Chapter 13
Cadherins in the Auditory Sensory Organ

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Abstract The exquisite sensitivity and frequency tuning of hearing depend on the correct structure and functioning of the auditory sensory hair cells, the neighbouring supporting cells, and the homeostasis of their ionic environment. The increasing number of adhesion proteins identified as causing hearing impairment in humans and mice when defective is consistent with a critical role for cell–cell junctions between neighbouring epithelial cells of the cochlea, and of fibrous links within the hair bundle, the sensory hair cell structure responsible for sound reception. Classical cadherins and/or associated adherens-junction proteins, such as p120-catenin or nectin 3, have been shown to be essential for establishment of the regular mosaic cellular pattern of the auditory sensory epithelium. Two cadherin-related proteins, protocadherin-15 and cadherin-23, are key components of both lateral links and tip-links in hair bundles; they are essential components of the mechanoelectrical transduction machinery. Studies of the role of these adhesion proteins and of the pathogenesis of the forms of deafness caused by defects of these proteins have provided considerable insight into the development and functioning of the auditory sensory epithelium, and of the hair cells in particular.

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13.1 Introduction

Our ability to discriminate sounds in noisy environments and to understand speech involves some unique properties of the mechanoreceptive sensory cells of the auditory sense organ (the cochlea), the outer hair cells (OHCs), which amplify the sound stimulus, and the inner hair cells (IHCs), the genuine sensory cells responsible for transmitting the sound-induced electrical signal to the primary auditory neurons (Fig. 13.1a–c). In mammals, hair cells are organised in a regular mosaic pattern running from the base to the apex of the snail-shaped cochlea. The requirement for strict control over ion homeostasis, and for the adhesive strength and stability of cell–cell contacts renders the cochlea very sensitive to defects of adhesion-related proteins (see El-Amraoui and Petit 2010; Richardson et al. 2011). Defects of several genes encoding adhesion proteins have been found to be responsible for hearing impairment in humans (see Table 13.1) and/or mice (http://hearingimpairment.jax.org/). The genes implicated in hearing impairment in humans include those encoding cadherin-23 (*CDH23*) and protocadherin-15

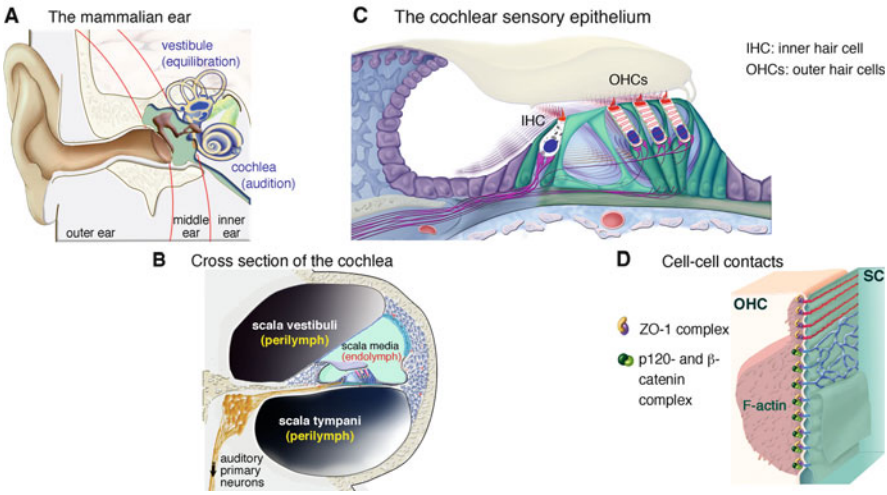

Fig. 13.1 The mammalian ear, the inner ear, and the hair cells, the sensory transducers. (**a**) The inner ear contains three semi-circular canals, the utricle and the saccule (balance organs) and the cochlea (auditory organ). (**b**) A cross-section through the cochlea showing the three fluid-filled compartments: scala vestibuli, scala tympani, and scala media. (**c**) The scala media house the auditory sensory epithelium, the organ of Corti (OC), consisting of one row of inner hair cells (IHCs), three rows of OHCs, and various types of supporting cells. (**d**) At the atypical cell–cell junctions between OHCs and Deiters cell (DC), catenin complexes are distributed together with claudin-containing domains (Adapted from Nunes et al. 2006)

