is absent from apical cells by E14. Consistent with chicken utricle and mouse cochlea (Shin et al., unpublished), staining within the stereociliary bundles is concentrated in taller stereocilia and excluded from the base of the bundle.

Plastin-1 is enriched in the middle and excluded from the base of mature stereocilia

Fascin-2 and plastin-1 antibodies and phalloidin were used to triple label E20 chicken utricular hair cells. Fascin-2 was enriched at the top and plastin-1 was enriched in the middle of stereociliary bundles (**Fig. 5**). Both fascin-2 and plastin-1 were completely absent from some smaller bundles, which were probably immature bundles (data not shown). In 14 bundles in three utricles, fascin-2 fluorescence relative to actin was highest near the tips, whereas plastin-1 fluorescence relative to actin was highest midway along the bundle (**Fig. 5b**). This plastin-1 localization pattern was consistent with that seen with our peptide antibodies (data not shown). However, the contrast of

fascin-2 and plastin-1 localization is most apparent in images that exploited rabbit anti-fascin-2 and mouse anti-plastin-1 co-labeling.

The relative abundance of important crosslinkers fluctuates greatly during stereocilia development

There were substantial developmental changes in the relative abundance of various actin crosslinkers in the basilar papilla by quantitative PCR of fascin-1, fascin-2, plastin-1, plastin-2 and plastin-3 mRNA (**Fig. 6**). Fascin-2 mRNA increased steadily through the period of stereocilia formation and elongation, a finding that is supported by qPCR experiments by Jung-Bum Shin in mouse cochlea and immunohistochemistry in mouse and chicken cochlea (Shin et al., unpublished). Fascin-1, in contrast, was by far the most abundant crosslinker early in stereocilia development at E7, but decreased steadily through embryonic development. The downregulation of fascin-1 over development is consistent with the qPCR experiments done in mouse. This reciprocal expression pattern of fascin-2 and fascin-1 could indicate that fascin-2 replaces fascin-1 in the bundle.

The three plastin crosslinkers identified in the utricle mass spectrometry analysis also had a complex pattern of expression during this time period. Plastin-3 was the most abundant mRNA and remained constant during stereocilia development; its abundance is surprising because it only accounts for a small fraction of the plastin protein in E20 utricle stereocilia. Plastin-3 is expressed transiently during development in rat cochlea stereocilia (Daudet et al. 2002). Plastin-1 increased with development, suggesting an important role in mature bundles. Plastin-1 has long been thought of as the dominant crosslinker in mature stereocilia and is upregulated during rat cochlea stereocilia development (Zine et al., 1995). Intriguingly, plastin-2 expression, which prior to our mass spectrometry had never been identified in the ear, increased nearly ten fold

between E7 and E12, but then decreased between E12 and E21. Thus, plastin-2 may be important for stereocilia development but not for mature bundle function.

Chapter 4. Discussion

Fascin-2 is important for tall stereocilia at later stages of bundle development

Fascin-2 is the most abundant actin crosslinker in chicken utricle at E20 and the second most abundant in mouse utricle at P4-6 (Shin et al., unpublished). I found that fascin-2 mRNA and protein increase during stereocilia development and that fascin-2 is enriched in the distal parts of tall stereocilia. My findings, along with the early onset hearing loss in fascin-2 mutant mice (Shin et al., unpublished), indicate that fascin-2 is essential for the maintenance of tall stereocilia. There are several characteristics of fascin-2 structure and regulation that could contribute to its function in stereocilia.

Fascin-2 may promote stereocilia elongation

Although both fascin-2 mRNA and protein levels increased at every point examined during stereocilia growth, the most substantial increases in fascin-2 protein in whole basilar papilla occurred when the stereocilia are undergoing rapid elongation. Because imaging of fascin-2-antibody-labeled basilar papilla demonstrated that fascin-2 is restricted to hair cells, the increases in mRNA and protein are attributable to hair cells, not other cells in the basilar papilla prep. During the two distinct phases from stereocilia elongation from E12 to E14 and again from E16 to E21, fascin-2 protein levels surged.

The stereocilia crosslinker espin causes elongation in stereocilia, so fascin-2 may play a similar role in directing elongation. It could be that fascin-2 alone is enough to achieve elongation. Fascin-2 over-expression causes lengthening of filopodia and stereocilia in tissue culture cells and zebrafish hair cells (McDermott et al.; personal communication with Dr. Peter Gillespie). However, unlike espin over-expression, fascin-1 over-expression in CL4 cells does not produce long microvilli (Loomis et al., 2003).

This could be due to a functional difference between fascin-1 and fascin-2 or a difference between zebrafish and mammalian CL4 cells.

Alternatively, fascin-2 could facilitate espin-mediated elongation. Perhaps fascin-2 incorporation is necessary for espin binding at the tips of stereocilia to direct elongation. For example, fascin-2 may set up the correct actin filament spacing for espin to bind. Conversely, fascin-2 incorporation could depend on espin binding at the tips, which could explain the increase in fascin-2 when espin-mediated elongation would be most active.

Fascin-2 may incorporate most readily into newly polymerized regions of filamentous actin. At least in young animals, the actin core of each stereocilium treadmills, with monomers incorporating at the apical ends of actin fascicles and dissociating at the base. Fascin-2 preferential incorporation into rapidly growing filaments would account for its increase during actin filament elongation and for its predominance in taller stereocilia, which treadmill faster than short stereocilia in the same bundle (Rzadzinska et al., 2004). Fascin is a low-affinity crosslinker with a fast off-rate (Aratyn, 2007). If it incorporates at the tips as new monomers are added but dissociates quickly, it would be restricted to the distal ends of stereocilia. In the fastest treadmilling actin fascicles, fascin-2 incorporated at the tips will travel further along the length of the stereocilia before dissociating. We observed fascin-2 enrichment in the tallest stereocilia and at the distal ends of short stereocilia.

