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To cite this version:

Aziz El-Amraoui, Christine Petit. Usher I syndrome: unravelling the mechanisms that underlie the cohesion of the growing hair bundle in inner ear sensory cells. Journal of Cell Science, 2005, 118 (20), pp.4593-4603. 10.1242/jcs.02636 . pasteur-03926862

HAL Id: pasteur-03926862

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Usher I syndrome: unravelling the mechanisms that underlie the cohesion of the growing hair bundle in inner ear sensory cells

Aziz El-Amraoui and Christine Petit

Unité de Génétique des Déficits Sensoriels, INSERM U587, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris Cedex 15, France
(e-mail: elaz@pasteur.fr; cpetit@pasteur.fr)

Journal of Cell Science 118, 4593-4603 Published by The Company of Biologists 2005
doi:10.1242/jcs.02636

Summary

Defects in myosin VIIa, the PDZ-domain-containing protein harmonin, cadherin 23 and protocadherin 15 (two cadherins with large extracellular regions), and the putative scaffolding protein Sans underlie five genetic forms of Usher syndrome type I (USH1), the most frequent cause of hereditary deafness-blindness in humans. All USH1 proteins are localised within growing stereocilia and/or the kinocilium that make up the developing auditory hair bundle, the mechanosensitive structure receptive to sound stimulation. Cadherin 23 has been shown to be a component of fibrous links interconnecting the growing stereocilia as well as the kinocilium and the nearest tall stereocilia. A similar function is anticipated for protocadherin 15. Multiple direct interactions between USH1 proteins have been demonstrated. In particular, harmonin b can bind to the cytoplasmic regions of cadherin 23 and protocadherin 15, and to F-actin, and thus probably anchors these cadherins to the actin filaments filling the stereocilia. Myosin VIIa and Sans are both involved in the sorting and/or targeting of harmonin b to the stereocilia. Together, this suggests that the disorganisation of the hair bundles observed in mice mutants lacking orthologues of USH1 proteins may result from a defect of hair-bundle-link-mediated adhesion forces. Moreover, several recent evidences suggest that some genes defective in Usher type II syndrome also encode interstereocilia links, thus bridging the pathogenic pathways of USH1 and USH2 hearing impairment. Additional functions of USH1 proteins in the inner ear and the retina are evident from other phenotypic abnormalities observed in these mice. In particular, myosin VIIa could act at the interface between microtubule- and actin-based transport.

Key words: Usher syndrome, Myosin VIIa, Harmonin, Cadherin 23, Protocadherin 15, Sans, Usherin, Vlgr1

Introduction

In recent years, unravelling the molecular bases of hereditary diseases has revealed numerous functional modules underlying developmental and physiological processes. A good example is the study of genetically heterogeneous syndromes that include primary cilium abnormalities, such as polycystic kidney disease, primary ciliary dyskinesia, nephronophtisis, Bardet-Biedl syndrome and oro-facio-digital syndrome. Studies of these syndromes have helped uncover the mechanosensory function of the primary cilium and how ciliary proteins are targeted to and transported within this organelle (Snell et al., 2004). Here, we consider the contribution of studies of Usher syndrome type I (USH1), a hereditary sensorineural deafness combined with retinitis pigmentosa, to our understanding of the development of the mechanosensitive auditory hair bundles receptive to sound. Previously, we had proposed that the various forms of USH1 result from a common pathogenic process: the absence of the interstereocilia links (or connectors) that maintain auditory hair bundle cohesion (Petit, 2001). This proposal is now supported by additional evidence, which sheds light on the underlying mechanisms.

Usher syndrome type I genes

Usher syndrome is the most frequent cause of hereditary deafness and blindness in humans, affecting one child in 25,000. Three clinical subtypes, USH1, USH2 and USH3, can be defined according to the severity of the hearing impairment, the presence or absence of vestibular dysfunction and the age of onset of retinitis pigmentosa (Petit, 2001). USH1 is the most severe form, characterised by severe to profound congenital deafness, balance deficiency and prepubertal-onset retinitis pigmentosa. USH2 patients have a moderate-to-severe hearing impairment that is in most cases stable, normal vestibular function and loss of vision after puberty. USH3 patients have a progressive hearing impairment, variable vestibular dysfunction and retinitis pigmentosa that can occur at various ages. Retinitis pigmentosa initially manifests as night blindness and a loss of peripheral vision. The progressive degeneration of photoreceptor cells also causes other retinal symptoms, including the accumulation of intra-retinal pigment deposits, from which the disorder gets its name.

Each USH subtype is genetically heterogeneous. Linkage analyses of USH1 families have so far revealed seven distinct USH1 loci (USH1A-USH1G). Five of these genes have been identified (see http://webhost.ua.ac.be/hhh/) (Fig. 1A). *USH1B*

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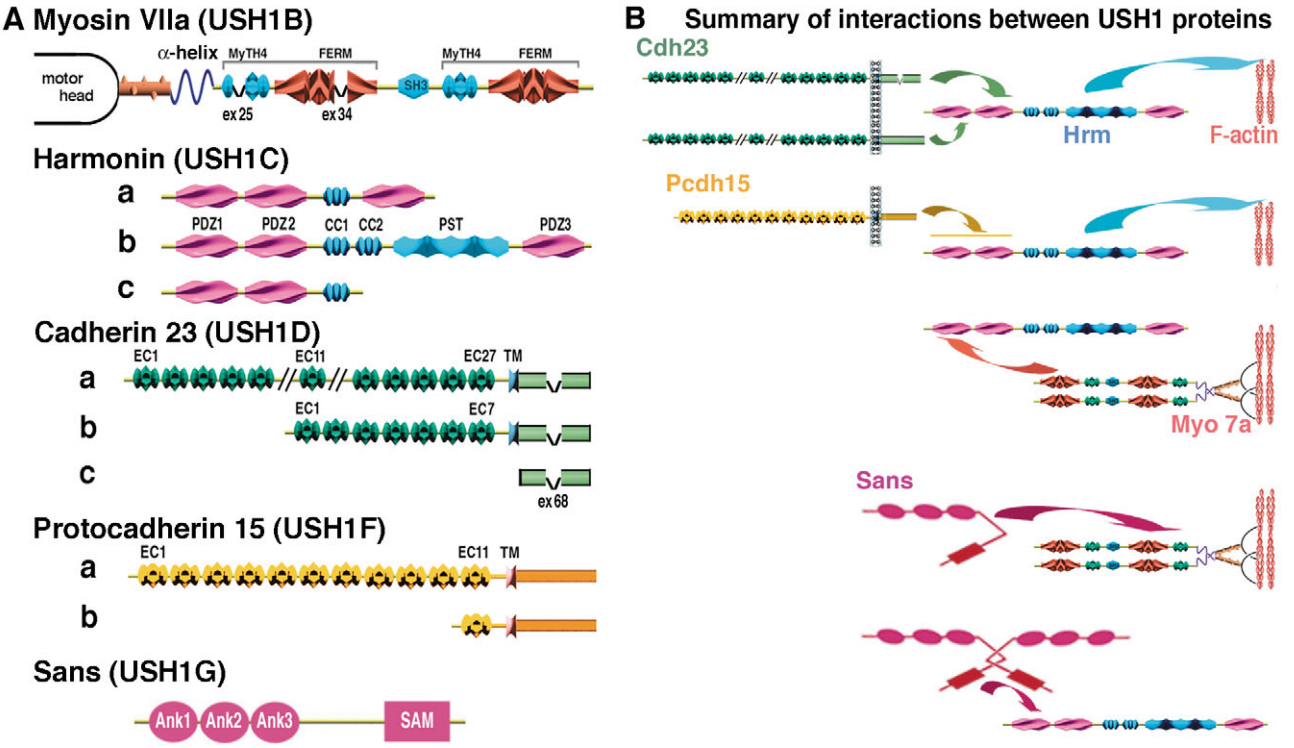