Table 13.1 Cadherins and cell–cell junction proteins involved in inherited human diseases

Proteins present in cell-cell contacts
***CLDN14*, claudin-14** (OMIM 605608)
Sensorineural recessive deafness, DFNB29 (OMIM 614035)
TRIC, MARVELD2 (OMIM 610153)
Sensorineural recessive deafness, DFNB49 (OMIM 610572)
***ILDR1*, immunoglobulin-like domain containing receptor 1** (OMIM 609739)
Sensorineural recessive deafness, DFNB42 (OMIM 609646)
TJP2, tight-junction protein 2 (OMIM 607709)
Sensorineural dominant deafness, DFNA51 (OMIM 6613558)
Proteins present in hair bundles
***CDH23*, cadherin-23** (OMIM 605516)
Sensorineural recessive deafness, DFNB12 (OMIM 601386) and Usher syndrome type 1D (OMIM 601067)
***PCDH15*, protocadherin-15** (OMIM 605514)
Sensorineural recessive deafness, DFNB23 (OMIM 609533) and Usher syndrome type 1F (OMIM 602083)
Proteins present in cell-cell contacts & hair bundles
***VEZT*, vezatin, an adherens junction protein**
Sensorineural recessive deafness, DFNBi (Bahloul et al. 2010)

The OMIM numbers contain the links between the indicated molecules and the related diseases

(*PCDH15*), which are responsible for syndromic (Usher syndrome) and isolated forms of deafness (Ahmed et al. 2001; Alagramam et al. 2001b; Bolz et al. 2001; Bork et al. 2001); vezatin (*VEZT)*, which causes an autosomal recessive form of deafness (DFNBi; Bahloul et al. 2009); and several tight junction proteins, such as claudin-14 (*CLDN14),* tricellulin (*TRIC*), and immunoglobulin-like domain-containing receptor 1 (*ILDR1*), responsible for the DFNB29 (Wilcox et al. 2001), DFNB49 (Riazuddin et al. 2006), and DFNB48 (Borck et al. 2011) recessive forms of deafness, respectively. In addition, mutations of the gene encoding tight-junction protein 2 (*TJP2)* cause the DFNA51 dominant form of deafness (Walsh et al. 2010).

The Ca^{2+}-dependent adhesion molecules of the cadherin superfamily play a key role in cell–cell adhesion, acting in concert with other intercellular or matrix-associated proteins. About 114 cadherins and cadherin-related molecules have been identified in humans (Hulpiau et al. 2013). During development and at mature stages, epithelial and neuronal cells express a "cadherin code" involved in cell fate specification, morphogenetic and cellular rearrangements, the maintenance of tissue integrity, and intracellular signal transduction (Nelson 2008; Harris and Tepass 2010; Takeichi 2011; Hirano and Takeichi 2012; El-Amraoui and Petit 2013). This review focuses on recent advances towards understanding the contribution of cadherins to the development and functioning of the auditory sensory organ. The detailed molecular mechanisms underlying the role of cadherins in the early steps of otic organogenesis, differentiation of the inner ear sensory epithelia, and cochlear compartment morphogenesis are not well understood. By contrast, large

amounts of data have been accumulated concerning the role of two cadherin-related proteins, cadherin-23 and protocadherin-15, in developing and mature hair cells.