Fascin-2 may strengthen long stereocilia

Fascin-2 might play a structural role in stiffening the exposed ends of the longest stereocilia. Immunolocalization in chicken basilar papilla, chicken utricle, *Xenopus* sacculus and mouse cochlea all show that fascin-2 protein is enriched in the distal region of the stereocilia and in the longest rows of stereocilia (Shin et al., unpublished).

Stereocilia move as rigid levers when they are deflected (Tilney et al., 1980), allowing deflections to be directly transferred to the tip links. The distal regions are the most vulnerable to stress during bundle deflection because they are less stabilized by neighboring stereocilia and, in some organs, are embedded directly into overlying membranes that produce shearing forces on the bundle. These regions of stereociliary bundles need to be exceptionally strong to prevent deformation.

Both fascin and plastin family proteins can produce stiff bundles because they have relatively short crosslinking distances. Short crosslinking distance is correlated with increased bundle stiffness because of tighter packing of actin filaments. Fascin-1 binds actin filaments with a spacing of about 8-9 nm (Ishikawa et al., 2003) and plastin-1 binds actin filaments with a spacing of about 12 nm (Volkemon et al., 2001). The even tighter packing afforded by fascin crosslinking may be important for stiffening the distal regions of long stereocilia. The ratio of the stereocilia width at the tip compared to its widest point approximates the ratio of the actin bundling distances of fascin and plastin. The enrichment of plastin-1 in the middle and fascin-2 at tips of hair bundles is therefore a reasonable explanation for the previously reported narrowing of bullfrog sacculus hair cells from 550 nm near the base to about 300-400 nm at the tips (García et al., 1998).

The incorporation of fascin-2 in stereocilia could coincide with stereocilia elongation because elongating stereocilia may require fascin-2 to maintain structural integrity. Fascin-2 stiffens established actin bundles late in development in other systems. In the *Drosophila* bristle, the crosslinker protein forked is thought to help form actin bundles. Subsequent fascin incorporation stiffens and straightens bristles (Tilney et al., 1998); without fascin, these bristles are more flexible (Tilney et al., 2005). The rapid dissociation rate of fascin-2 could be a disadvantage in creating sturdy bundles, but even with continuous exchange, fascin is able to stiffen bundles during rapid deflections (Aratyn et al., 2007). Fascin-2 spacing and dynamics would increase bundle

stiffness and strength during deflection but allow exchange for other crosslinkers as the actin filaments treadmill.

Further studies of the effects of blocking or over-expressing fascin-2 on hair bundle stiffness and comparative studies of morphologically distinct hair cell types would help elucidate the role of fascin in stiffening stereocilia.

Several mechanisms could promote Fascin 2 incorporation at stereocilia tips

The localization of fascin-2 at the distal ends of stereocilia might be regulated in a number of ways. The two components to achieving the observed fascin-2 pattern are transport of fascin-2 to the ends of stereocilia and restriction of fascin-2 binding at the base of the stereocilia. Several mechanisms can account for this specific localization. These include trafficking by myosin motors, fascin-2 actin-binding affinity regulation by phosphorylation, and interference by other actin binding proteins. Although translational and transcriptional upregulation seem to be important during the developmental increases in fascin-2 within bundles, regulation of fascin-2 activity is essential for proper fascin-2 localization. In fact, it is likely that after initial stereocilia formation, most regulation of fascin-2 localization and incorporation into bundles is post-translational. In other cell types, fascin-1 in actin bundles cycles quickly from a soluble pool within the filopodia rather than requiring fascin-1 newly transported from the cell body (Aratyn, 2007); how this soluble pool is regulated determines where and how much fascin incorporates into actin fascicles.

Myosin motors, a number of which are already implicated in the specific transport and anchoring of stereocilia proteins, may regulate the soluble pool of fascin-2. For example, the actin crosslinker espin is trafficked to stereocilia tips by myosin-IIIa (Salles et al., 2009). Myosin-IIIb, which we identified in stereocilia by mass spectrometry (Shin et al., unpublished), is a candidate for specific trafficking of fascin-2. Myosin-IIIb is

localized to the distal ends of stereocilia, similar to fascin-2 (Peter Gillespie, unpublished data). Myosin-IIIa over-expression and myosin IIIb over-expression are both implicated in longer stereocilia (Salles et al., 2009; Merritt et al., 2009). Myosin-IIIb is able to transport espin and partially compensate for mutant myosin-IIIa (Merritt et al., 2009). Their functions may be partially overlapping, but differential expression and localization patterns indicate that they are specific to distinct cargos.

Fascin-2 actin-binding and actin-bundling are diminished by phosphorylation at serine 39 (Lin-Jones & Burnside, 2007), so regulation by phosphorylation may also help determine fascin-2 localization. Because the phosphorylation site is contained in one of two fascin actin-binding sites, it reduces actin binding and actin crosslinking. A kinase that is active toward the base of stereocilia could reduce the actin-binding affinity of fascin-2, preventing it from forming stable crosslinks in these areas. Even if soluble fascin-2 is present in these regions, if it is phosphorylated, its binding will not be able to compete with other crosslinkers such as plastin-1 that are also present in the bundle and have higher actin-binding affinity. The effect of phosphorylation on fascin-2 localization could be tested by over-expressing fascin-2 with the phosphorylation site mutated to either prevent phosphorylation or mimic phosphorylation. Phosphorylation may also be a component of myosin regulation. Both myosin-IIIa and myosin-IIIb have functional kinase domains. If myosin-IIIb does interact with fascin-2, it could phosphorylate fascin-2 to prevent binding to actin during transport to the stereocilia tips. The ability of myosin-IIIa and myosin-IIIb to phosphorylate fascin-2 could be tested in vitro.

