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Review

Espins and the actin cytoskeleton of hair cell stereocilia and sensory cell microvilli

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Abstract. The espins are novel actin-bundling proteins that are produced in multiple isoforms from a single gene. They are present at high concentration in the parallel actin bundle of hair cell stereocilia and are the target of deafness mutations in mice and humans. Espins are also enriched in the microvilli of taste receptor cells, solitary chemoreceptor cells, vomeronasal sensory neurons and Merkel cells, suggesting that espins play important roles in the microvillar projections of vertebrate sensory cells. Espins are potent actin-bundling proteins that are not inhibited by Ca^{2+}. In cells, they efficiently elongate parallel actin bundles and, thereby, help determine the steady-state length of microvilli and stereocilia. Espins bind actin monomer via their WH2 domain and can assemble actin bundles in cells. Certain espin isoforms can also bind phosphatidylinositol 4,5-bisphosphate, profilins or SH3 proteins. These biological activities distinguish espins from other actin-bundling proteins and may make them well-suited to sensory cells.

Keywords. Espin, actin, hair cell, stereocilia, microvilli, sensory, deafness, taste.

The stereocilia and microvilli of vertebrate sensory cells and their actin-bundling proteins

Many classes of vertebrate sensory cells detect chemical or mechanical stimuli through microvilli or their derivatives, such as stereocilia. These relatively long-lived, fingerlike specializations of the plasma membrane are built around a common cytoskeletal element – the parallel actin bundle (PAB) [1]. PABs consist of tightly packed collections of actin filaments cross-linked by actin-bundling proteins. The filaments are aligned along their longitudinal axis and display a uniform polarity with respect to their preferred ends for actin monomer addition, which in microvilli and stereocilia is positioned at the distal tip. The PAB displays hallmarks of a supramolecular scaffold that determines the placement, dimensions, flexibility and signaling properties of microvilli and stereocilia. Although filopodia also contain a PAB at their core and are believed to sense the local environment, they are considerably more dynamic and differ significantly from microvilli and stereocilia in their molecular composition and biogenesis [2].

Mirroring the findings of landmark studies of PAB-containing structures in *Drosophila* [3], it is increasingly evident that microvilli and stereocilia owe many characteristics to the actin-bundling proteins that cross-link their PAB. Microvilli and stereocilia contain multiple actin-bundling proteins in different combinations. To the extent examined, these different actin-bundling proteins accumulate at different times during PAB assembly or maturation, and mutation of different actin-bundling proteins has distinct phenotypic consequences. Three major

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classes of actin-bundling protein have been detected in the microvilli or stereocilia of vertebrate cells: villin, fimbrins/plastins and espins [1]. Last to be identified among these actin-bundling proteins, espins are concentrated in the PAB-containing projections of a variety of chemosensory and mechanosensory cells, including hair cell stereocilia [4–7]. Espins differ significantly from villin and fimbrins/plastins in their interactions with actin and other ligands. The espins come in multiple isoforms that are expressed in a cell type- or developmental stage-specific fashion and are capable of affecting the actin cytoskeleton and sensory transduction in a variety of ways [4, 6]. The discovery that a mutation in the espin gene causes hereditary deafness and vestibular dysfunction accompanied by stereociliary shortening in the jerker mouse [7] revealed that espins play critical roles in stereocilium length regulation and integrity. This connection has been strengthened with the identification of two recessive and four dominant mutant alleles of the espin gene associated with deafness in humans [8, 9]. What is it, precisely, that espins do for the PABs of hair cell stereocilia, and to what extent do these roles extend to the actin-based microvillus-type specializations of other sensory cells?

Introduction to espin structure and activities

The prototype espin was first identified as a ~110-kDa actin filament-binding protein in the PABs of the junctional plaque of the Sertoli cell ectoplasmic specialization [10, 11]. It is from this peculiar and neglected intercellular junction that espins get their name: espin = ectoplasmic specialization + -in. Shortly thereafter, a smaller, ~30-kDa, espin isoform was identified as a relatively minor, albeit high-affinity, actin-bundling protein in the PABs of brush border microvilli in intestine and renal proximal tubule [12]. It is now evident that espins constitute a family of novel actin-bundling proteins (Fig. 1A). Encoded by a single gene, espins are produced in multiple isoforms that differ markedly in size and in their complement of ligand-binding sites [4, 6] (Fig. 1A). Different transcriptional start sites give rise to four major espin isoform size classes, which range from ~110 to ~25 kDa, and are designated 1–4, in order of decreasing size; splice variants are further specified alphabetically [4] (Fig. 1A). Although present in multiple actin-rich structures, espins are especially prevalent in the PABs of hair cell stereocilia and the microvilli of other sensory cells, where they appear to be distributed uniformly along the PAB at the core [4, 5, 7].

All known espin isoforms contain a 116-amino acid C-terminal actin-bundling module (ABM) (Fig. 1A), which is necessary and sufficient for the potent actin-bundling [11, 12] and microvillar PAB elongating [13] activities of the espins. All espin isoforms also contain a Wiskott-Aldrich Syndrome protein homology 2 (WH2) domain (Fig. 1A) that binds actin monomer and is required for espin-mediated actin bundle formation [4, 13, 14]. The major differences between espin isoforms is in their N-terminal peptides, which differ primarily because of differential transcription start-site selection [4] (Fig. 1A). Depending on isoform, the espin N terminus can include