Fig. 1. (A) Predicted domain structures of the USH1 proteins. Myosin VIIa consists of a spectrin-like SH3 subdomain followed by the motor head, a neck region composed of five IQ (isoleucine-glutamine) motifs and a large tail. The tail starts with an α-helical domain, followed by two large repeats, each containing a myosin tail homology 4 (MyTH4) and a 4.1, Ezrin, Radixin, Moesin (FERM)-like domain. These are separated by a poorly conserved Src homology 3 (SH3) domain. Positions of the spliced exon 25 (ex 25) and 34 (ex 34) are indicated. There are three classes of harmonin isoform, depending on the presence of two or three PDZ domains and the presence or absence of a second coiled-coil (CC) domain associated with a proline-, serine- and threonine-rich (PST) domain. The largest cadherin 23 and protocadherin 15 isoforms have 27 extracellular cadherin (EC) repeats and 11 EC repeats, respectively. Sans is composed of three ankyrin (ANK)-like repeats and a sterile alpha motif (SAM) domain. (B) Summary of the direct interactions between USH1 proteins. The domains involved in each interaction are indicated by arrows. Harmonin is able to bind to any of the other USH1 proteins. The cytoplasmic regions of cadherin 23a isoforms containing or lacking the exon 68-encoded peptide (ex 68 in A) preferentially bind to the harmonin PDZ1 or PDZ2 domain, respectively. Harmonin can bind to the cytoplasmic region of protocadherin 15 through its first two PDZ domains. It can also bind to the myosin VIIa tail through a PDZ1–C-terminal-MyTH4-FERM domain interaction. Harmonin can also bind to Sans through a PDZ1-SAM region interaction. Finally, the Sans central region can bind to the myosin VIIa N-terminal MyTH4-FERM domain. Harmonin (not shown) and Sans can also form homodimers.

encodes the unconventional myosin VIIa (Weil et al., 1995). *USH1C* encodes harmonin, a PDZ-domain-containing protein (Verpy et al., 2000). *USH1D* and *USH1F* encode two large cadherin-related proteins, cadherin 23 and protocadherin 15, respectively (Ahmed et al., 2001; Alagramam et al., 2001b; Bolz et al., 2001; Bork et al., 2001). *USH1G* encodes a putative scaffold protein, Sans, that contains three ankyrin repeats and a sterile alpha motif (SAM) domain (Weil et al., 2003).

Analysis of the transcripts of these genes in the inner ear and retina, and biochemical studies suggest the existence of several isoforms of myosin VIIa, harmonin, cadherin 23 and protocadherin 15 (Fig. 1A). Splice variants of *USH1B* lacking some exons [e.g. exons 8, 9 and 13 (the motor head), exon 25 (the first MyTH4 domain) and exon 34 (the first FERM domain)] have been detected; they are all predicted to conserve the myosin VIIa reading frame (Weil et al., 1996) (Fig. 1A). A shorter *USH1B* transcript isolated from a human testis cDNA library is predicted to encode a myosin VIIa isoform ending after the first MyTH4 domain (Chen et al., 1996). Nevertheless, there is no evidence from northern blots or at the protein level that confirms the existence of this shorter isoform. Alternatively spliced *USH1C* transcripts allow us to predict at least ten harmonin isoforms. These form three subclasses (a, b, c), according to their protein domain composition (Verpy et al., 2000) (Fig. 1A). *USH1D* encodes at least six cadherin 23 isoforms: transmembrane isoforms containing 27 (a isoforms) or seven (b isoforms) extracellular cadherin (EC) domains, each with two different intracellular regions, and two cytosolic subtypes (the c isoforms) (Lagziel et al., 2005; Michel et al., 2005) (Fig. 1A). Finally, *USH1F* transcripts are predicted to encode at least two transmembrane isoforms containing 11 (a isoform) or one (b isoform) extracellular cadherin (EC) domain(s) (Ahmed et al., 2003) (Fig. 1A).

Mutations in four of the USH1 genes have also been reported to cause isolated recessive (DFNB) or dominant (DFNA) forms of deafness: DFNB2 and DFNA11 (*USH1B*) (Liu et al., 1997a; Liu et al., 1997b; Weil et al., 1997), DFNB18 (*USH1C*) (Ahmed et al., 2002; Ouyang et al., 2002), DFNB12 (*USH1D*) (Bork et al., 2001) and DFNB23 (*USH1F*) (Ahmed et al., 2002). The mutations causing these isolated forms of deafness are usually expected to be less deleterious for the protein activities than those observed in USH1 patients.

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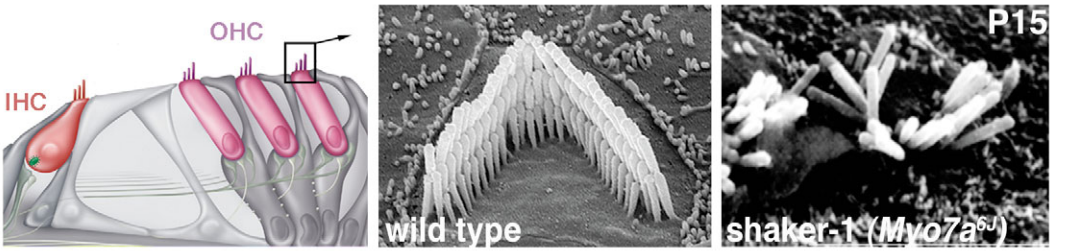

Fig. 2. The mammalian auditory epithelium, the organ of Corti. The sensory inner (IHC) and outer (OHC) hair cells are flanked by various types of supporting cell. Viewed from the top of the organ of Corti, the scanning electron microscopy images show the organisation of the OHC hair bundle from wild-type (source: Marc Lenoir, Montpellier, France), and Myo7a^{6J} (Arg241Pro) shaker-1 mutant mice at P15. In the shaker-1 mutant, the stereocilia form clusters of a small number of stereocilia arranged in diverse patterns and orientations (adapted with permission from Self et al., 1998).