Fascin actin-binding is prevented by direct interference at its binding site. The interference of other high affinity crosslinkers could prevent fascin-2 binding, restricting it to regions devoid of competing crosslinkers, namely the newly formed ends of actin filaments. Other actin binding proteins can interfere with fascin binding (Yamashiro-Matsamuro & Matsamuro, 1986). Tropomyosin, for example, might play a role in

preventing fascin-2 binding in the cuticular plate of mature cells because its expression is restricted to that region (Drenkhahn et al., 1991). It is likely that interference of high affinity actin-binding proteins along with specific transportation and regulation of fascin-2 work in conjunction to establish the specific localization pattern.

Fascin-1 and fascin-2 may have distinct functions or simply distinct temporal expression

Most of what is known about fascin-2 function is inferred from fascin-1 function. The abundance of fascin-1 mRNA early in stereocilia development and its subsequent downregulation during the period in which fascin-2 is upregulated is therefore surprising. This reciprocal expression pattern indicates that they may not be as similar as previously thought.

Endogenous fascin-1 does not fully compensate for fascin-2 in fascin-2 mutant mice. Fascin-2 mutant mice develop well but have early onset hearing loss and hair bundle degeneration (Shin et al., unpublished). In these mice, fascin-2 is correctly localized to the distal regions of stereocilia, so it may retain some of its function. It may be able to bind to actin and be correctly localized within the stereocilia but have reduced ability to crosslink and support the bundles. It could be that fascin-1 compensates for fascin-2 early but the decrease in fascin-1 expression over development limits complete compensation in mature cells. If fascin-1 is able to compensate for fascin-2, driving expression of fascin-1 in mature DBA/2J mice would prevent hair cell degeneration.

One explanation for the switch from fascin-1 to fascin-2 expression could be simply that they are regulated together with other genes that are important for each phase of development but that their function is actually redundant. Fascin-1 is closely genetically linked to beta-actin and fascin-2 is closely linked to gamma-actin, so it is possible that the expression patterns are driven by actin gene regulation. Gamma-actin

has a similar expression pattern to fascin-2 and a gamma-actin mutation causes deafness with a similar onset pattern to the DBA/2J mutant mice (Belyantseva et al., 2009).

Bundle crosslinker composition changes dramatically during bundle development

The relative abundances of actin crosslinkers expressed in the basilar papilla fluctuates during stereocilia development. Fascin-2 expression increases concurrently with stereocilia growth, but the changing complement of crosslinkers cannot be explained simply by a direct upregulation of crosslinkers in response to growing filaments. The actin core grows in diameter by the addition of new actin filaments and in length by the polymerization of new monomers. Additionally, actin fascicles treadmill rapidly by the addition of monomers at the distal end and the de-polymerization at the taper region. In fact, actin treadmilling in hair cells might be a mechanism for allowing the bundle to reorganize and turn over its population of crosslinkers during each phase of development.

Specific crosslinkers must be necessary for specific steps in development. Between E14 and E16 there is a minimal increase in fascin-2 protein, yet individual stereocilia incorporate more actin fascicles as they increase in diameter. During this period, other crosslinkers may be preferentially incorporated into the growing stereocilia. For example, plastin-2 might be particularly important for the addition of new actin filaments because its expression increases during the early phases of stereocilia development and subsequently decreases. Each of these actin crosslinkers must be studied in depth beginning with immunolocalization over development to begin to assess their functional contributions to hair bundles.

Crosslinker composition varies by species and organ

Fascin-2 was by far the most abundant crosslinking protein identified by mass spectrometry of chicken utricle stereocilia at E20, but plastin-1 was more abundant in a similar experiment using early postnatal rat stereocilia (Shin et al., unpublished) and *fascin-1* mRNA is the most abundant transcript in E20 chicken utricle. Because of the differences in developmental state of hair cells in each of these organs, it is difficult to make predictions based on these initial comparisons. Our findings, in addition to other known differences between vestibular and auditory hair cells and between different species, call for systematic comparative investigations of different hair cell samples. Mass spectrometry, the technique our lab has already applied to chicken utricle stereocilia, allows for the identification of the entire consortium of proteins in a given sample. Running comparative analyses of samples from vestibular and auditory stereocilia from different species at a range of developmental stages will help drive predictions about the applicability of findings in each system. Proteins that are different between organs or at different times during development are good candidates for further study.

Chapter 5. Conclusions

In this thesis, I developed several tools that will be useful for researchers studying actin crosslinkers in multiple systems. I cloned and sequenced chicken fascin-1 and chicken fascin-2 and developed antibodies for fascin-2. Peptide antibodies for fascin-1 are under production but were not ready in time for these studies. Through the combined approach of mass spectrometry and quantitative RT-PCR, our lab has begun a comprehensive comparative study of functionally related groups of stereocilia proteins. By looking at the entire set of proteins expressed at different ages and in different organs, and then following up on specific families of proteins, we can begin to understand the essential components of all hair cells.

I found that multiple actin crosslinkers are expressed during the period of stereocilia development. Their distinct expression patterns indicate that they are each important for different phases in development or in mature bundles. It is likely that their unique properties, such as binding affinity, crosslinking distance, and posttranslational regulation, make them suitable for crosslinking actin in different regions of bundles or at different times during stereocilia growth. Fascin-2, which is necessary for long-term maintenance of stereocilia, increases in expression during stereocilia growth. Because of its localization to the tips of tall stereocilia and its known functions in other cell types, fascin-2 presumably stiffens the longest, most exposed portions of tall stereocilia of mature bundles. Crosslinker temporal and sub-cellular localization presented here command further studies addressing the function and regulation of fascin-2 and the other stereocilia crosslinkers.