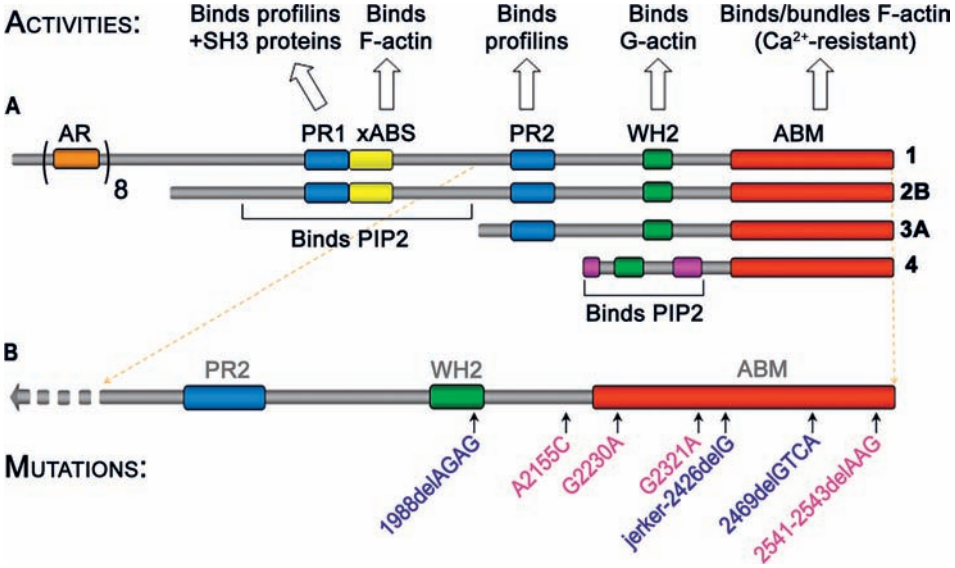

Figure 1. Espins: isoforms, structure-activity relationships and deafness mutations. (*A*) Stick-Figure diagram of representative examples of the four size-classes of espin isoform (1, 2B, 3A and 4) highlighting their structures, domains and activities. ABM, actin-bundling module; AR, ankyrin-like repeat; PIP2, phosphatidylinositol 4,5-bisphosphate; PR, proline-rich peptide; SH3, Src homology 3; WH2, WASP homology 2 domain; xABS, additional F-actin-binding site; magenta domains, peptides encoded by exons that are unique to espin 4. (*B*) Expanded stick-Figure diagram of an espin C-terminal peptide fragment noting positions of the designated, naturally occurring mutations associated with recessive (blue) or dominant (red) deafness.

eight ankyrin-like repeats, an additional binding site for F-actin, a binding site for phosphatidylinositol 4,5-bisphosphate (PIP2) and one or more proline-rich peptides, which can bind the profilins or certain SH3 domains [4, 15] (Fig. 1A). Notably, these upstream motifs or binding sites can bind ligands that have been implicated in membrane-cytoskeletal interaction, actin cytoskeletal regulation or signal transduction. In most instances, the natural ligands for these upstream binding sites remain to be identified. Nevertheless, the fact that different cell types express distinct espin isoforms or combinations of isoforms [4, 10–12] and appear to do so in complex spatiotemporal patterns during development [6] argues forcefully that the differences between espin isoforms are biologically meaningful.

Obvious espin orthologs are present in vertebrates from pufferfish to human, but have not been detected in the sequenced genomes of prokaryotes, yeast or nematodes. Espins show no obvious amino acid sequence similarity to other actin-binding proteins, with the exception of the forked proteins, which have been implicated in the formation of PABs in the developing neurosensory bristles of *Drosophila* pupae [3]. The sequence similarity is limited to 66-amino acid peptide in the espin ABM, which shows 39% identity to a peptide in the forked proteins, and to the ankyrin-like repeats found in espin 1 (Fig. 1A) and the largest forked isoforms [1].

Espins in hair cell stereocilia

Shortly after their discovery, espins were detected at high levels in hair cell stereocilia in the cochlea and vestibular system [7] (Fig. 2A–C). Hair cells are mechanosensory cells that detect sound or motion. In fact, it is the espin-containing, oriented, staircase-like collection of cross-linked stereocilia atop of each hair cell – the stereociliary bundle (or 'hair bundle') (Fig. 2A) – that makes hair cells mechanosensitive to sound, motion or gravity [16]. Briefly, deflection of the stereociliary bundle in the direction of the tallest stereocilia increases the gating of ion channels in the stereociliary plasma membrane. The resulting influx of cations, such as K^+ and Ca^{2+}, elicits downstream events that leads to neurotransmitter release

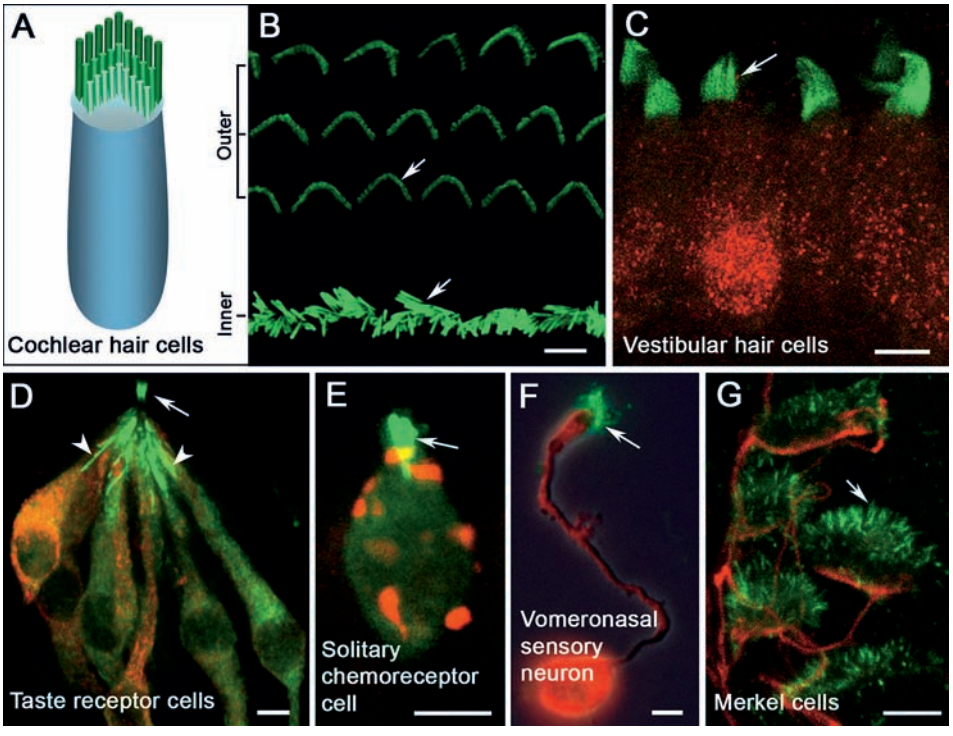

Figure 2. Espins in hair cell stereocilia and the microvilli of other sensory cells. (*A*) Diagram of cochlear hair cell, showing espin (green) in the staircase-like collection of stereocilia in the stereociliary bundle. (*B–G*) Immunofluorescence localization of espin (green; arrows or arrowheads). (*B*) Stereocilia of inner and outer hair cells viewed from above in cochlear surface preparation. (*C*) Stereocilia of vestibular hair cells in vestibular macula (double-labeled with antibody to calretinin to highlight the hair cell cytoplasm; red). (*D*) Microvilli and extended PAB rootlets of type II taste receptor cells (arrowheads) and microvilli of type III taste receptor cells (arrows) in a taste bud in lingual cirvumvallate papilla (double-labeled with antibody to α-gustducin, which stains the cytoplasm of a subset of taste receptor cells; red). Note espin-deficient zone between the two populations of labeled microvilli. (*E*) Microvilli of a solitary chemoreceptor cell in the convex face of vomeronasal organ (double-labeled with antibody to class III β-tubulin to highlight associated nerve fibers; red). (*F*) Dendritic microvilli of an isolated vomeronasal sensory neuron (double-labeled with antibody to calretinin to highlight the cell body and dendrite; red). (*G*) Microvilli of Merkel cells in hard palate (double-labeled with antibody to class III β-tubulin to highlight associated nerve fibers; red). Bars, 5 µm.

from the hair cell base at synapses with afferent nerve fibers. In the mammalian cochlea, deflections of the stereociliary bundle result from sound-induced deformations of the basilar membrane on which the hair cells rest. In the vestibular system, deflections are initiated when a gelatinous mass on top of the stereocilia is displaced via linear or angular acceleration of the head.