13.2 Structure of the Mammalian Auditory Organ: Focus on Membrane–Membrane Contacts

The mammalian inner ear consists of various sense organs: the utricle, the saccule, and three semi-circular canals (forming the balance organ), which detect linear and angular accelerations, and the cochlea, which detects sound waves (Fig. 13.1a). The cochlea consists of three fluid-filled compartments of different ionic compositions: the scala vestibuli and the scala tympani, both filled with perilymph, and the scala media (cochlear duct) filled with endolymph (Fig. 13.1b). Endolymph is an unusual fluid with a high K^+ concentrations and a low Na^+ concentration (Fig. 13.1b, c), whereas perilymph has an ion composition more typical of extracellular fluids (high Na^+ concentration and low K^+ concentration) (Wangemann 2006). The apical surface of the hair cells and supporting cells is bathed in endolymph, whereas the cell bodies of these cells are surrounded by perilymph. The absence of paracellular permeability between the endolymphatic and perilymphatic compartments of the cochlea is crucial for hearing. The cells of the sensory epithelium experience prolonged mechanical stress due to the sound-induced motion of this epithelium. A resilient epithelial barrier is required to withstand such mechanical stress and to prevent the leakage of fluid between adjacent cells of the auditory sensory epithelium. Evolution has led to adaptive changes in structure at the junctions between OHCs and their neighbouring supporting cells (Deiters cells) (Togashi et al. 2011; Fukuda et al. 2014; Fig. 13.1d), to maintain the basoapical axial forces involved in OHC electromotility, membrane potential-driven changes in cell length mediating the frequency-tuned cochlear amplification (Dallos et al. 2008). Specialised junctions connect OHCs with their four neighbouring supporting cells. These large hybrid junctions (about 3–5 μm high) are unique in that they combine the ultrastructural features of tight junctions and adherens junctions, hence their name of ‘tight-adherens junctions’ (see Fig. 13.1d). At these junctions, claudins are arranged into claudin-14 and claudin-9/6 subdomain strands. At least three adherens junction proteins, p120-catenin, and the α- and β-catenins, colocalise with the claudin-9/6 subdomain and recruit a dense cytoskeletal actin network (Nunes et al. 2006).

The integrity of cadherin-mediated extracellular contacts between opposing membranes of the same cell also are essential for normal hearing. Indeed, fibrous extracellular links connect the cell apical membranes in a highly organised structure within the hair cell, the hair bundle. The hair bundles are the site at which sound waves are converted into electrical signals by a process of mechanoelectrical transduction (MET) (Corey and Hudspeth 1983) (Fig. 13.2a, b). The hair bundle, a V-shaped structure that crowns the apical surface of the hair cell, consists of 20–300 actin-filled, stiff microvilli, the stereocilia, organised into three rows with a