Initially, complementary studies to determine the developmental and sub-cellular localization of all of the known crosslinkers should be completed. This analysis should

include additional time points at E18 and during postnatal development. Crosslinker levels fluctuate throughout embryonic development, and it would be good to know what the final mature hair bundle composition is. As fascin-2 localization suggests a role in stiffening tall stereocilia, the localization of each crosslinker will drive predictions about its specific function. These studies require specific antibodies, which are under development.

Manipulations of crosslinkers will elucidate their functional roles. The roles of each type of crosslinker in stereocilia can be assessed by knocking down, over-expressing or expressing mutant crosslinkers. Both fascin and plastin are regulated by phosphorylation, and plastin is regulated by calcium (Ono et al., 1997; Lin et al., 1994); disrupting regulation could change the distribution of crosslinkers within hair bundles. Additionally, if the crosslinkers interact with myosin motors, disrupting these interactions could also change crosslinker localization.

Changes in stereocilia composition could also affect the length, diameter and stiffness of individual stereocilia. Measuring the morphological effects of changes in crosslinker composition requires high definition imaging. Increasing the incorporation of shorter crosslinkers should increase bundle stiffness, and increasing the incorporation of longer crosslinkers should decrease bundle stiffness. Bundle stiffness can be determined by measuring the force required to displace a bundle.

Expressing crosslinkers at inappropriate times during development could cause increased stereocilia elongation or widening outside of the normal developmental periods. Because of the complex developmental expression patterns documented here, functional studies will need to take into account the developmental stage of the hair bundles and the complex interactions of the array of crosslinkers present. Variation between hair cell type and species are also important considerations for future experiments.

Figures

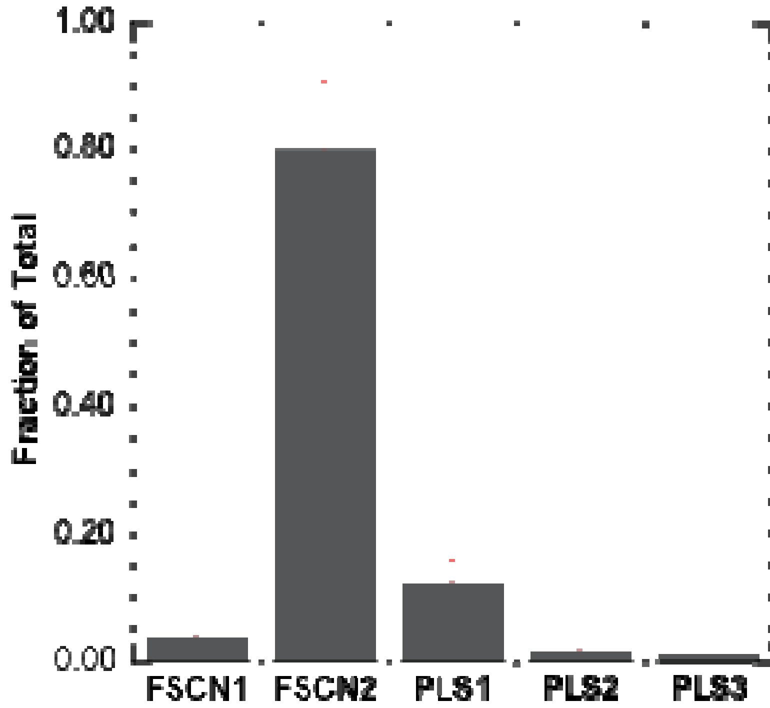


Figure 1. Fascin-1, fascin-2, plastin-1, plastin-2 and plastin-3 are expressed in stereocilia in the chicken utricle. Mass spectrometry analysis of E20 chicken utricle stereocilia preparation. Intensity factor was used to determine relative abundance of fascin-1, fascin-2 and total plastin (plastin-1, plastin-2 and plastin-3 combined). Relative abundance of plastin isoforms (which share common peptides) is based on the number of unique peptides identified normalized to protein size.

a

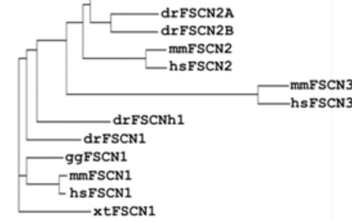


Figure 2. Fascin sequences are highly conserved across species. a. Multiple protein sequence alignments. Black shading labels residues that are identical to the consensus sequence residue and gray shading labels residues that are similar to the consensus sequence residue. Serine 39, which is a regulatory phosphorylation site, is highlighted in yellow. Arginine 109, which is mutated to a histidine in DBA/2J mice, is highlighted in red. Gene abbreviations: ggFSCN2 (*Gallus gallus* fascin-2), xtFSCN2 (*Xenopus tropicalis* fascin-2), drFSCN2A (*Danio rerio* fascin-2A), drFSCN2B (*Danio rerio* fascin-2B), mmFSCN2 (*Mus musculus* fascin-2), hsFSCN2 (*Homo sapiens* fascin-2), mmFSCN1 (*Mus musculus* fascin-1), hsFSCN1 (*Homo sapiens* fascin-1), ggFSCN1 (*Gallus gallus* fascin-1), xtFSCN1 (*Xenopus tropicalis* fascin-1), drFSCN1 (*Danio rerio* fascin-1), drFSCNh1 (*Danio rerio* fascin-homolog-1), mmFSCN3 (*Mus musculus* fascin-3), hsFSCN3 (*Homo sapiens* fascin-3), **b.** Phylogenetic tree of Fascin genes. Branch lengths are proportional to the amount of evolutionary distance between related genes.