Stereocilia can be thought of as highly specialized microvilli. They are made in part through a programmed elongation and thickening of microvillus-like precursors, changes that mirror alterations in the dimensions of the PAB scaffold at their core [16–18]. Although derived from microvilli, stereocilia differ from microvilli in key respects. For example, most stereocilia are longer than microvilli and contain more actin filaments. In addition, stereocilia and their PABs taper near their base; only a central core of actin filaments extends a rootlet into the apical cytoplasm of the hair cell, where it is embedded in a dense meshwork of actin filaments termed the cuticular plate [16, 18]. The formation of the stereociliary bundle from microvillus-like precursors is a complex process involving emergence and initial elongation, thickening, further elongation to make a staircase and resorption of excess [16–18]. In the chicken cochlea, these phases of assembly are separated in developmental time, and the process is largely completed around the time of hatching. In the rodent cochlea, the phases are more difficult to resolve, and the process extends into the first 2 weeks of postnatal development. Remarkably, stereocilia show an apparently stable and regularly graded increase in length from row to row within the stereociliary bundle, even though their PAB appears to undergo actin treadmilling, at least in transfected hair cells in cultured cochlear explants [19]. To signal properly, the stereociliary staircase on each hair cell must adopt a specific orientation relative to anatomical landmarks within the cochlea or vestibular system, a feat accomplished in part through the planar cell polarity pathway [20, 21]. The overall length of the stereociliary bundle, commonly scored by measuring the length of the tallest stereocilia within a bundle, varies in a reproducible way according to hair cell type or position. For example, the length of the tallest stereocilia increases ~3-fold from base to apex along the cochlear spiral in rodents (or along the proximal-distal axis in the chicken) [13, 17, 18, 22, 23], and inner hair cells have longer stereocilia than outer hair cells (Fig. 2B), except at the extreme base [13, 22, 23]. There can also be regular, place-specific variations in the slope of the stereociliary bundle [24]. A detailed accounting of stereocilum actin filament number and length in the chicken cochlea reached the startling conclusion that each cochlear hair cell invests roughly the same amount of actin into filaments [18].

Espins are associated with stereociliary PABs throughout their formation [6, 25]. In fact, the level of espin protein accumulation in hair cells and its extent of compartmentalization to stereocilia increase dramatically throughout stereociliogenesis [6, 25]. In rats, this overall increase in espin protein level has been shown to involve a complicated spatiotemporal pattern of expression of different espin isoforms, suggesting that specific espin isoforms function preferentially in certain hair cell types or during discrete phases of stereociliary bundle formation [6]. For example, the espin 1 isoform, which in the inner ear of adult rats appears confined to the stereocilia of vestibular hair cells [4], accumulates in the inner ear during late embryonic and early postnatal development coincident with the elongation and maturation of stereocilia on vestibular hair cells [6]. In contrast, the espin 4 isoforms accumulate in rat inner ear between postnatal days 6 and 10 [6]. This relatively late timing implicates espin 4 isoforms in the postnatal maturation of stereocilia on cochlear hair cells. This could include the differential elongation that establishes the increasing gradient in stereocilium length from base to apex along the cochlear spiral [6] or other aspects of postnatal maturation, such as staircase assembly or the remodeling of links between stereocilia [26]. In addition to espins, hair cell stereocilia contain isoforms of the fimbrin/plastin class of actin-bundling protein [18, 27].

Jerker deafness mutation in the mouse espin gene

At roughly the same time that espins were localized to hair cell stereocilia, the mouse espin gene (*Espn*) was mapped to the distal end of mouse chromosome 4 by in situ hybridization [7]. This was the same general region of chromosome 4 to which a naturally occurring autosomal recessive deafness mutation – the jerker mutation – had been ascribed through classical linkage analysis. First described in an article in 1941, the story of the jerker mutation has its origins in a ‘dancing’ mouse held in the collection of a mouse fancier [28]. Mice that are homozygous for the jerker mutation (jerker mice) exhibit peculiar head-jerking movements and, as is true for many mouse deafness mutations, rapid circling. Early histological analyses of jerker mice revealed a loss of hair cells in the cochlea and a defect in the tectorial membrane [29], the flap of fibrillar extracellular matrix positioned above cochlear hair cells. Assessments of hearing physiology showed that jerker mice were totally deaf from the first time point such measurements could be carried out [30, 31]. Electron microscopic studies in the early 1990s revealed a pronounced shortening and degeneration of stereocilia in the cochlear hair cells of jerker mice, defects that became pronounced near the onset of hearing around postnatal day 10 [32, 33]. Occasional defects, such as protrusion and folding of the cuticular plate, were also noted in the cochlear hair cells of newborn jerker mice. The stereocilia of newborn jerker mice may also be significantly shorter and thinner at birth [34]. Degeneration

involving disintegration of the cuticular plate, stereociliary disarray and fusion and hair cell expulsion was noted for the vestibular hair cells of jerker mice, but these changes happened later than in the cochlea, and hair cells in the cristae ampullares were largely spared [35]. Phenotypically, mice that are hetereozygous for the jerker mutation (jerker heterozygotes) appear relatively normal. Although aged jerker heterozygotes have been reported to show stereociliary and hair cell degeneration [32, 33], it is presently unclear whether these defects are related to the jerker mutation or are associated with age-related hearing loss, which is evident in a number of inbred mouse strains [36]. Unfortunately, the reports of cochlear defects in aged jerker heterozygotes [32, 33] used mice of a different background strain (CBA) as wild-type controls, and to our knowledge wild-type mice of the standard inbred jerker mouse strain (JE/LeJ, stock number 000249) obtained from the Jackson Laboratory (Bar Harbor, Maine, USA) have not yet been tested for age-related hearing loss.