Animal models of USH1

Mice that have defective myosin VIIa (shaker-1) (Gibson et al., 1995), harmonin (deaf circler) (Johnson et al., 2003), cadherin 23 (waltzer) (Di Palma et al., 2001; Wilson et al., 2001), protocadherin 15 (Ames waltzer) (Alagramam et al., 2001a) or Sans (Jackson shaker) (Kikkawa et al., 2003) have been described. These all exhibit severe hearing impairment and vestibular dysfunction but, surprisingly, none displays signs of retinitis pigmentosa. EM scanning analysis of the hair bundles of the auditory sensory cells in mutant mice lacking any of USH1 proteins showed that this subcellular structure is disorganised, and the bundles lack their characteristic cohesion (see Fig. 2). A detailed chronological analysis of morphological abnormalities in the hair bundles of shaker-1 and waltzer mutants has revealed that their fragmentation can already be detected at embryonic day 18 (E18) in both mutants (Self et al., 1998; Holme and Steel, 2002).

Zebrafish mutants possessing defective myosin VIIa or cadherin 23 have also been described. These also have disorganised hair bundles, which can easily be observed in the lateral line sensory system (Ernest et al., 2000; Sollner et al., 2004). Two zebrafish orthologues of the mammalian protocadherin 15 gene, *pcdh15a* and *pcdh15b*, encoding proteins that have highly divergent intracellular domains, have recently been described. Mutations in *pcdh15a* result in disorganisation of the hair bundle but cause no visual abnormalities, whereas *pcdh15b* inactivation causes only retinal anomalies: mutants exhibit abnormal alignment of photoreceptor outer disks with the pigment epithelial cells and abnormal interdigitation of the outer disks (Seiler et al., 2005).

Morphogenesis of the hair bundle

The hair bundle is located at the apical surface of the auditory hair cell (see Fig. 2). It comprises between 20 and 300 actin-filled, stiff microvilli – the stereocilia – which contain the mechanoelectrical transduction (MET) machinery and a single cilium, the kinocilium (Fig. 3A). As in other microvilli, actin filaments are uniformly polarized, the fast growing ends being located at stereocilia tips (Schneider et al., 2002). However, stereocilia in the adult hair bundle have certain specific morphological features (Figs 2, 3): (1) they are wider and longer than the microvilli of epithelial cells and, in some species, can contain up to 2000 actin filaments (Revenu et al., 2004); (2) they taper off at their base, where they insert into the apical surface of the cell; (3) they are arranged in three to four rows, the height the rows rising towards the kinocilium (Fig. 3A); and (4) they are connected by many lateral links and one tip link. The kinocilium is also connected to the nearest tall stereocilia by fibrous extracellular links, i.e. kinociliary links (Fig. 3A).

Four stages in the formation of the chick auditory hair bundles have been defined by Tilney et al. (reviewed in Tilney et al., 1992) (see also Fig. 3A, left panels). First, the stereocilia emerge at the apical surface of the sensory cell as a homogeneous group of small, equally sized microvilli, clustered around a single central kinocilium. Second, the kinocilium migrates to the peripheral edge thus dictating the orientation of the hair bundle (i.e. its planar polarity); this is followed by a selective and sequential elongation of the stereocilia row (starting with the row closest to the kinocilium) to form a staircase pattern of height-ranked rows. Third, the stereocilia stop elongating and a few central actin filaments of the stereocilia extend rootlets deep into a horizontal meshwork of actin filaments – the cuticular plate – located beneath the apical cell membrane. Then, the stereocilia become wider with the addition of new actin filaments. Finally, the stereocilia complete their elongation to reach their adult length and any stereocilia not incorporated into the bundle regress. These steps have also been seen in mammals (reviewed in Frolenkov et al., 2004), although some differences do exist; for example, elongation and widening of the stereocilium processes, which are separated in time in chick, occur concomitantly in mammals, and the kinocilium disappears in hair bundles of mammalian auditory cells.

Distinct specialised links connect the stereocilia (Fig. 3A). The tip link is a single, three-stranded interrow link that connects the tip of one stereocilium to the side of the nearest tall stereocilium. It has a unique direction, following the functional axis of the hair bundle. It is already detectable at E17 in the mouse and persists throughout life (Geleoc and Holt, 2003; Goodyear et al., 2005). Pickles et al. have proposed that these tip links are a specialisation of the links that join immature stereocilia laterally near their tips (Pickles et al., 1991). Indeed, a dense network of links that connect stereocilia within the growing hair bundle and show dramatic changes between its early developmental and mature stages. An abundant and uniform fibrous network is progressively substituted by distinct projecting dense links organised at different points over the length of the stereocilia, connecting

them within and between adjacent rows. Top connectors couple stereocilia just below the tip link, shaft links connect part or all the length of the stereocilia, and ankle links connect the stereocilia at the base (Goodyear and Richardson, 1999; Goodyear et al., 2005) (Fig. 3A). The ankle links are first detectable in the mouse at P0, but they are completely lost at P12 (Goodyear et al., 2005). Most of these links are recognised by specific antibodies and characterised by distinct biochemical properties (e.g. resistance to the calcium chelator BAPTA or the protease subtilisin). Whereas the ankle links are sensitive to both treatments, the tip link is only sensitive to BAPTA (see Goodyear et al., 2005). With the exception of the tip link (see below), the roles of the links in the growing and mature hair bundles are unknown. However their conservation throughout evolution, from fish (Sollner et al., 2004; Seiler et al., 2005), mice (Alagramam et al., 2001a; Di Palma et al., 2001; Wilson et al., 2001) to humans (Bork et al., 2001; Ahmed et al., 2002), strongly argues for their functional relevance. Their molecular composition is a key element because they will behave as more or less elastic connectors when submitted to the force of the mechanical stimulation, depending on their extracellular domain composition, their possible oligomerisation and binding to other ligands.

The mechanoelectrical transduction process

The highly elaborate organisation of the hair bundle is evolutionarily conserved in vertebrates and probably plays a crucial role in auditory and balance transduction. The time constant of the transduction process is too short (a few microseconds) to involve a second messenger. In response to sound or acceleration stimuli, the hair bundle deflects and pivots around the basal insertion points of the stereocilia. This causes variations in the opening probability of MET channels in the plasma membrane. These thought to result from changes in the tension of the tip link, which are proposed to be linked to these channels (Howard and Hudspeth, 1988) (see Fig. 3A). The rapid influx of cations, mostly K^+ but also Ca^{2+} leads to hair cell depolarisation followed by neurotransmitter release. Two TRP channels have been proposed to be components of the MET channel: NompC in zebrafish (Sidi et al., 2003) and TRPA1 (also called ANKTM1) in mammals (Corey et al., 2004).