Figure 3. Fascin 2 mRNA and protein increases during stereocilia formation and elongation. a-b. Depiction of stereociliary bundle development adapted by Peter Gillespie from Tilney, 1992. **c.** Quantitative RT-PCR showing *fascin-2* mRNA elevation between embryonic day 7 (E7) and embryonic day 21 (E21) in whole basilar papilla.

Average +/- SEM of three separate samples run with two different primer sets are plotted. Each sample had between 6 and 30 basilar papillae. **d.** Immunoblot detection of fascin-2 in whole basilar papilla with fascin-2 antibody #36. Between 10 and 30 basilar papillae were pooled for each sample. The same blot was also probed for GAPDH as a loading control. Each lane from E7 to E21 contains 20μg of total protein. Dilutions of the E21 protein sample were loaded on the right side for quantification. **e.** Fascin-2 protein quantification based on immunoblots of two separate samples. Pixel intensity of each band was measured in ImageJ and normalized to GAPDH. Fascin-2 protein was quantified relative to the dilution series of the E21 sample. The average +/- the range for the two samples is plotted.

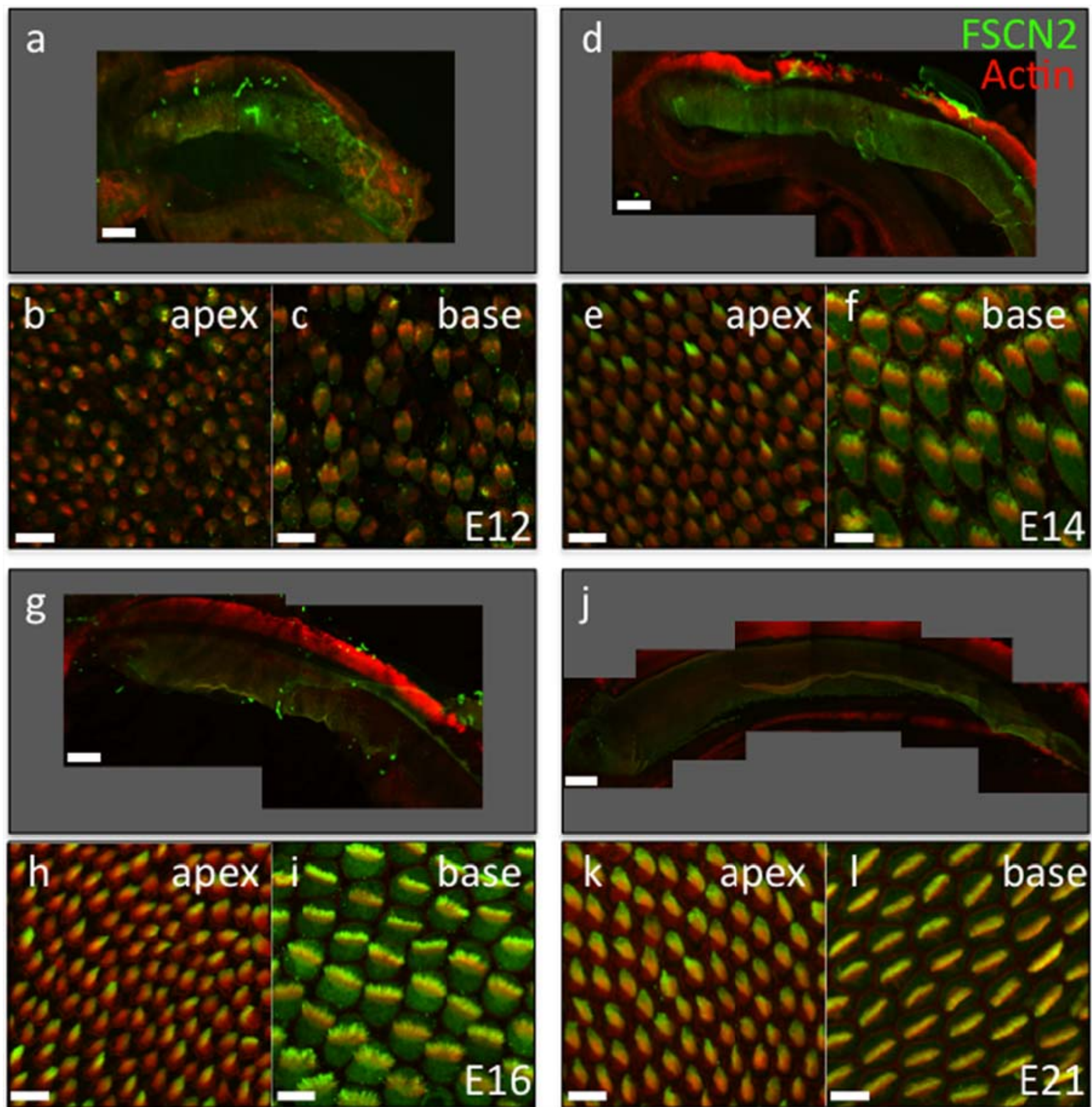


Figure 4. Fascin 2 is present in the cell bodies and stereocilia of immature hair cells and is restricted to the distal region of stereocilia. Basilar papillae were labeled with fascin-2 in green and actin in red. Images are maximum projections of z-stacks taken at 0.5 μm intervals. **a, d, g, j.** Low magnification images of the entire basilar papilla at each age show fascin-2 staining in hair cells. The low magnification image of the E21 basilar papilla was taken with a 20X objective and the others were taken with a 10X objective, but they are all presented at the same scale. Scales bars

are 200 μm. **b, e, h, k.** High magnification images taken near the apex. Scale bars are 10 μm. **c, f, i, l.** High magnification images taken near the base. Scale bars are 10 μm. **a-c.** E12. **d-f.** E14. **g-i.** E16. **j-l.** E21.