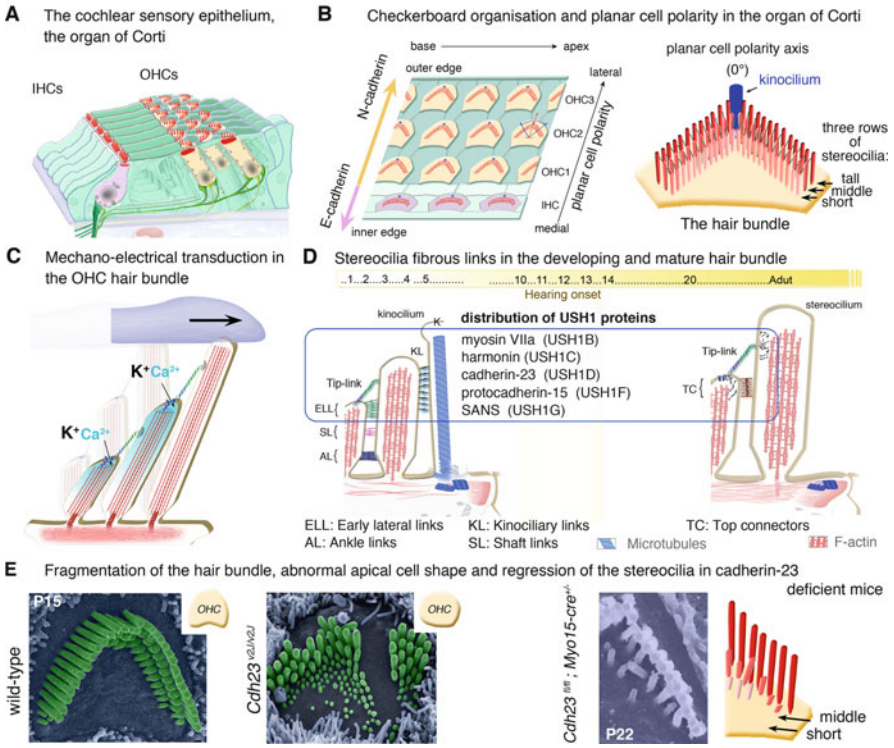

Fig. 13.2 The planar cell polarity of the auditory sensory epithelium, and hair bundle structure. (**a**) A top view of the organ of Corti showing the typical checkerboard-like organisation of the auditory sensory epithelium. (**b**) Positioning of the kinocilium, which directs the staircase pattern of the hair bundle, and determines the planar cell polarity axis. (**c**) Deflection of the hair bundle in the direction of the tall stereocilia leads to MET channel opening, K^+ influx and cell depolarisation. (**d**) Within the hair bundle, the stereocilia are connected by different types of transient and persistent extracellular links. The early transient lateral links (*ELL*), shaft links (*SL*), ankle links (*AL*), and kinociliary links (*KL*) are progressively replaced by the apical top connectors (*TC*) found in mature outer hair cells. The tip-links appear during development and persist in mature hair cells. (**e**) *Left* and *middle panels*: hair bundle disorganisation in a cadherin-23 deficient mouse ($Cdh23^{v2j/v2j}$). The *insets* are *top* views of the cell apical circumference, illustrating the loss of shape transition in $Cdh23^{v2j/v2j}$ mice: the cadherin-23-deficient OHC is round rather than having a convex (typically hexagonal) circumference. *Right panels*: in a mouse with postnatal loss of cadherin-23 (conditional knockout mouse, $Cdh23^{fl/fl}Myo15\text{-}Cre^{+/-}$), the tip-links disappear, and the stereocilia of the *short* and *middle rows* have regressed by P22

gradient in height, yielding a staircase-like structure. A single primary cilium, the kinocilium, and its basal body are located at the vertex of the V-shaped hair bundle (Fig. 13.2b). The kinocilium is thought to serve as a 'guidepost', determining the orientation and morphology of the hair bundle. Along the longitudinal axis of the cochlea, all the vertices of the hair bundles point in the same direction, towards the lateral wall of the scala media. This establishes a uniform polarity in the auditory sensory epithelium, the organ of Corti (Jones and Chen 2008; Kelly and Chen 2009;

Ezan and Montcouquiol 2013) (Fig. 13.2b). The kinocilium has been lost from the mature hair bundles of mammalian auditory hair cells (see Fig. 13.2c, d).

13.3 Cadherins, Organogenesis, and Planar Cell Polarity in the Cochlea

The auditory sensory epithelium has, over the years, proved very useful as a model system for studying planar cell polarity (PCP). Several excellent reviews have been published on hair cell polarity, PCP signalling pathways, and the relationships between these processes and the concomitant remodelling of apical junction complexes in the hair cells and the supporting cells (Ezan and Montcouquiol 2013; Jones and Chen 2008; Kelly and Chen 2009; Montcouquiol et al. 2006). The organ of Corti acquires a checkerboard-like pattern due to the arrangement of hair cells into a single row of IHCs and three rows of OHCs, interspersed with several types of supporting cells: the inner and outer pillar cells, Deiters, and phalangeal cells (see Fig. 13.2a). In the mouse, this organisation into different rows of cells occurs via a convergent extension process (Ezan and Montcouquiol 2013; Jones and Chen 2008; Kelly and Chen 2009; Montcouquiol et al. 2006). On embryonic day 14.5 (E14.5), all postmitotic hair cells and supporting cells are tightly packed and confined to a small region at the base of the cochlear duct. From E14.5 to E18.5, these differentiating cells undergo extensive morphogenetic movements along the elongating cochlear duct, leading to a thinning and lengthening of the epithelium due to cell intercalations, and the remodelling of cell–cell junctions (Keller et al. 2000; Kelly and Chen 2007; Etournay et al. 2010; Lepelletier et al. 2013).

The integrity and strength of the physical coupling between hair cells and supporting cells have been shown to affect hair cell polarity and the morphology of the hair bundles (Choi and Peifer 2011; Fukuda et al. 2014). A lack of nectin 3, an adherens junction protein of supporting cells, has been shown to cause cellular mispatterning in the organ of Corti, to alter the positioning of kinocilia and to result in an abnormal orientation and structure of the hair bundles (Fukuda et al. 2014). The role and spatiotemporal pattern of expression of the classical cadherins, E-cadherin and N-cadherin, have been studied in the developing organ of Corti and at early postnatal stages (Leonova and Raphael 1997; Mahendrasingam et al. 1997; Nunes et al. 2006; Simonneau et al. 2003; Whitlon 1993). Further investigations of the cellular and subcellular distribution of these cadherins showed that E- and N-cadherins had complementary expression patterns, delineating a sharp boundary between specific cell populations within the auditory sensory epithelium (see Fig. 13.2b) (Chacon-Heszele et al. 2012; Etournay et al. 2010). Indeed, E-cadherin has been detected in the outer pillar cells, OHCs, Deiters cells, and Hensen cells, whereas N-cadherin is restricted to cells in the medial IHC region, including the inner pillar cells, IHCs, and inner border cells (Etournay et al. 2010). Based on the differences in their properties, it has been suggested