Through adaptation processes, the hair bundle preserves its high sensitivity to extrinsic stimuli, i.e. the MET channel opening probability (Po) tends to be restored to its resting level while the mechanical stimulus persists. Fast and slow adaptation processes have been described in the hair cells. Fast adaptation is mediated by Ca^{2+} influx through the MET channel, which provides a feedback signal acting on the MET channel itself (Fettiplace and Ricci, 2003). Ca^{2+} also regulates a slower component of adaptation, which has been demonstrated to involve myosin 1c (Holt et al., 2002). Myosin VIIa has also been proposed to contribute to adaptation (Kros et al., 2002) (see below).

USH1 protein properties and interactions

The tails of unconventional myosins are thought to position

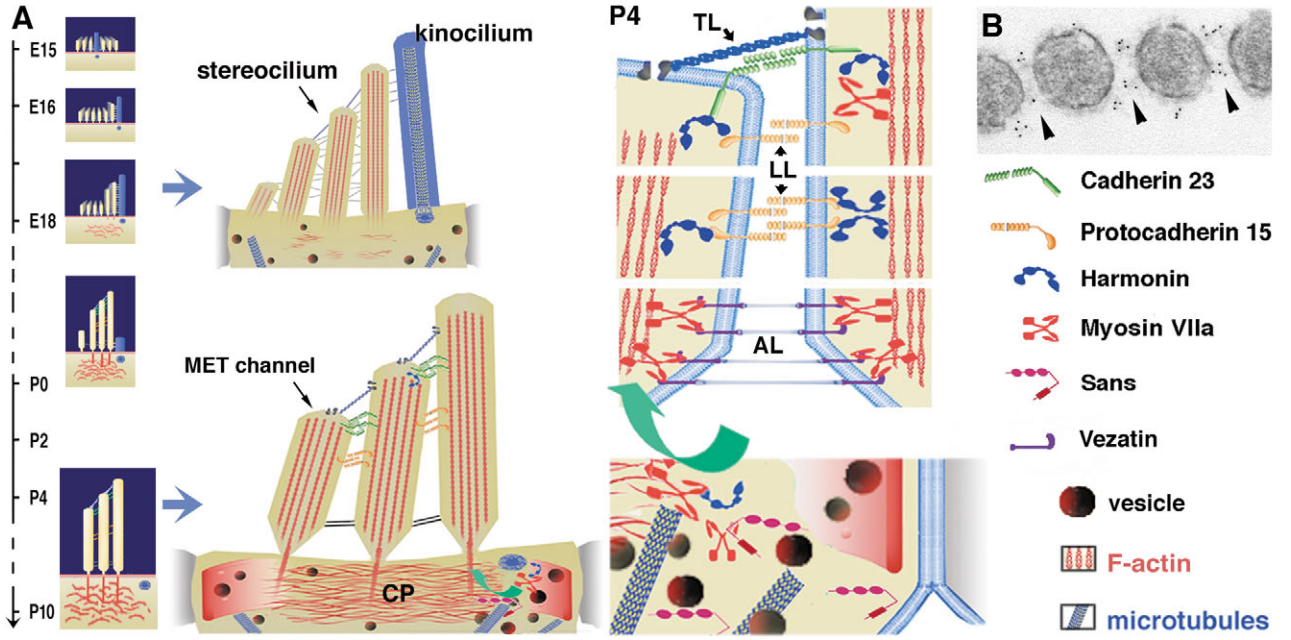

Fig. 3. (A) Early (E15-P0) and late (P4-P10) stages of auditory hair bundle maturation in mice. The stereocilia that form the hair bundle are held together by various side-links. The number and structure of these links vary during the course of development (top) and in mature (bottom) cells. At late developmental stages, the most central actin filaments of stereocilia insert their rootlets into another actin-rich structure, the cuticular plate (CP). Note that the kinocilium is no longer present in mature auditory hair cells, although its basal body persists. The tip link (TL) is believed to gate the mechanoelectrical transduction (MET) channel. (B) Immunogold labelling showing cadherin 23 is associated with links connecting growing stereocilia (adapted with permission from Michel et al., 2005). Protocadherin 15 also probably makes interstereocilia lateral links (LL) in the differentiating hair bundle. During hair bundle maturation, the binding of harmonin b to cadherin 23, protocadherin 15, and F-actin should anchor the interstereocilia links to the stereocilia actin core. Myosin VIIa is required for transfer of harmonin b up to the stereocilia (green arrow). Myosin VIIa also interacts with vezatin, a ubiquitous component of adherens junctions, which is associated with the ankle links (AL).

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these motors at a given intracellular location by interacting with protein(s) and/or lipid(s); the motor activity of the head is then harnessed to exert tension on the tethered molecules. These molecules (and possibly associated subcellular compartments) can then be moved or not and, in certain cases, can undergo conformational changes. Myosin VIIa is an actin–plus-end-directed unconventional myosin expected to move from the base to the tip of stereocilia in the hair bundle. It has been described as a dimeric motor (Inoue and Ikebe, 2003), but whether (as in the case of myosin VI) it can also function as a monomer is unknown. All myosins exert their motile activity during a cyclic interaction with actin filaments and ATP. The enzymatic characteristics of motor proteins are key determinants of their motile activity and underlie their various cellular roles (see De La Cruz and Ostap, 2004). Although the speed of myosin VIIa has been estimated at ~160 nm/second (Inoue and Ikebe, 2003), some of its kinetic parameters are yet to be studied. Hence, whether or not it is a processive motor remains to be established. How proteins associated with its tail might influence its activity is another unanswered question.

Myosin VIIa has a much higher affinity for ADP (K_{ADP}=7 μM) than for ATP (K_{ATP}=200 μM) and requires high ATP concentrations for its motile activity. ADP markedly increases the binding of myosin VIIa to actin filaments (Inoue and Ikebe, 2003). Therefore, in subcellular compartments that have high ADP concentrations, myosin VIIa may maintain tension rather than move cargo.

The involvement of type VII myosins in tension-mediated processes seems to be evolutionarily conserved in metazoan organisms, because inactivation of myosin VII in *Dictyostelium* amoeba inhibits particle ingestion (Titus, 1999) owing to defects in the adhesion of particles to the cell surface (Tuxworth et al., 2001). Tuxworth and Titus have proposed two non-exclusive roles for this motor protein (Tuxworth et al., 2001). First, myosin VII dimers capture membrane proteins 'floating' at the surface of the cell, allowing their clustering at the sites of contact formation. Second, through their motor domains, myosin VIIa molecules may act locally as force sensors, determining the strength of the interaction at the contact zone. For every receptor that can withstand the strain imposed by myosin, a non-motile protein anchored to the cytoskeleton replaces the myosin. The receptor thus becomes engaged in a stiff link until the contact area reaches a certain limit. Through recruitment of additional receptors and their coupling to the cytoskeleton, myosin VIIa would thus contribute to the mechanical stability of the adhesion sites required for efficient cell phagocytosis. Direct evidence for this model is still missing; however, it can explain the role of the direct interaction between mammalian myosin VIIa and vezatin, a component of adherens junctions (Kussel-Andermann et al., 2000), in the entry of *Listeria monocytogenes* into cells (Sousa et al., 2004). Myosin VIIa might have a similar role in the auditory hair bundle (see below).