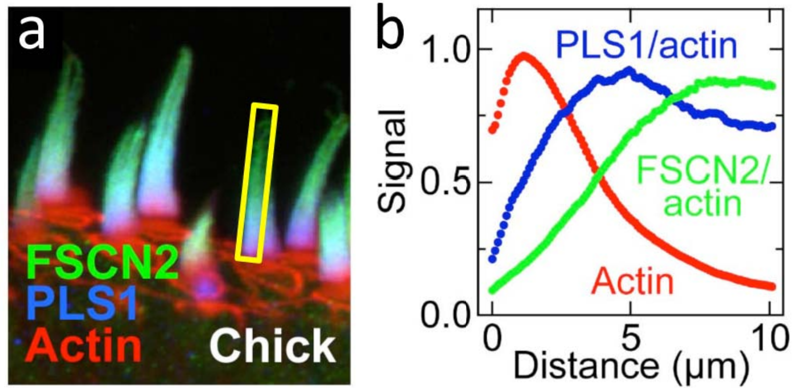


Figure 5. Fascin-2 is enriched at the distal ends of stereociliary bundles and plastin-1 is enriched in the middle of bundles. a. Maximum projection of a folded E21 chicken utricle imaged in profile shows fascin-2 concentrated at distal ends of bundles and plastin-1 concentrated lower down. Both crosslinkers are absent from the base of the bundle. **b.** Quantitation of fluorescence intensity for actin, fascin-2/actin and plastin-1/actin from the base to the tip of the bundle. Profiles of 14 cells from 3 utricles were averaged. The yellow box in (a) shows a typical region chosen for analysis.

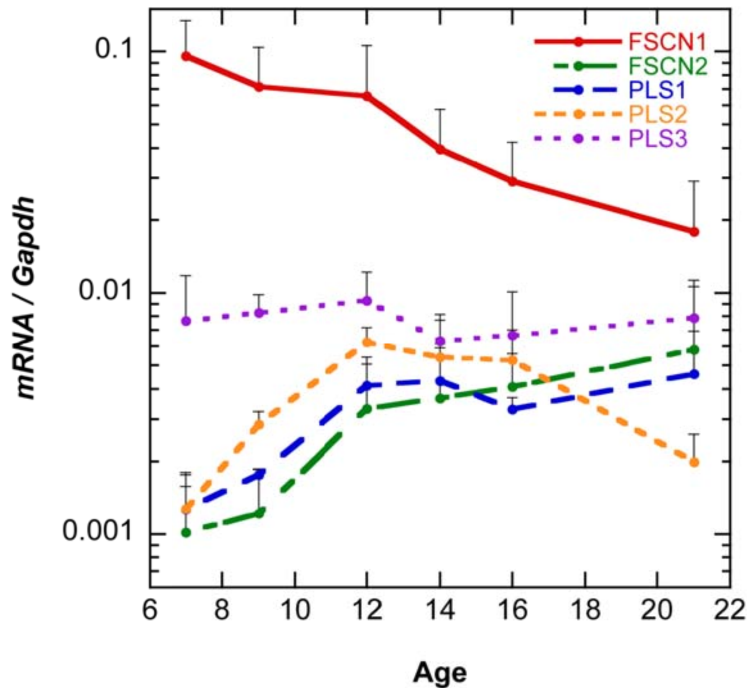
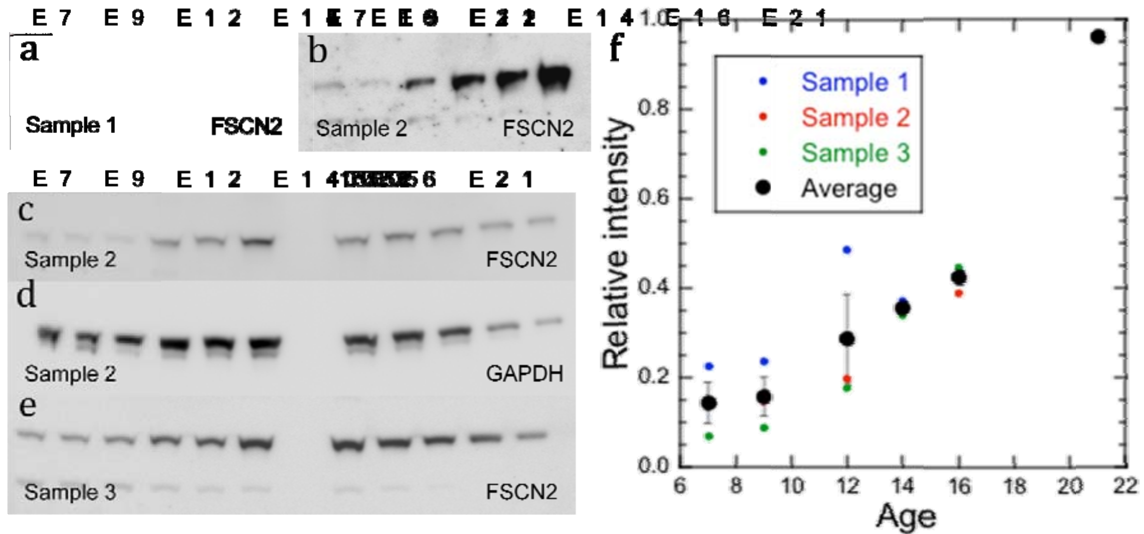


Figure 6. Fascin and plastin crosslinkers have a complex expression pattern over stereocilia development. Quantitative RT-PCR of fascin-1, fascin-2, plastin-1, plastin-2 and plastin-3 in whole basilar papilla from embryonic day 7 (E7) to embryonic day 21 (E21). Note that fascin-1 is very abundant and decreases over development, while fascin-2 and plastin-1 expression increase over development. Plastin-2 expression increases and then decreases and plastin-3 expression remains relatively constant. Three separate preps using between 6 and 30 basilar papillae each were averaged for each sample.