Through Western blotting and DNA sequencing of RT- and genomic PCR fragments, the jerker mutation was traced to the mouse espin gene [7] (Fig. 1B). The jerker mutation (*Espn*^{je}) is the deletion of a G residue from an exon that encodes part of the espin ABM (2426delG, in the nucleotide numbering scheme for mouse espin 1) and is, therefore, expected to affect all known espin isoforms. Deletion of this G residue causes a frameshift that affects roughly the C-terminal half of the espin ABM, replacing the final 63 amino acids with a novel 39-amino acid peptide (the jerker peptide) that is rich in positively charged amino acids, especially arginine, but also serine [7, 14]. The effect of the jerker mutation on espin protein expression is profound. Although the tissues of jerker mice contain wild-type levels of mutated espin mRNAs, espin proteins are virtually undetectable in every cell or tissue that would normally express an espin, including hair cells [7]. This deficiency of espin protein, which is evident using antibodies directed against different parts of the espin proteins, makes jerker mice appear espin null at the protein level. Jerker heterozygotes contain roughly half wild-type levels of espin protein. Concordance between the espin gene and the jerker mutation has been confirmed by detailed physical mapping studies [37, 38].

The molecular basis for the espin protein deficiency in jerker mice remains to be established. One possibility is that the loss of the C-terminal half of the ABM, which would make the mutated espins poor actin-bundling proteins [12], destabilizes the proteins. The amino acid sequence of the jerker peptide shows significant similarity to those of certain nuclear and nucleolar proteins, such as protamines, the lamin B receptor, the Bcl2-associated transcription factor and the RS domains of SR protein splicing factors [14]. Although jerker espins fail to accumulate in the tissues of jerker mice, they can be expressed in transfected cells. In transfected cells, the jerker peptide acts as a potent nucleolar localization signal, which efficiently targets jerker espins, as well as multiple heterologous proteins, to nucleoli [14]. Thus, another possibility is that nucleolar targeting mediated by the jerker peptide may destine jerker espins for premature degradation.

Deafness and mutations in the human espin gene

Since the mapping of the jerker mutation to *Espn*, six deafness mutations have been ascribed to the espin gene of humans (*ESPN*) (Fig. 1B), which is located at chromosome 1p36.3 [8, 9]. Two recessive mutations, 1988delAGAG and 2469delGTCA (numbering refers to human espin 1) (Fig. 1B), were discovered in Pakistani families, where they were associated with a prelingual, profound sensorineural hearing loss, delayed independent ambulation and vestibular areflexia. Both are frameshift mutations [8]. The 1988delAGAG mutation introduces a premature stop codon near the end of the WH2 domain (Fig. 1B). Because this premature termination codon is >55 nucleotides upstream of a splice-generated exon-exon junction, these mutated mRNAs are probably destroyed by nonsense-mediated decay [39] and, therefore, never produce significant protein. The 2469delGTCA mutation replaces the C-terminal 31 amino acids of the wild-type human espins with a novel 21-amino acid peptide [8] (Fig. 1B). In this case the premature termination codon is downstream of the 3′-most exon-exon junction. Therefore, these mutated mRNAs are probably spared from nonsense-mediated decay [39] and could produce mutated espin proteins. Although the frameshifted peptide introduced by the 2469delGTCA mutation bears no sequence resemblance to the jerker peptide, it, too, obliterates the putative C-terminal F-actin-binding site of the ABM (Fig. 1B).

ESPN mutations have also been identified in patients affected by autosomal dominant hearing loss without apparent vestibular involvement [9] (Fig. 1B). These mutations affect different conserved amino acids present in all espin isoforms [9]. Severe, bilateral sensorineural hearing loss is associated with two of the mutations: the 2541-2543delAAG mutation, which deletes a single K residue from the putative C-terminal F-actin-binding site in the ABM (delK848), and the G2230A mutation, which substitutes an N for a D in the N-terminal part of the ABM (D744N) (Fig. 1B). Mild-to-moderate, late-onset bilateral hearing loss is associated with two other mutations: the G2321A mutation, which substitutes a Q for an R in the N-terminal part of the ABM (R774Q), and the A2155C mutation, which substitutes an R for an S in the peptide between the WH2 domain and the ABM (S719R) (Fig. 1B). Although no information is available about how these mutations affect stereocilia or hair cells in these patients,

three of the mutations cause discernible defects in microvillar elongation or organization when the mutated espin proteins are expressed in transfected epithelial cells [9].

Espins in the microvillar processes of other sensory cells

In addition to hair cell stereocilia, espins are detected at high levels in the microvillar PABs of a number of other sensory cell types, including taste receptor cells, solitary chemoreceptor cells, vomeronasal sensory neurons and Merkel cells [4, 5] (Fig. 2D–G). Although the microvilli of these various sensory cell types differ from each other in size, shape and arrangement, each appears to be a primary site of sensory transduction. Specifically, either the binding of a chemical ligand to its cognate receptors in the microvillar plasma membrane or the mechanical deformation of the microvilli is believed to trigger a series of downstream signaling events that includes an increase in the concentration of intracellular Ca^{2+} and results in release of neurotransmitter onto afferent nerve fibers.

Taste receptor cells are the peripheral receptors of the vertebrate gustatory system and help detect nutrients and avoid toxic substances. They are organized with supporting cells and progenitor cells in taste buds, which are distributed at multiple specific locations in the oral and pharyngo-laryngeal epithelium. In a taste bud ~50–100 slender, polarized taste receptor cells and supporting cells are packed tightly together [40]. They extend microvilli from their tapered apical surface into the taste pit and pore, which reflect a small, recessed opening in the surrounding epithelium. On the basis of microscopic features and specific markers, the sensory cells of taste buds are presently categorized as type II or type III cells, whereas the supporting cells are referred to as type I cells [40–42]. A majority of the sensory cells in taste buds are type II cells, which are believed to detect bitter, sweet and umami stimuli [42, 43]. Tastant molecules bind to G protein-coupled receptors in the plasma membrane overlying their relatively short, thick microvilli, which project into the lower half of the taste pit. The PABs of type II cell microvilli often extend rootlets deep into the taste cells [44] (Fig. 2D, arrowheads). Type III cells appear to represent a relatively minor population of taste bud cells and are believed to display a single blunt apical microvillus that projects far up into the taste pore [41, 44, 45] (Fig. 2D, arrow). The microvilli of the supporting (type I) cells appear to take up a position intermediate between those of the type II and type III cells [44]. The actin-bundling proteins villin and fimbrin/plastin have been detected in the microvilli of supporting (type I) and sensory (type II) cells in taste buds [46]. In contrast, espins appear to be expressed at high levels only in the sensory cells, specifically the type II and type III cells [4, 5] (Fig. 2D). A consequence is that, when labeled with espin antibody, taste buds often reveal a multi-tiered apical cytoskeletal apparatus, in which the espin-containing microvillar/rootlet PABs of the type II cells appear separated from the espin-containing microvillar PAB of type III cells by an espin-deficient zone, which presumably contains the microvilli of type I cells [5] (Fig. 2D).