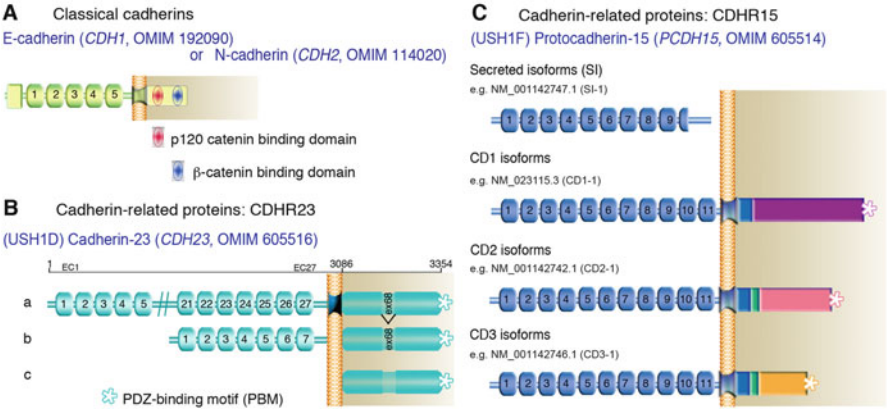

Fig. 13.3 Inner ear classical cadherins and cadherin-related proteins (**a**) All cadherins have an extracellular region consisting of extracellular cadherin (EC) domains, which are ~110 amino acids in length. (**a**) E- and N-cadherins are classical cadherins consisting of an extracellular region with five EC domains, a transmembrane domain (TM), and a cytoplasmic domain capable of binding two catenins, p120-catenin and β-catenin. (**b**, **c**) Cadherin-23 and protocadherin-15 both have an unusually large number of EC domains, and their cytodomains display no sequence similarity either to each other or to those of classical cadherins; in particular, they lack the consensus motif for binding to p120- or to β-catenins. (**b**) Three classes of cadherin-23 isoforms (*a*, *b*, and *c*) have been described. (**c**) The protocadherin-15 splice isoforms are grouped into four distinct classes: integral membrane isoforms with different cytodomains (CD), CD1, CD2, CD3, and secreted isoforms (SI)

that N-cadherin plays a major role in the early steps of cochlear extension, favouring cell mobility, whereas E-cadherin is involved in the establishment of stable cell–cell contacts (Chacon-Heszele et al. 2012). Evidence for cadherin involvement in the rapid changes in cell–cell contact occurring during cochlear extension was provided by the altered convergent extension process due to low N- and E-cadherin cell contents in the absence of p120-catenin (Chacon-Heszele et al. 2012). Classical cadherins interact principally with two catenins: p120-catenin, and β-catenin (see Fig. 13.3a). β-catenin anchors cadherins to the actin cytoskeleton by interacting with α-catenin, whereas p120-catenin is required for cadherin stabilisation at the plasma membrane (see Kourtidis et al. 2013; Nelson 2008).

A lack of p120-catenin has been shown to cause impaired cellular patterning, but does not lead to the misorientation of hair cells (Chacon-Heszele et al. 2012). Thus, different mechanisms underlie cochlear extension and hair cell polarity. It was recently shown that the establishment of hair cell planar polarity is influenced by kinocilium/basal body positioning and by junctional complex remodelling at the contacts between hair cells and their supporting cells (see Ezan and Montcouquiol 2013; Jones and Chen 2008; Kelly and Chen 2009). Indeed, core PCP signalling is required to establish the complementary compartmentalisation at the apical surface of the hair cell of two polarised protein complexes, the Pins (Partner Inscutable)/

Gαi (G-protein alpha i) and aPKC/PAR6/PAR3 complexes (Ezan et al. 2013; Tarchini et al. 2013). Pins//Gαi has been found to be restricted to a stereocilium-free lateral region also known as the 'bare zone', whereas aPKC occupies a medial zone that does not overlap with the core PCP protein Vangl2 region. These well-defined and delimited zones guide kinocilium positioning at the vertex, and the subsequent shaping and orientation of the hair bundle. Video microscopy studies monitoring movements of the kinocilium basal body (mother centriole) and its daughter centriole ex vivo established that the gradual displacement of the kinocilium to the cell periphery occurs at the same time as the convergent cochlear extension process (Lepelletier et al. 2013). The structural constraints ensuring kinocilium confinement to the vertices of the hair cells and the cytoskeletal mechanisms underlying kinocilium migration are not understood.