Supplemental Figure 1. Immunoblot analysis including three separate experiments, which includes an outlier at age E12. Similar to the immunoblot data presented in figure 3d and 3e, but including an additional run that was performed with no loading control. The point at E12 for sample 1 is an extreme outlier, which is probably due to a loading error. Because of this, sample 1 was excluded from figure 3. Excluding the aberrant point at E12, sample 1 follows the same expression pattern as samples 2 and 3. All immunoblots were probed with fascin-2 antibody #36. **a.** Developmental series sample 1. **b.** Developmental series sample 2. **c.** Sample 2 run with dilutions of E21 sample for relative quantitation. **d.** The same blot as (c) probed with GAPDH antibody as a loading control. **e.** Developmental series sample 3 run with dilutions of E21 sample for relative quantitation. **f.** Fascin-2 protein quantification based on immunoblots of three separate samples. Pixel intensity of each band was measured in ImageJ and normalized to GAPDH except for Sample 1, which was not probed for GAPDH. Fascin-2 protein was quantified relative to the dilution series of the E21 sample. The average +/- the SEM for the two samples is plotted in addition to the results for each sample.

Appendix

Primers used for cloning and sequencing *Gallus gallus* fascin 1 and fascin 2 from whole temporal bone cDNA

Gene	Primer name	Sequence
FSCN1	ggFSCN1fornoATG	CACCGAGACGGAGCACTTTG
	ggFSCN1revstop	TTAATATTCCCAGAGGGTGGAG
FSCN2	Fascin2 Deg For2	CAGRTKCTGAARATMCAGTTYGGC
	ggFSCN2completeForNoATG	CACCCCAACGAATGGGATCC
	ggFSCN2revstop	TCAGTACTCCCAGAGCGTGGC

Fascin-1 nucleotide sequence

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>ggFSCN1
ATGACGGCGAACGGAACGGCGGAGCCGGTGCAGATCCAGTTCGGGCTGATCAACTGCGGCAACAA
GTACCTGACGGCGGAGGCGTTCGGCTTCAAGGTGAACGCGTCGGCCGCCAGCATGAAGAAGAAGC
AGATCTGGACGTTGGAGCAGGACGGGGAGGATTCCAGCGCGGTGCTGCTCAAGAGCCACCTGGGC
CGCTACCTGGCGGCCGACAAAGACGGCCGCGTGAGCTGCGACAGCGAGGAGCCGGGCCCCGACTG
CCGCTTCCTGGTGGTGGCCCACGGCGACGGGCGGTGGTCGCTGCAGTCCGAGCCGCACCGCCGCT
TCTTCGGCGGCACCGAGGACCGCCTGTCGTGCTTCGCCCCGTCCGTGTCCCCCGCCGAGAAGTGG
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CCTGGGGCCGCGCCGCGACGAGCTGGCCGTGGACCGCGACGTGCCGTGGGGCGTGGACGCGCTCA
TCACGCTGCTCTTCGTGGAGCAGCGCTACAGCCTGCAGAGCTGCGACCACCGCCTGCTGCGGGCC
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GTGCTGCGCGCTGGGAACGACCGGAACGTGTCCACCCGGCAAGGGATGGACCTGTCGGCCAACCA
GGACGAGGAGGGCGACCAGGAGACCTACCAGCTGGAGATCAACAAGGACACCAAGAAATGTGCCT
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CAACGGCAAATACGTGACGGCAAAGAAGAACGGGCAGCTGGCAGCCTCCATGGAGACAGCAGGTG
AGACGGAGCACTTTGTGATGAAGCTGATCAACAGACCCATCATCGTGCTGCGTGGAGAGCACGGC
TTCATCGGCTGCCGGAAGGTGACCGGCACCTTGGACTCCAACCGCTCCTCCTACGACGTCTTCCA
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ATCCGCCGACGCCATCGACGCCTCCACCCTCTGGGAATATTAA
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Fascin-2 nucleotide sequence

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GCGCTTCAGCATCATCACGCAGTCGGACGGGCGCTGGGCGCTGCAGTCGGCGCCGCACCGGCGCT
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TGCACACCAACGCTGGCAGCTACTGGACCCTGGTGGCCCACGGGGGCATCCAGGCTGTGGCCACC
GAAGTTGCTGCCAACACCATGTTTGACATCGAGTGGCGCGGGCGGCGCGTGGCCCTGCGTGCCAG
CAACGGCCGCTATGTGTGCACCAAGAGGAACGGGCAGCTGGCGGCCGTCAGCGACGCCGTGGGGG
AGGACGAGGAGTTCACCCTGAAGCTGATCAACCGGCCCATGCTGGTGCTGCGGGGCGAGCACGGC
TTCGTCTGCTACCACCGCGGCTCCAACCTGCTCGACTCCAACCGCTCCGTCTACGACGTCTTCCA
CGTGGGCTTCAGCGACGGCGCCTACCAAATCCGAGGTCAGGGGGGAAAGTTCTGGTACGTGGCGA
GCAGCGGGGCGGTCTGCAGCGACGGGGACCTCTCCGAGGATTTCTTCTTTGAGTTCCGGGAGCGC
GGGCGCGTCGCCATCAAAGGGAAGAACGGGCGGTACCTGCGTGGGGACCCGGCCGGCACCCTGCG
GGCCGACAGCGAGTCGGTGCTGCGGGCCACGCTCTGGGAGTACTGA