Harmonin b, the longest harmonin isoform subclass, directly binds to F-actin through its C-terminal second coiled-coil and/or its PST domain (see Fig. 1A). When overexpressed in cells, it induces the formation of large F-actin bundles resistant to cytochalasin D or latrunculin A, which suggests that harmonin b stabilises actin filaments. It also induces bundling of actin filaments in vitro (Boëda et al., 2002).

Cadherin 23 and protocadherin 15 differ from the classical cadherins (e.g. E- and N-cadherins) by possessing numerous EC repeats (Fig. 1A). Transfected L929 cells expressing full-length cadherin 23 (L-CDH23 cells) form Ca^{2+}-dependent cell-cell contacts, indicating that cadherin 23 can mediate Ca^{2+}-dependent, homophilic cell-cell adhesion (Siemens et al., 2004). Whether protocadherin 15 also undergoes homophilic interactions is unknown. These two cadherin cytoplasmic regions share no homology with any known protein. In particular, they lack the consensus motif for binding to β-catenin (Bershadsky, 2004). This raises the question of how these two cadherins are linked to the actin cytoskeleton.

Yeast two-hybrid screenings combined with biochemical analyses have shown that the five known USH1 proteins each directly interact with at least one other USH1 protein (Fig. 1B). Harmonin can bind to any of the other USH1 proteins through its PDZ domains (Boëda et al., 2002; Siemens et al., 2002; Weil et al., 2003; Adato et al., 2005). With the exception of myosin VIIa, the four other USH1 proteins harbour class I PDZ-binding motifs at their C-terminal ends. However, harmonin's in vitro interactions with either of these proteins is not interrupted by the deletion of these C-terminal PDZ-binding motifs (Adato et al., 2005). Therefore, the PDZ interactions of harmonin with USH1 proteins are not characterized by simple classical binding of C-terminal motifs.

The Sans central region is involved in the Sans–myosin-VIIa interaction and Sans homomerisation, whereas the SAM domain binds to harmonin. The existence of the two distinct and independent interaction motifs is supported by the formation of an in vitro Sans-Sans-harmonin tripartite complex (Adato et al., 2005) (see Fig. 1B). The common binding domains of harmonin and Sans for several other USH1 proteins, as well as their involvement in homo-oligomerisation (Sans) or hetero-oligomerisation (harmonin isoforms) strongly suggest that some of these interactions are competitive (Adato et al., 2005).

USH1 proteins and the cohesion of the growing hair bundle

USH1 proteins are expressed in the hair cells throughout life, although their subcellular distributions in the stereocilia vary dramatically during development up to the mature stage (Fig. 4). All are expressed in the growing auditory hair bundle. However, whereas myosin VIIa, cadherin 23, harmonin and protocadherin 15 are present within the stereocilia (and some are also within the kinocilium), Sans has been detected only transiently within the auditory kinocilium.

Harmonin, cadherin 23 and protocadherin 15 are present in the hair bundle as soon as it emerges at the apical surface of the sensory cells (Boëda et al., 2002; Ahmed et al., 2003). Harmonin b is present mainly at the tips of stereocilia during early postnatal stages (Boëda et al., 2002). Cadherin 23 first occupies the entire length of the emerging stereocilia and then becomes progressively restricted to the tip region (see Fig. 4). Cadherin 23 can no longer be detected in the auditory hair bundle after postnatal day 14, but the labelling persists along the kinocilium of the vestibular organs, where it may contribute to kinociliary links (Lagziel et al., 2005; Michel et al., 2005). Protocadherin 15 has been detected uniformly distributed along the growing stereocilia (Ahmed et al., 2003)

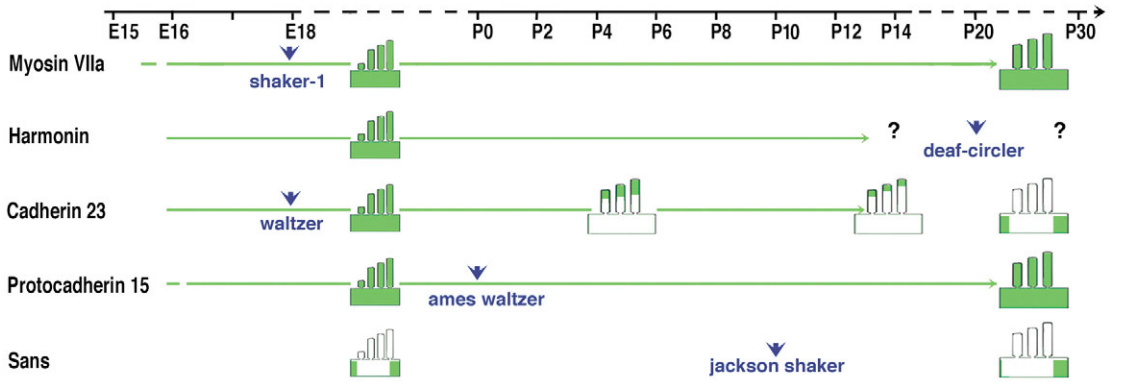

Fig. 4. Distributions of USH1 proteins in developing and adult hair cells throughout life, their spatial and temporal subcellular distribution vary for each molecule. Green arrows indicate the presence of a USH1 protein in the stereocilia. Myosin VIIa (Hasson et al., 1997) and protocadherin 15 (Ahmed et al., 2003) are present along the entire length of the stereocilia. The precise distribution of harmonin in the stereocilia at the postnatal stages remains to be established. Notice that, at P30, cadherin 23 disappears from the stereocilia and appears to move to the pericuticular necklace (Lagziel et al., 2005; Michel et al., 2005). Sans is no longer present in the stereocilia (Adato et al., 2005). Arrowheads refer to the stage when first signs of hair bundle anomalies have been reported in USH1 mouse mutants; E18 in shaker-1 (Self et al., 1998) and waltzer (Holme and Steel, 2002) mutants, and P0 in Ames waltzer (Washington et al., 2005). In Jackson shaker and deaf-circler mutants, the hair bundle disorganisation has been reported only from P10 (Kitamura et al., 1992), and P21 (Johnson et al., 2003), respectively.

(see Fig. 4). Myosin VIIa is present in the emerging hair bundles (El-Amraoui et al., 1996; Boëda et al., 2002) and also within the cuticular plate and the pericuticular necklace region, which is characterised by a dense ring of vesicles (Hasson et al., 1997). Sans is especially abundant in a vesicle-rich region in the immediate vicinity of the basal body of the kinocilium (Adato et al., 2005) (see Fig. 3A, Fig. 4). For harmonin and protocadherin 15, their precise subcellular localisations in the course of auditory hair bundle differentiation remain to be determined.