During early postnatal development in the mouse, a further refinement of hair bundle orientation and of the establishment of V-shaped OHC hair bundles has been shown to be correlated with a change in the shape of the apical circumference of these cells, which are initially round, but then develop a convex (typically hexagonal) circumference (Etournay et al. 2010). This shape transition is impaired in the absence of some hair bundle proteins, such as cadherin-23 (see insets in Fig. 13.2e, and Etournay et al. 2010) in mice. Several proteins of the apical junctional complex, including myosin II, myosin VIIa, shroom2, and F-actin, have been shown to be asymmetrically redistributed during the remodelling of the OHC apical circumference (Etournay et al. 2010). Consistent with the importance of actin- and myosin-based mechanisms in epithelial remodelling (see Takeichi 2014), a study of convergent extension in myosin II-deficient mice revealed that this type of myosin was required for cell patterning and alignment within the cochlear sensory epithelium (Yamamoto et al. 2009). Furthermore, the inhibition of myosin II activity in the auditory sensory epithelium has been shown to deform cells, changing their apical shape, and to lead to an overall expansion of the epithelium (Ebrahim et al. 2013). Along the apical junctional complex, nonmuscle myosin II sarcomeres are arranged in precise pairs across the junctional barrier between hair cells and adjacent supporting cells, forming an integrated, transcellular contractile network across the apical epithelial surface (Ebrahim et al. 2013). Connections between stereociliary rootlets and the apical junctions have been described (Etournay et al. 2010), but additional studies are required to clarify the mechanisms by which crosstalk between the protein complexes and the signalling pathways in the hair bundle and at the tight and adherens apical junctions between hair cells and supporting cells ensure correct planar cell polarity.

13.4 Cadherins in the Differentiating and Mature Auditory Hair Cells

13.4.1 The Sound-Receptive Structure of Hair Cells, the Hair Bundle

The transformation of sound waves into electrical signals (mechanoelectrical transduction; Fig. 13.2c), takes place in the hair bundle of cochlear hair cells (Corey and Hudspeth 1983). In developing hair cells, the stereocilia of the hair bundle are connected to each other, and some are connected to the kinocilium. Five different types of extracellular lateral links have been identified: early transient lateral links, kinociliary links, shaft links, ankle links, and the tip-link (Goodyear et al. 2005; El-Amraoui and Petit 2014; Pepermans and Petit 2015; Michalski and Petit 2015; Fig. 13.2d). In the mature hair bundle, two types of links persist: the tip-link and apical links, referred to as top connectors in OHCs (Fig. 13.2d). The tip-link, which is 5 nm in diameter and 125–250 nm long, is an oblique intertwined filament that connects each stereocilium of the short and middle rows to the side of the adjacent taller stereocilium (Pickles et al. 1984). Ultrastructural analyses have shown that there are electron-dense regions at the upper and lower insertion points of the tip-link (Corey and Hudspeth 1983; Furness and Hackney 1985; Furness et al. 2008) (see Fig. 13.2c).

According to the 'gating-spring' model of MET (Corey and Hudspeth 1983), a positive displacement of the bundle (towards the tall stereocilia) increases the tension in the tip-links, thereby increasing the open probability of the MET channels located at tips of stereocilia. The resulting influx of cations (K^+ and Ca^{2+}) sets up a depolarising receptor potential in OHCs and IHCs, eventually leading to neurotransmitter release by IHCs and signalling to the brain (Howard and Hudspeth 1988; Hudspeth et al. 2000). The steady-state tension at the tip-link has been estimated at ~8 pN in frogs (Jaramillo and Hudspeth 1993), but is unknown in mammals. Indirect estimates suggest that, during hair-cell stimulation, the tip-link tension probably lies in the range of 10–40 pN, although higher values cannot be excluded (Jaramillo and Hudspeth 1993).