Solitary chemoreceptor cells resemble taste receptor cells in many respects, but are distributed more diffusely, at varying densities, within the epithelium of the respiratory and digestive systems [47]. They may be a subpopulation of the 'brush cells' identified in these epithelia [48]. Solitary chemoreceptor cells can contain tastant receptor and key proteins of the gustatory transduction cascade, such as phospholipase C β2, inositol-4,5-trisphosphate receptor III and α-gustducin [47]. Although the size and shape of solitary chemoreceptor cells varies according to the thickness of the epithelium in which they reside, they display a collection of stout, espin-rich microvilli that emanate from a narrow apical neck (Fig. 2E). These microvilli have also been reported to contain villin and fimbrin/plastin [49]. The function of solitary chemoreceptor cells has been examined in greatest detail for those in the nasal cavity, where they appear to respond to the inhalation of irritating substances by initiating protective reflexes, such as sneezing or apnea [47]. The presence of espins in solitary chemoreceptor cells has been documented most thoroughly in the nasal cavity and in the convex face of the vomeronasal organ [4].

The vomeronasal organ is a bilateral, cigar-shaped structure positioned at the base of the nasal septum. It is primarily concerned with the detection of pheromones that regulate sexual, hormonal and reproductive function in mammals [50, 51]. Chemosensory stimuli enter the crescent-shaped lumen of the vomeronasal organ through its opening into the nasal cavity and are detected by the sensory neurons that densely populate the concave face [50]. The vomeronasal sensory neurons extend a single, long dendritic process toward the lumen [50]. There, the dendrite expands into an apical dendritic knob, which is covered with espin-rich microvilli [4] (Fig. 2F). Villin has also been detected in the dendritic microvilli [52]. The binding of pheromone to receptors in the microvillar plasma membrane is believed to initiate an action potential that travels along the axon to the accessory olfactory bulb. Neurons that have their cell bodies positioned apically display different pheromone receptors and G-proteins than those with their cell bodies positioned basally [50, 51]; however, both populations contain high concentrations of espin in their dendritic microvilli [4]. The microvilli of neighboring supporting cells are deficient in espin and villin, but appear to contain fimbrin/plastin [4, 52].

Merkel cells display the properties of mechanoreceptors [53, 54]. They are found in a complex with enlarged

terminal endings of nerve fibers and are believed to be sensitive touch receptors. Merkel cell-neurite complexes are typically found in the basal layers of the epidermis or contiguous mucosae, *e.g.* the masticatory mucosa, and are especially prevalent in the sinus hair follicles of nonhuman mammals [53]. Merkel cells have long, spiky microvilli [53]. These microvilli contain high concentrations of espins [4] (Fig. 2G) and have also been reported to contain villin [55]. A favored model is that, by analogy to hair cells, mechanical deformation of Merkel cell microvilli initiates Ca^{2+} influx and a signaling cascade that results in neurotransmitter release onto the affiliated neurite. In support of this model, Merkel cells are excitable, contain small dense-core and clear vesicles and are enriched in key presynaptic proteins and neurotransmitters [53, 54]. Interestingly, a mechanosensory function has also been postulated for another class of cells with espin-containing microvilli: epithelial cells in renal proximal tubule [12]. In this case, an increase in the hydrodynamic torque on the microvilli is believed to increase the efficiency of absorption of Na^{+} to maintain glomerulotubular balance [56]. Kidney epithelial cells appear to be able to sense flow via their primary cilia, deformation of which causes increases in intracellular Ca^{2+}[57].

Binding and bundling F-actin in a Ca^{2+}-resistant fashion

The prevalence of espins in hair cell stereocilia and the microvilli of other sensory cells begs the question of what they do for these cells and their PAB-containing structures. *In vitro* and in transfected cell models, espins display a number of biological activities that distinguish them from other actin-bundling proteins. Many of these activities stem from the espin ABM and its superior ability to bind and bundle actin filaments.

In cosedimentation binding assays, espin 2B binds preformed filaments of rabbit skeletal muscle actin with a K_d of ~70 nM [11]. This affinity is 1–2 orders of magnitude greater than that observed for most other actin-bundling proteins. In fact, espins bind with even higher affinity to filaments of nonmuscle actin [12]. Espins are also extraordinarily efficient at cross-linking actin filaments into parallel bundles. In cosedimentation bundling assays, purified recombinant espin 2B converts all F-actin into sedimentable bundles when present at a molar ratio as low as 1 espin for every 20–50 actin monomers [11]. This efficient cross-linking is confirmed by negative staining electron microscopy [11, 12] (Fig. 3A).

The F-actin-binding and -bundling activities of espins are not inhibited by Ca^{2+} [4, 11, 12,]. This makes espins different than the other actin-bundling proteins found in stereocilia or the microvilli of vertebrates. Fimbrins/plastins and villin are inhibited by Ca^{2+} [1]. In fact, villin becomes an actin-severing protein in the presence of Ca^{2+} [58]. Thus, one possible explanation for why espins are abundant in stereocilia and the microvilli of sensory cells is that they supply Ca^{2+}-stable cross-links to the PAB at the core. This could be crucial for sensory cells, because transient local increases in the concentration of intracellular Ca^{2+} accompany signal transduction in each of the

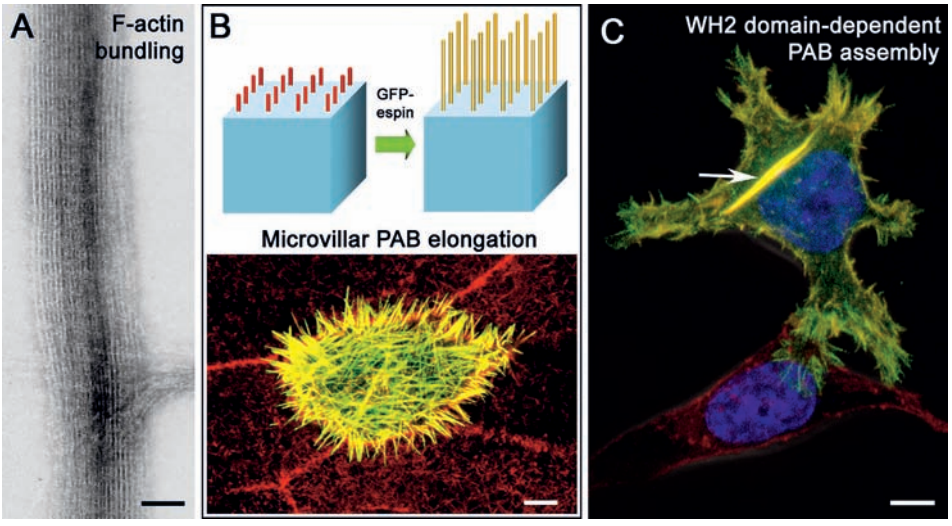

Figure 3. Major activities of espins. (*A*) High-affinity, Ca^{2+}-resistant bundling of actin filaments *in vitro* illustrated by negative-staining electron microscopy. (*B*) Microvillar PAB elongation illustrated for epithelial cells transfected with GFP-espin in diagrammatic form (upper) and in confocal image of microvillar surface region of an LLC-PK1-CL4 monolayer (lower) double-labeled for F-actin with Texas Red-phalloidin (red). The elongated microvilli of the transfected cell are yellow because they contain GFP-espin and F-actin. (*C*) WH2 domain-dependent PAB assembly in cells illustrated in confocal image of a PC12 cell transfected with GFP-espin and double-labeled with Texas Red-phalloidin (red). The arrow points to a large GFP-espin-rich PAB that forms in association with centrosomal region. Lower levels of GFP-espin and F-actin are also present in filopodia-like structures at the cell periphery. The lower cell is not transfected. Bars, 100 nm (*A*) or 5 µm (*B*, *C*).