On the basis of the in vitro direct interaction between cadherin 23 and harmonin, the co-localisation of cadherin 23 and harmonin b in the forming hair bundle and the actin binding activity of harmonin b, we have proposed that cadherin-23-containing stereocilia connectors are anchored to the actin core of the stereocilium through harmonin b, thereby ensuring hair bundle cohesion during early morphogenesis (Fig. 3A) (Boëda et al., 2002). Hair bundle disorganisation has since been reported in the deaf circler 2J mouse mutant, which lacks harmonin b isoforms (Johnson et al., 2003). Also, almost all DFNB18 patients carry mutations in *USH1C* that selectively affect the harmonin b subclass (Ouyang et al., 2002). Moreover, ultrastructural studies have recently established that cadherin 23 is a component of both interstereocilia and kinociliary links of the growing hair bundle (Michel et al., 2005) (see Fig. 3A,B). Protocadherin 15, which can bind to harmonin b (Adato et al., 2005), may play a role similar to that of cadherin 23 in hair bundle morphogenesis (see Fig. 3A). Whether protocadherin 15 and cadherin 23 can form links through heterophilic interactions is not yet known. Notice that, genetic evidence in mice and humans supports the involvement of these two cadherins in the same pathway, although it is unclear whether the late effect reported reflects the early roles of the two cadherins (Zheng et al., 2005).

Since it is present throughout the entire hair cell, myosin VIIa probably has several functions. We analysed harmonin b distribution in the Myo7a^{4626SB} shaker-1 mice, which carry a premature stop codon in the motor domain of myosin VIIa (Gln520→Stop) and have severely disorganized hair bundles (see Kros et al., 2002). In these mice, harmonin b cannot be detected at the stereocilia tip and accumulates beneath the hair bundle (Boëda et al., 2002). This indicates that myosin VIIa is involved in the sorting and/or targeting of harmonin b to the hair bundle. In Jackson-shaker mutants, harmonin b is also absent from the hair bundle (Gaëlle Lefèvre, personal communication), which suggests that Sans, which interacts with myosin VIIa, is also involved in the sorting of harmonin b. These two proteins may also be involved in the sorting of other hair bundle proteins. Within the hair bundle, myosin VIIa might have other functions. It may cluster cadherin-23–protocadherin-15–harmonin complexes, thereby organising the spatial distribution of the hair bundle links. Myosin VIIa may also act as a sensor, providing a dynamic link between interstereocilia connectors and the actin filaments, through which the cell may measure the tension force at a given position along the stereocilium membrane.

The crucial role of interstereocilia and kinociliary links in the cohesion of the growing hair bundles discussed here manifests itself when stereocilia have no or underdeveloped actin rootlets. At that time, the connection between the hair bundle and the apical region of the hair cell body seems mainly to involve microtubules that arise from the kinocilium basal body (Tilney et al., 1992) (see Fig. 3A). Subsequently, the anchoring of stereocilia actin rootlets in the cuticular plate confers additional stability upon the hair bundle; fibrous links that connect these rootlets to horizontal actin filaments in the cuticular plate horizontal actin filaments also participate (Tilney et al., 1992). Unconventional myosins, including myosin VIIa, in the cuticular plate may regulate the stability of the stereocilium–cuticular-plate anchor.

In addition, myosin VIIa can bind to vezatin, a transmembrane protein that colocalises with ankle links and an ubiquitous protein component of adherens junctions (Kussel-Andermann et al., 2000). Myosin VIIa may also strengthen the adhesion at the junctions between the hair cells and supporting cells and thus contribute to the stability of the cuticular plate.

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How these elements are subsequently involved in the development and/or the maintenance of hair bundle cohesion remains to be clarified.

Other roles of USH1 proteins in the hair cell

Seven mutations in the *Myo7a* gene have been described and have varying degrees of phenotypic severity (Mburu et al., 1997). In the original shaker-1 mutant, the *Myo7a*^{sh1} mutation is an Arg502Pro substitution in a poorly conserved surface loop of the motor domain. This change is associated with the mildest of the pathological effects seen among shaker-1 alleles (Self et al., 1998). EM scanning analysis of the hair bundles in these Myo7a^{sh1}shaker-1 mutants revealed normal stereocilia development in contrast to other shaker-1 alleles – for example, Myo7a^{6J} or Myo7a^{4626SB} (Self et al., 1998; Kros et al., 2002). Also, whereas in Myo7a^{4626SB} mice (Gln520→Stop) harmonin b is absent from the stereocilia, normal harmonin b distribution is observed in the stereocilia tip of Myo7a^{sh1} mutants (A.E.-A., unpublished). The mutant myosin VIIa molecule in the Myo7a^{sh1} mice seems to have conserved sufficient activity to support normal early development of the stereocilia and proper harmonin b targeting. This further supports the correlation between the presence of harmonin b in the stereocilia and normal hair bundle organisation. However, the progressive hearing loss manifested by Myo7a^{sh1} mutant mice (Self et al., 1998) indicates that, besides its function in stereocilia development, myosin VIIa plays unidentified roles in the hair cell.

Kros et al. have shown that, in Myo7a^{6J} and Myo7a^{4626SB} shaker-1 mice, the auditory mechanotransduction slow-adaptation process is impaired at an early postnatal stage (Kros et al., 2002). Whether the defective adaptation in these two mutants is due to a failure of myosin VIIa function at the mechano-electrical transduction machinery itself or from a more general perturbation of the dynamic properties of the entire hair bundle is not known. Irrespective of the exact underlying mechanism, it will therefore be interesting to characterise hair cell adaptation in Myo7a^{sh1} shaker-1 mutants.

The various isoforms of cadherin 23 may also perform additional functions beyond the maintenance of the growing hair bundle cohesion. Two recent studies using antibodies directed against part of its cytoplasmic region localized cadherin 23 to the tip of stereocilia in the mature hair bundle, which suggests that cadherin 23 dimers may form the tip links (Siemens et al., 2004; Sollner et al., 2004). By contrast, as mentioned above, two other groups failed to detect cadherin 23 at the tip of stereocilia from mature hair bundles by antibodies raised against an extracellular epitope [EC11, epitope N1 (Michel et al., 2005)] or other parts of its cytoplasmic region (Boëda et al., 2002; Lagziel et al., 2005; Michel et al., 2005). Notice that cadherin 23 does not completely disappear from the auditory hair cell, because labelling is observed in the vesicle-rich pericuticular region of the hair cell. This suggests that it still plays a role in adult (Michel et al., 2005). Definition of the temporal and spatial subcellular localisation of the different cadherin 23 isoforms might help clarify the above observations.