Accession numbers for sequences used in the alignment

Gene	Reference
ggFSCN2	GU907099
xtFSCN2	gi|154147674|ref|NP_001093724.1
drFSCN2A	gi|184186129|ref|NP_001116988.1
drFSCN2B	gi|175363512|gb|ACB72459.1
mmFSCN2	gi|266456824|ref|NP_766390.2
hsFSCN2	gi|6912626|ref|NP_036550.1
mmFSCN1	gi|113680348|ref|NP_032010.2
hsFSCN1	gi|4507115|ref|NP_003079.1
ggFSCN1	GU952756
xtFSCN1	gi|45360983|ref|NP_989128.1
drFSCN1	gi|115494998|ref|NP_001070028.1
drFSCNh1	gi|122890525|emb|CAM13035.1
mmFSCN3	gi|31982710|ref|NP_062515.2
hsFSCN3	gi|9966791|ref|NP_065102.1

ggFSCN2 amino acid sequence alignment scores

xtFSCN2	77
hsFSCN2	70
mmFSCN2	70
drFSCN2B	70
drFSCN2A	68
hsFSCN1	61
ggFSCN1	60
mmFSCN1	59
drFSCN1	59
drFSCNh1	56
xtFSCN1	54
hsFSCN3	28
mmFSCN3	28

Primers used for quantitative PCR analysis

Gene	Forward	Reverse	Exon span
FSCN1	GACTCCAACCGCTCCTCCTACGAC	AAAAAGAAGTCCACGGGCGTATCG	4–5

	GTCCACCGCCTCCACAAAGAAC	TCCGTCTCACCTGCTGTCTCCAT	1-2
FSCN2	GTGGGGGAGGACGAGGAGTTCAC	GTAGACGGAGCGGTTGGAGTCG	4
	CAGCCAACGGCAGATTCGTCTC	CTGGAAGGTCTCGTGGTTCAGC	1-2
PLS1	GTTGCTCGAAAAATTGGTGCTCGT	CCTCTTCCCATCAAACATGCAAACA	16
	TGAAATGACTCGTGTGCCAGTTGAC	CCAGCAATACCGACCAGGGAGAAT	12-13
PLS2	TTGGTGGTGGAGAGAAGGTGAACG	ATGCTGGTGCTGATTTTGCCATC	14-15
	TCGTCACAGCCACTGATGTTGTCC	TCCTCTCTTCCCTTGTCTCACCTTC	9-10
PLS3	ATTCTCCCTGGTTGGCATTGGA	ATGTCGTCGTTGGCTTTCTGACC	13-14
	CAGTGAGGGAACGCAGCACTCATA	CAGGGTCATTTTCCAGGCTTTGT	3-4

Peptides for FSCN2 antibodies, highlighted in the ggFSCN2 sequence

34035 [C]QDEADSSVVFLKSH
34036 [C]ADSELVLRATLWEY
34037 [C]YTLEFKAGKLAFKD
34038 [C]GKNGRYLRGDPAGT

>ggFSCN2
MPTNGIHQVLKIQFGLINCESRYLTAESFGYKVNASAPSLKRKQIWTLEQDEADSSVVFLKSHLG
RYLGADKDGQVRCEAEQPGRDERFSIITQSDGRWALQSAPHRRFFGGREDRLSCFAPSITEGELW
TVHLAMHPQANLLSVSRRRYAHLSAHEDEIATDSNLPWGVDALITLCFQDKKYSLRTADERYLRC
DGILVPEPGARTGYTLEFKAGKLAFKDCDGKYLAPTGPTGTLKSGRSSKPGKDELFDLEESHPQV
VFTAANGRFVSIRQGVNVSANQDEELNHETFQLQIDRDTKKCSLHTNAGSYWTLVAHGGIQAVAT
EVAANTMFDIEWRGRRVALRASNGRYVCTKRNGQLAAVSDAVGEDEEFTLKLINRPMLVLRGEHG
FVCYHRGSNLLDSNRSVYDVFHVGFSDGAYQIRGQGGKFWYVASSGAVCSDGDLSEDFFFEFRER
GRVAIKGKNGRYLRGDPAGTLRADSELVLRATLWEY

Peptides for FSCN1 antibodies, highlighted in the ggFSCN1 sequence

34441 [C]AVDRDVPWGVDALIT
34442 CRKVTGTLDSNRSSYD
34443 [C]YLKGDHAGVLKAS

>ggFSCN1
MTANGTAEPVQIQFGLINCGNKYLTAEAFGFKVNASAASMKKKQIWTLEQDGEDSSAVLLKSHLG
RYLAADKDGRVSCDSEEPGPDCRFLVVAHGDGRWSLQSEPHRRFFGGTEDRLSCFAPSVSPAEKW
SVHLAMHPQANLYSLARKRYAHLGPRRDELAVDRDVPWGVDALITLLFVEQRYSLQSCDHRLLRA
DGRLVPSAEPGTAFTLEFRCGKVAFRDGEGRYLAPSGPSGTLKAGKSSKVGKDELFALEQSCPQV
VLRAGNDRNVSTRQGMDLSANQDEEGDQETYQLEINKDTKKCAFRTYTGKYWTLTSNGGIQSTAS
TKNASCYFDIEWCDKRITLRAANGKYVTAKKNGQLAASMETAGETEHFVMKLINRPIIVLRGEHG
FIGCRKVTGTLDSNRSSYDVFQLEFNDGAYNIKDTTGKYWMVGSESSVTSSSDTPVDFFFEFCDY
NKVAIKINGKYLKGDHAGVLKASADAIDASTLWEY

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