espin-containing sensory cell types. For example, a transient increase in Ca^{2+} concentration is observed within the stereocilium upon deflection of the stereociliary bundle in the direction of the longest stereocilium [59]. In fact, the stereociliary shortening and degeneration noted in jerker mice could be interpreted as a collapse of the PAB when mechanotransduction begins in earnest at the time of hearing onset. A role in protecting cross-linked actin filaments could also explain why espins are present in the dendritic spines of Purkinje cells in the cerebellum, but apparently not in those of other spiny neurons in the central nervous system [15]; among spiny neurons, Purkinje cell dendritic spines experience some of the largest Ca^{2+} transients [60].

When examined by analytical ultracentrifugation, purified recombinant espins are monomeric in solution [11, 12]. This most likely means that their bundling activity reflects the presence of at least two actin-binding sites per espin monomer. Mutagenesis studies have traced the actin-bundling activity of espins to the 116-amino acid ABM at the distal C terminus of all known isoforms [12] (Fig. 1A). Incremental truncation of the ABM from either end eliminates actin-bundling activity and, thus, identifies two candidate F-actin-binding sites disposed roughly at either end of the ABM [12]. These peptide regions show a high degree of amino acid sequence conservation among espins from different species and are the target of deafness mutations (Fig. 1B).

Espin 1 and the espin 2 isoforms contain an additional F-actin-binding site [11]. This additional F-actin-binding site was identified when peptide fragments substantially upstream of the ABM were observed to also bind to F-actin *in vitro*. These peptide fragments bind with a K_d of ~1 μM, an affinity more comparable to that observed for other actin-bundling proteins, and decorate F-actin stress fiber-like structures in transfected cells [11]. Through mutagenesis, the additional F-actin-binding site was mapped to a 23-amino acid peptide immediately C-terminal to the N-terminal proline-rich peptide of these isoforms [11] (Fig. 1A). This peptide bears little obvious sequence similarity to known actin-binding sites. It is unclear how the additional F-actin-binding site contributes to filament binding/bundling for these isoforms, although it may account for the increment in affinity noted for the larger isoforms in F-actin-binding assays [11].

Increasing the steady-state length of PABs

Beyond providing Ca^{2+} stable cross-links, espins increase the steady-state length of PABs. This activity was discovered when examining the effects of espins on the brush border microvilli of LLC-PK1-CL4 epithelial cells [13]. Espins expressed exogenously in these cells are efficiently targeted to the microvilli and dramatically increase their steady-state length by causing the barbed-end elongation of the PAB scaffold at their core [13] (Fig. 3B). The magnitude of the lengthening is dependent on the level of espin expression: the more espin, the longer the microvillar PABs. For example, when expressed at relatively high levels under the control of the cytomegalovirus promoter, espins increase the steady-state length of LLC-PK1-CL4 cell microvillar PABs an average of ~6-fold, from ~1.3 μm to ~7.9 μm, although extreme examples of cells showing >10-fold elongation are also evident [13]. This span, 1.3–7.9 μm, covers the range of lengths observed for the microvilli and stereocilia of most vertebrate cells. Moreover, on the basis of antibody labeling intensity, this microvillar PAB elongation appears to take place over a physiologically relevant range of espin concentrations. Transiently transfected cells are typically examined the following day, yet significant microvillar elongation is evident only 4 h after transfection. The ABM is necessary and sufficient for the espin-mediated elongation of PABs, suggesting that the lengthening stems directly from espin cross-links within the PAB [13]. Deletion of either putative F-actin-binding site from the ABM destroys elongation activity. Potent microvillar PAB elongating activity is exhibited by all known espin isoforms and appears relatively specific to espins among the actin-bundling proteins of stereocilia and microvilli [13]. Fimbrins/plastins cause a barely discernible increase in microvillar length, and villin causes moderate levels of microvillar elongation. Fascin appears to have no effect on microvillar length and is, instead, targeted to filopodia at the basal surface of LLC-PK1-CL4 cells.

Inhibition by low-dose cytochalasin D indicates that the espin-mediated lengthening response involves net elongation at the barbed-end of the actin filaments making up the PAB [13]. Interestingly, however, fluorescence-recovery-after-photobleaching (FRAP) of GFP-β-actin in the microvilli suggests that PAB actin filaments are undergoing treadmilling at approximately the same rate, ~0.25 μm/min (or ~1.5 actin monomers per second), before and after the elongation [13]. A simple calculation illustrates that the dramatic lengthening observed in response to espin in LLC-PK1-CL4 cells likely reflects only a relatively small change in the rate of actin polymerization or depolymerization. When treadmilling at ~0.25 μm/min, a transient change in the rate of actin polymerization or depolymerization of only ~10% will increase microvillar PAB length from 1.3 to 7.9 μm in 4 h. One can envision how espin cross-links might retard actin depolymerization at the pointed end of filaments by ~10%. In fact, *in vitro* experiments suggest that espin cross-links might cause a slight (<30%) inhibition of actin filament depolymerization [13]. However, it is also theoretically possible that espin cross-links stimulate actin polymerization (decrease critical concentration) at their barbed end by ~10%. The fact that the microvillar

PABs do not elongate indefinitely suggests that the cell adapts to the presence of espins and adjusts the rates of actin polymerization/depolymerization to return to the steady-state, possibly via a 'balance-point' mechanism, like that believed to control the steady-state length of eukaryotic flagella [61]. Besides espins, other proteins have been implicated in stereociliary length regulation, notably the unconventional myosin motor myosin XVa and its PDZ domain-containing ligand whirlin [62]. Thus, it is also possible that the presence or absence of espin cross-links in a stereociliary PAB affect steady-state length indirectly through their effects on the delivery, stability or activities of other proteins like myosin XVa and whirlin.