USH1 proteins in the retina

Although none of the mice that have mutations in USH1 orthologues display retinitis pigmentosa, minor electroretinographic abnormalities (a reduction of the amplitude of the wave response to light) have been reported in some shaker-1 and waltzer mutants (Libby et al., 2003). These have not been seen in Ames waltzer (Ball et al., 2003) and deaf circler mutant mice (Johnson et al., 2003). Slight peripheral retinal degeneration has been found in 9-month-old deaf circler mutants, however (Johnson et al., 2003).

Because opsin accumulates in the photoreceptor inner segment and connecting cilium in shaker-1 mice, myosin VIIa could function in the transport of opsin (Liu et al., 1999) (Fig. 5A). Moreover, in the absence of myosin VIIa, there is a significant decrease in outer disk phagocytosis by pigment epithelial cells (Gibbs et al., 2003). The renewal of photoreceptor outer disk membranes involves continual addition of new disks at the base of the outer segment, the shedding of the most distal ones and their phagocytosis by the pigment epithelial cells (Fig. 5). In pigment epithelial cells of shaker-1 mice, the transfer of ingested phagosomes from the apical cell processes to the cell body is delayed (Gibbs et al., 2003). Also, EM studies in shaker-1 mice showed that melanosomes are not spread throughout the apical projections of these cells but cluster around the nucleus. This suggests that myosin VIIa is necessary for the transfer of these organelles from the cell periphery to the apical microvilli (Liu et al., 1998).

MyRIP/Slac2c, a protein that binds to myosin VIIa tail and directly interacts with Rab27a (El-Amraoui et al., 2002; Fukuda and Kuroda, 2002) and with F-actin (Desnos et al., 2003) might be involved in this transport process (see Fig. 5B). Among the 18 classes that constitute the myosin superfamily, myosins I, II, V and VI have been implicated in the transport of organelles such as vacuoles, recycling endosomes, lysosomes, secretory granules and melanosomes (reviewed in Seabra and Coudrier, 2004). In particular, actin-dependent capture of the melanosomes has been shown to involve a myosin V motor in skin melanocytes (see Seabra and Coudrier, 2004). In these cells, a complex formed by Rab27a, a MyRIP homolog called melanophilin and myosin Va is involved. In melanocytes isolated from mice that have defective Rab27a (*leaden* mutants), melanophilin (*ashen* mutants), or myosin Va (*dilute* mutants), melanosomes are abnormally clustered around the nucleus, as in retinal pigment epithelial cells in shaker-1 mutants. In melanophilin-depleted skin melanocytes, ectopic overexpression of myosin VIIa alone does not rescue the clustering phenotype, whereas overexpression of myosin VIIa together with MyRIP restores normal peripheral melanosome distribution (Kuroda and Fukuda, 2005). MyRIP thus acts as a myosin VIIa linker in vivo, and a Rab27A–MyRIP–myosin-VIIa tripartite complex appears to function in the transfer of retinal melanosomes to the actin cytoskeleton, a prerequisite for their entry into the apical microvilli.

Despite the apparent diversity of the phenotypic abnormalities observed in shaker-1 mice, we can speculate that some of these abnormalities are related to the same myosin VIIa activity. An attractive possibility is that the failure of protein/organelle translocation (harmonin b sorting, opsin transport, melanosome delivery and phagosome transfer) is due to a defect in the transport role of myosin VIIa at the interface between microtubules and actin filaments (green arrows, Figs 3, 5). Indeed, myosin VIIa has been reported to interact with

the microtubule-associated protein MAP2B (Todorov et al., 2001). The MyTH4-FERM domains of myosin X interact directly with microtubules (Weber et al., 2004). The myosin VIIa MyTH4-FERM domains might therefore bind to microtubules in a similar way.

The presence of all USH1 proteins at the photoreceptor synaptic active zone strongly suggests they contribute to the trafficking of synaptic vesicles (Reiners et al., 2003; Reiners et al., 2005). The bulk of protocadherin 15 labelling is, however, present in the photoreceptor outer segments in the monkey and human retina (Ahmed et al., 2003), which is consistent with its having a role in the organisation of the photoreceptor outer disks. During photoreceptor outer disk outgrowth, protocadherin 15, through its extracellular domain, may provide the cell with information about its environment, in particular its proximity to the apical microvilli of the pigment epithelial cells. Targeted ablation of a photoreceptor-specific member of the cadherin superfamily, prCAD, results in outer disk disorganisation and fragmentation. PrCAD is confined to the base of the photoreceptor outer segment, where it may interact either with extracellular matrix components or with photoreceptor membrane proteins (Rattner et al., 2001). Protocadherin 15 is, by contrast, present along the entire length of the outer segments (Ahmed et al., 2003). Protocadherin 15 may thus connect adjacent outer disks through homophilic interactions.

Protocadherin 15 may also engage in heterophilic interactions with an as yet unknown cadherin present at the membrane of pigment epithelial cells (see Fig. 5C). In the latter case, protocadherin 15 may contribute to photoreceptor-RPE adhesion by anchoring the membranes of these two cell types. Whether it also interacts with other cell surface or extracellular matrix proteins cannot be excluded. The failure of such interactions may explain the abnormal alignment of photoreceptor outer disks with the pigment epithelial cells in the pcdh15b-deficient zebrafish (Seiler et al., 2005). Although little is known about the cytoskeletal elements and/or associated proteins within the outer disk, harmonin b has been shown to be restricted to the photoreceptor outer segment (Reiners et al., 2003). Therefore, it is worth considering the possibility that protocadherin-15–harmonin-b complexes play a role in the organisation of the hair bundle in the inner ear (Fig. 3A) and the photoreceptor outer disks in the retina (Fig. 5C).