Although a transient ~10% effect of espin cross-links on the rate of actin polymerization or depolymerization in a PAB may not seem large, the effects of espins on microvillar and stereociliary PAB length are dramatic. Transfection with espin constructs elicits pronounced microvillar PAB elongation both in epithelial cell lines in culture and in epithelial cells in tissue explants [13, 34]. A significant lengthening effect can even be observed for the stereociliary PABs of transfected hair cells [34], even though they already express a large amount of espin endogenously. Conversely, as mentioned above, a major consequence of the espin deficiency noted in jerker mice is a marked shortening of stereocilia. It is possible that the stereociliary shrinkage observed in jerker mice in early postnatal life can also be traced to an ~10% difference in rates of actin polymerization and depolymerization in their PAB. The fact that stereociliary bundles form in jerker mice [32–34] suggests a greater requirement for espins in the postnatal elongation and steady-state length maintenance than in stereocilium formation *per se*. The correlation between espin protein level and stereociliary length also extends to hair cells in normal animals. For example, at a given position along the cochlear spiral, inner hair cells have higher espin levels and longer stereocilia than the corresponding outer hair cells [6, 13] (Fig. 2B). There is also an increasing gradient in hair cell espin levels from base to apex along the cochlear spiral that is correlated with the known increasing gradient in stereocilium length [6, 13]. In fact, both gradients appear to arise simultaneously during early postnatal development [6]. It is presently unclear how espin-mediated PAB elongation might figure into building the stereociliary staircase, for which stereocilium length must vary in a regular way from row to row in a single cell. Staircase assembly could involve espin targeting or local regulation in the hair cell.

As exemplified by *Drosophila*, PAB length in the invertebrates appears to be determined in large part by the end-to-end juxtaposition of relatively short PAB modules [3]. However, this form of modular construction has never been reported for the PABs of vertebrate cells. Thus, espins may fill a special niche – one of steady-state length regulation for the microvillar and stereociliary PABs of vertebrate cells. In theory, as actin-bundling proteins, one might think that espins should also be able to affect bundle diameter by incorporating greater numbers of filaments into a PAB. In fact, in the LLC-PK1-CL4 cell transfection model the larger espin isoforms, which include the 23-amino acid additional F-actin-binding site (Fig. 1A), make long microvillar PABs that appear wider [13]. Although the stereociliary bundle of the hair cell affords an extreme example of PAB dimension regulation in the vertebrates, it is important to point out that many types of vertebrate sensory cells exhibit a characteristic size and shape for their microvillus-based sensory projections.

WH2-mediated actin monomer binding and actin bundle assembly

In addition to the ABM, all espin isoforms contain a WH2 domain [4, 13, 14] (Fig. 1A). This domain is highly conserved among espins. The first 30 amino acids of the peptide encoded by the WH2 domain exon, including the 17-amino acid actin monomer-binding core of the WH2 domain, are identical in espins from pufferfish to human. WH2 domains bind ATP-actin monomer [63]. In addition to being present in the WASP family proteins, from which it derives its name, the WH2 domain is present in a variety of other actin cytoskeletal proteins, such as the β-thymosins, ciboulot, verprolin/WASP-interacting protein (WIP), Srv2/cyclase-associated protein (CAP), the Missing in Metastasis (MIM) proteins and Spir [63]. Espins can bind ATP-actin monomer *in vitro* in pull-down assays, and deletion of the 17-amino acid WH2 domain core eliminates this binding [4, 13, 14]. In contrast, deletion of the WH2 domain has no effect on espin's actin-bundling activity *in vitro* [12] or its ability to elongate microvillar PABs in transfected LLC-PK1-CL4 cells [13].

The espin WH2 domain appears to also bind actin monomer *in vivo*. This activity was first noted in FRAP studies of long microvilli in LLC-PK1-CL4 cells co-transfected with plasmids encoding GFP-β-actin and untagged espin [13]. After photobleaching, a rapid recovery of GFP-β-actin fluorescence occurs throughout the entire length of the long microvilli. This behavior is uncharacteristic for PAB-based structures, which, if dynamic, generally undergo actin treadmilling. Deletion of the 17-amino acid core of the espin WH2 domain reduces the rapid GFP-β-actin recovery and reveals the ~0.25 μm/min actin treadmilling in the microvillar PAB [13]. This result suggests that espins associated with a microvillar PAB in a living cell can bind actin monomer via their WH2 domain and that this bound actin monomer is rapidly exchangeable. The physiological role of this interaction and its contribution to the incorporation of GFP-β-actin observed in transfected hair cells [19] are presently unclear. However, at a minimum, by binding actin monomer to the WH2

domain, espins can increase the local concentration of polymerizable actin monomer. This could position actin monomer advantageously for polymerization or repair.

The espin WH2 domain has also been implicated in a novel activity of the espins: the ability to assemble a large PAB *de novo* when targeted to a specific cellular location [14]. Thus far, this activity has been observed for wild-type espins that were targeted to the centrosome in transfected neuronal cells and for jerker espins that were targeted to the nucleolus in a wide variety of transfected cell types as a result of the nucleolar localization sequence in the jerker peptide [14]. In neuronal cell lines and primary hippocampal neurons, espin expression results in a large PAB that grows in association with the centrosome [14] (Fig. 3C). Inhibition by dynamitin overexpression or nocodazole implicates the dynein-dynactin motor complex and microtubles in the delivery of espin to the centrosomal region of these cells [14]. In the case of jerker espins, the result is a large wavy or donut-like nuclear actin bundle, which grows in association with the nucleolus [14]. This PAB-building activity, which appears specific to espins among actin-bundling proteins, requires two espin F-actin-binding sites and the actin monomer-binding activity of the espin WH2 domain, but evidently no other parts of the espins [14]. Well-characterized actin monomer-binding WH2 domains from other proteins, such as N-WASP, can substitute for the espin WH2 domain, and the WH2 domain functions even when placed, via mutagenesis, in an altogether different location in the espin molecule [14]. This flexibility strongly suggests that the role of the WH2 domain is to increase the local concentration of polymerizable actin monomer, thereby delivering the ATP-actin monomer needed to support local nucleation or elongation reactions. Espin's multiple F-actin-binding sites presumably cross-link any actin filaments and stimulate elongation by the same mechanism that causes the barbed-end elongation of preformed microvillar PABs [13] (Fig. 3B). One intriguing possibility is that the espin WH2 domain somehow works in concert with the espin F-actin-binding sites to form actin bundles in cells. Although purified recombinant espins do not activate the Arp2/3 complex or exhibit significant nucleation activity *in vitro* [14], it is still possible that espins could participate in the nucleation of actin polymerization in cells. The same type of novel PAB-building activity noted for the espins can be mimicked by adding a WH2 domain to an unrelated actin-bundling protein, villin [14]. Thus, when a WH2 domain is attached to a peptide that can bundle actin filaments – a situation that occurs naturally in the case of espins – the resultant protein can assemble large PABs when targeted to a specific location in cells.

This PAB-building activity serves as a second demonstration of the actin monomer-binding capability of the espin WH2 domain in cells. Nevertheless, relatively little is known regarding the role of this activity in situ. It is noteworthy, however, that espins are present in hair cells and taste bud cells relatively early in their differentiation, at a time when stereociliary or microvillar PABs are forming [5, 6]. In addition, it remains possible that espin WH2 domain-dependent PAB assembly occurs locally in cells in situ on a smaller scale, without resulting in the accumulation of such a large PAB. This type of activity could be tied to the more diffusely localized pools of espin protein that have been detected in the developing inner ear and in a variety of epithelia undergoing morphogenesis [6] or in the dendritic spines of Purkinje cell neurons [15]. It is even possible that this type of activity could play a role in the local repair of damaged actin filaments and bundles. One can, however, predict that a large PAB would form in cells if espins were to become suprathreshold at a particular location. This type of response may explain the formation of the 'cytocaud,' a pathological protrusion containing a large PAB that extends basally from the hair cells of mice with certain deafness mutations, specifically the inner hair cells and type-I vestibular hair cells of adult pirouette and shaker-2 mice and the type-I vestibular hair cells of the waltzing guinea pig [64, 65]. In addition to their progressive accumulation in the stereocilia of differentiating hair cells, espins are also present in a long, tail-like process – the espin-rich basal process or 'hair cell tail' – that emanates from the base of all developing hair cells in wild-type rodents during late embryonic development [6]. Thus, the WH2 domain-dependent PAB-building activity of the espins, working within the confines of the espin-rich basal process, may contribute to cytocaud pathology.

Binding PIP2 and ligands for proline-rich peptides

The espin 2 and 4 isoforms, but not the espin 3 isoforms, bind phospholipid vesicles containing PIP2 *in vitro* at physiological pH, ionic strength and temperature [4] (Fig. 1A). (Recombinant espin 1 is insoluble under these conditions and has not been tested.) The binding is specific in that it is not observed when using control vesicles made from phosphatidylcholine alone. Notably, distinct peptides from the N termini of these two isoforms are required for binding to the PIP2-containing vesicles [4] (Fig. 1A). In the case of espin 4, maximum binding requires the peptides encoded by the two exons that are unique to espin 4 (Fig. 1A, magenta). The PIP2-binding peptides of espins are rich in positively charged amino acids, but otherwise bear no obvious resemblance to the PIP2-binding domains of other proteins. PIP2 is ordinarily present in the cytoplasmic leaflet of the plasma membrane bilayer and is especially enriched in the plasma

membrane overlying structures that contain espins, such as stereocilia [66]. This raises the possibility that espins located on the outside of a stereociliary or microvillar PAB could function in part as linkers that help connect the PAB to the plasma membrane. In addition, PIP2 is a substrate for phospholipase C, which cleaves PIP2 into diacylglycerol and inositol 1,4,5-trisphosphate and is believed to play a prominent role in sensory transduction in cells that contain espins [42, 67, 68]. Thus, it is possible that espins regulate the availability of PIP2 in an isoform-specific way. The PIP2-binding espin isoforms may also be among the long list of actin cytoskeletal proteins that are regulated by PIP2 or its metabolites [69, 70].

All espins except the espin 4 isoforms contain proline-rich peptides. Espin 1 and the espin 2 isoforms contain two proline-rich peptides, whereas the espin 3 isoforms contain one (Fig. 1A). Proline-rich peptides are frequent mediators of protein-protein interaction in signaling cascades and multiprotein scaffolds [71]. Although proline-rich peptides figure centrally in actin cytoskeletal regulation [71], they are noticeably absent from other actin-bundling proteins found in stereocilia and microvilli. Thus far, two types of protein ligand have been found to bind the proline-rich peptides of espins: the profilins and the IRSp53 SH3 domain [4, 13, 15] (Fig. 1A). Profilins bind actin monomer and can stimulate actin polymerization at the barbed end of the actin filament [72]. Profilin-binding proline-rich peptides are present in a number of cytoskeletal regulatory proteins and are believed to function in part to increase the local concentration of profilin-actin complexes [72]. For example, binding of the profilin-actin complex to the proline-rich peptides of the formins increases their ability to support barbed-end elongation [73]. Although proline-rich peptides may supply espins with another way to increase the local concentration of actin monomer, profilin-espin interactions have not yet been documented *in situ*, and known espin activities such as microvillar PAB elongation and WH2 domain-mediated actin bundle assembly do not appear to require the proline-rich peptides [13, 14]. IRSp53 is an SH3 domain-containing scaffolding protein that links Rac1 and Cdc42 small GTPases to actin cytoskeletal regulators, such as WAVE2 and Mena [74, 75]. IRSp53 was identified as an espin ligand by yeast two-hybrid screening [15]. In pull-down assays, the IRSp53 SH3 domain binds exclusively to the N-terminal proline-rich peptide of the espins [4, 15] (Fig. 1A). Accordingly, the espin 3 isoforms, which lack this proline-rich peptide, do not bind the IRSp53 SH3 domain [4]. Although implicated in actin cytoskeletal regulation in lamellipodia, ruffles, filopodia and dendritic spines [15, 74–76], evidence linking IRSp53 to stereocilia and microvilli is currently lacking. It is possible that the espin proline-rich peptides interact with other SH3 domain-containing scaffolding proteins in stereocilia and microvilli.

Outlook

In conclusion, espins are novel, multifunctional actin-bundling proteins that show a special connection to hair cell stereocilia and the microvillar specializations of vertebrate sensory cells. The findings to date suggest major roles for espins in regulating the organization, dimensions, dynamics and signaling capabilities of these PAB-containing specializations with which vertebrates sense their world. Although many of the better-characterized activities of the espins stem from their ABM, much remains to learned about how espin cross-links affect actin filament and bundle structure and PAB dynamics. There is also a possibility that new activities of espins will be discovered. The expanding roster of espin deafness mutations is guiding rational mutagenesis in refined structure-function analyses and affords new opportunities to decipher the complex relationships between molecular pathology and phenotype. Mouse models and transfection studies promise to shed new light on the nature of the defects in humans with espin deafness mutations. There is a growing appreciation of how different cell types employ specific espin isoforms at distinct times. Nevertheless, relatively little is known about the subcellular compartmentalization of espin isoforms, the identity of natural ligands for their N-terminal binding sites and how these ligands affect espin localization and function. The presence of an actin monomer-binding WH2 domain appears unique to espins among actin-bundling proteins, and suggests additional roles for these proteins in actin monomer recruitment and PAB assembly, repair and pathology. In view of the profound effects of espin gene mutations, the importance of espins to hair cells and their stereocilia seems indisputable. Additional experiments will reveal how espin deficiency or mutation affects the structure and function of other classes of vertebrate sensory cells.

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