It is unclear why mice lacking orthologues of USH1 proteins

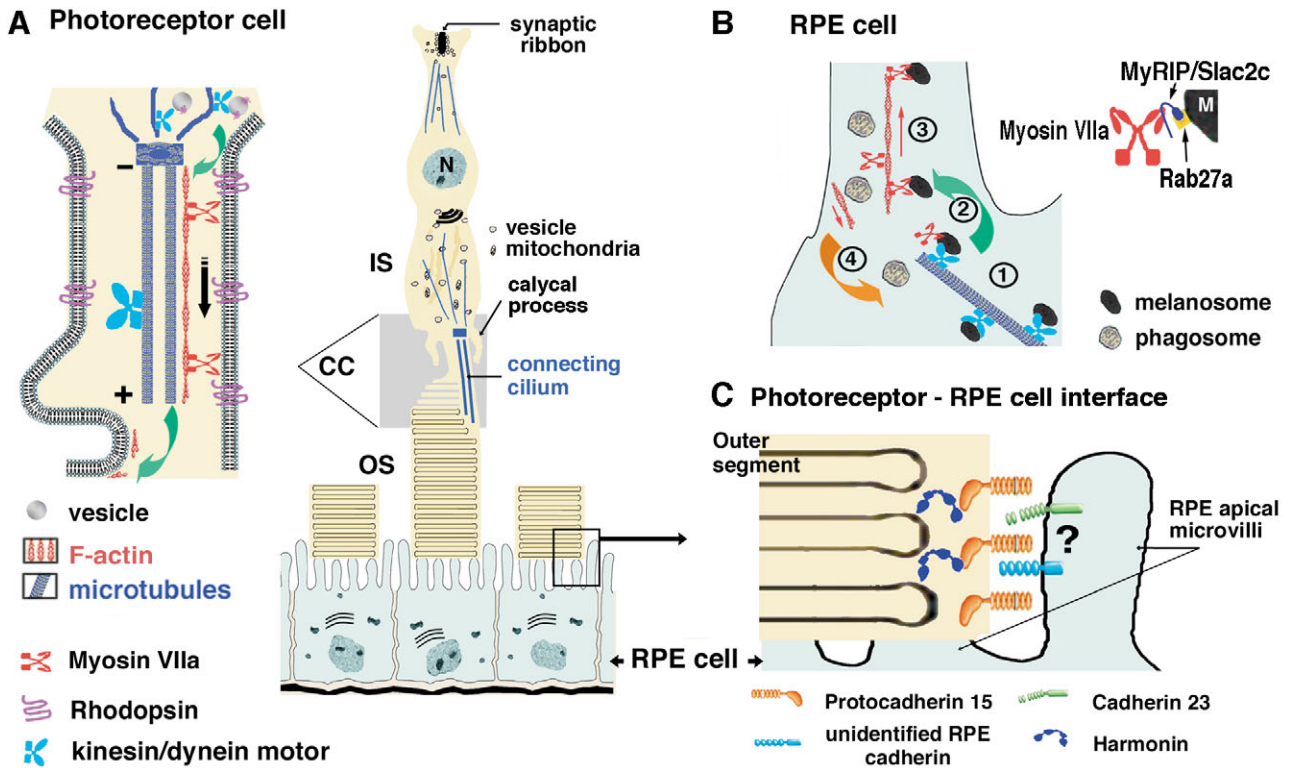

Fig. 5. (A) Photoreceptor and retinal pigment epithelial (RPE) cells. At the tip of the photoreceptor inner segment (IS), myosin VIIa may be involved in opsin transfer (green arrow) to the connecting cilium and its transport to the outer segments (OS). (B) In RPE cells, melanosomes (M) display fast, bidirectional microtubule-dependent long-range movements in the cell body driven by kinesin/dynein motor proteins (1). Upon reaching the plus end of the microtubule at the periphery, myosin VIIa may be involved in the transfer (green arrow) of these organelles towards the actin filaments (2). Rab27a, which is targeted to the melanosome membrane, interacts with its effector, MyRIP/Slac2c, which in turn binds to myosin VIIa. Myosin VIIa then enables the retention and/or local movement of the melanosomes along the actin filaments of the microvilli (3). Myosin VIIa also plays a role in the transfer of phagosomes from the microvilli to the cell body (4; orange arrow). (C) At outer-disk–RPE-cell interface, protocadherin 15 present at the membrane of photoreceptor outer segments may serve to sense its immediate environment. For instance, through its extracellular domain, it may engage in heterophilic interactions with unidentified cadherin(s) in RPE cells to ensure proper alignment of the outer disks and apical microvilli. Harmonin b expressed in the photoreceptor outer disks is expected also to play a role in disk structure. N, nucleus.

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do not suffer from retinitis pigmentosa. Several explanations could account for this difference between humans and mice. These include the shorter lifespan of the mouse, differences in light exposure experienced by humans and mice, functional redundancy of USH1 proteins in the human but not the mouse retina, or the presence of highly developed calycal processes in human but not mouse photoreceptor cells. The calycal processes are microvillus-like extensions of the photoreceptor inner segment that cup the base of the outer segment (see Fig. 5A) and are connected to the connecting cilium stalk plasma membrane by fibrous links. They share common components with hair bundle links. For instance, ALA (ankle link antigen), which associates with the ankle links in the hair bundle, also colocalises with links bridging the calycal processes and the connecting cilium (Goodyear and Richardson, 1999). The functions of calycal processes, first described more than 40 years ago (Cohen, 1963), have received little attention, however.

Perspectives

The study of USH1 proteins strongly suggests that some fibrous hair bundle extracellular links have a crucial role in the cohesion of the growing hair bundle during auditory hair cell differentiation. This raises the question of the respective contribution of interstereocilia and kinociliary links to the cohesion of the forming hair bundle. We must also establish that hair bundles are disorganised in the early developmental stages of all mutants that have defective USH1 proteins. Additional USH1 proteins will undoubtedly be discovered in the near future. This should help us further decipher this molecular network, which at present contains no known regulatory molecule. Moreover, recent evidence suggests that the transmembrane (TM) usherin isoforms, encoded by the *USH2A* gene, are found within other interstereocilia links (Avital Adato, personal communication). Furthermore, in the hair cells of mutant mice lacking Vlgr1 (very large G-protein-coupled receptor-1, also called Mass1), a transmembrane protein defective in USH2C (Weston et al., 2004), the hair bundle appear rounded and deformed, with a loss of stereocilia cohesion (Johnston et al., 2005). Similarly to TM usherin, Vlgr1 has a long extracellular modular ectodomain (~180 nm), and is thus qualified to form interstereocilia links. Whether the other USH2 or USH3 proteins are also involved in connecting stereocilia is similarly worth considering.

Adhesion contacts and associated cytoskeletal networks have been shown to operate as tension-sensing devices that trigger signal transduction to orchestrate the cellular response (see Ingber, 2003). Such signaling cascades, however, have not yet been addressed in adhesion sites at the level of stereocilia connecting links. It is worth considering that the developing hair bundle is a tensegrity-based structure and that link-mediated adhesion forces can act as active elements that affect the tensegrity force balance that controls and preserves hair bundle three-dimentional architecture, differentiation and function.

Most of the USH1 proteins are expressed in mature sensory cells, both in the inner ear and in the retina. Their roles in fully differentiated sensory cells are not known. However, this could also be addressed by genetic approaches, provided new tools are developed to circumvent the early abnormal phenotype we discuss here.

Finally, the studies discussed here are fuelled largely by the need for a deeper understanding of the roles played by USH1 proteins in the two sensory organs so that we can develop therapy or prevent (in the case of the visual defect) Usher syndrome in humans. Clarification of the pathogenesis of the retinal degeneration observed in humans suffering from USH1 is required and calls for more favourable animal models.

We are grateful to Jean-Pierre Hardelin, Gaelle Lefèvre and Raphaël Étournay for stimulating discussions, to Avital Adato for her critical reading of the manuscript, to Jacqueline Levilliers for help in the preparation of the document and to Marc Lenoir for the SEM image. We apologise for not citing all the relevant references because of space limitations. The work in the authors' laboratory is supported by the R. and G. Strittmatter Foundation, the A. and M. Suchert Forschung contra Blindheit-Initiative Usher Syndrom.

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