13.4.2 Two Cadherin-Related Proteins Form the Tip-Link, a Key Component of the MET Machinery

Studies of a form of human syndromic deafness, Usher syndrome type I (USH1), have provided considerable insight into the development and functioning of the hair bundle (see Caberlotto et al. 2011a; Petit and Richardson 2009; Richardson et al. 2011; Pepermans and Petit 2015). Usher syndrome has an estimated prevalence of 1/10,000, and seems to account for about 50 % of all cases of monogenic

deafness/blindness. USH1, the most severe form, is characterised by severe to profound congenital deafness, constant vestibular dysfunction, and retinitis pigmentosa beginning before puberty, eventually leading to blindness (reviewed by El-Amraoui and Petit 2014; Mathur and Yang 2015; Pepermans and Petit 2015). Six *USH1* genes have been identified; they encode the actin-based motor protein myosin VIIa (USH1B), the PDZ-domain–containing submembrane protein harmonin (USH1C), the scaffold protein Sans (USH1G), the integrin and calcium-binding protein CIB2 (USH1J), and the two Ca^{2+}-dependent adhesion proteins, cadherin-23 (USH1D) and protocadherin-15 (USH1F; reviewed by Petit and Richardson 2009; Pepermans and Petit 2015).

Animal USH1 models mimicking the abnormal hearing phenotype are available for all USH1 genes. Cadherin-23 is defective in the deaf *waltzer* mouse (Di Palma et al. 2001), and in the *sputnik* zebrafish (Sollner et al. 2004), whereas protocadherin-15 is defective in the *Ames waltzer* mouse (Alagramam et al. 2001a) and in the *orbiter* zebrafish (Seiler et al. 2005). These mutants have fragmented and disorganised hair bundles (see Fig. 13.2e) (Ahmed et al. 2003; Boeda et al. 2002; Lagziel et al. 2005; Michel et al. 2005; Senften et al. 2006; Siemens et al. 2002). A similar abnormal hair bundle phenotype has been observed in mutant mice defective for the other USH1 proteins (Johnson et al. 2003; Kikkawa et al. 2003; Self et al. 1998). This led us to suggest that USH1 proteins function together to establish the cohesiveness of the developing hair bundle (Lefevre et al. 2008; see Fig. 13.2e). An interdisciplinary study of these animal models, including an in-depth analysis of USH1 proteins, led to the suggestion that the physical coupling of cadherin-23– and protocadherin-15–mediated early transient interstereociliary and kinociliary links is required for the formation of individual, correctly shaped hair bundles (Boeda et al. 2002; Lagziel et al. 2005; Michel et al. 2005; Siemens et al. 2002, reviewed by El-Amraoui and Petit 2005; Mathur and Yang 2015; Richardson et al. 2011; Pepermans and Petit 2015). The tip-link, which has a helical structure (Kachar et al. 2000), consists of at least two cadherin heterodimers: *cis*-homodimers of cadherin-23 interact in trans with *cis*-homodimers of protocadherin-15 (Kazmierczak et al. 2007), to form the upper and lower parts of this link (Fig. 13.4). Crystallographic studies have shown that the two aminoterminal cadherin repeats (extracellular cadherin (EC) repeats 1 and 2) of protocadherin-15 and cadherin-23 interact in an 'extended handshake' manner to form an overlapping antiparallel heterodimer (Sotomayor et al. 2012). Molecular dynamics simulations and binding experiments have indicated that the bond formed by protocadherin-15 and cadherin-23 is mechanically strong enough to withstand the forces applied to the tip-link during hair bundle deflection (Sotomayor et al. 2012). The three-dimensional (3D) structures of the cadherin-23 and protocadherin-15 EC1 domains are similar to those of other cadherins (three Ca^{2+}-binding sites (1, 2, and 3) at the linker between EC1 and EC2), but with several unusual features, such as an elongated N-terminus stabilised at the tip by Ca^{2+}-binding site 0 (Elledge et al. 2010; Sotomayor et al. 2010). As in classical cadherins, Ca^{2+}-ion binding probably renders the cadherin extracellular domains more rigid, promoting *trans*-junctional interactions. The local Ca^{2+} concentration in the

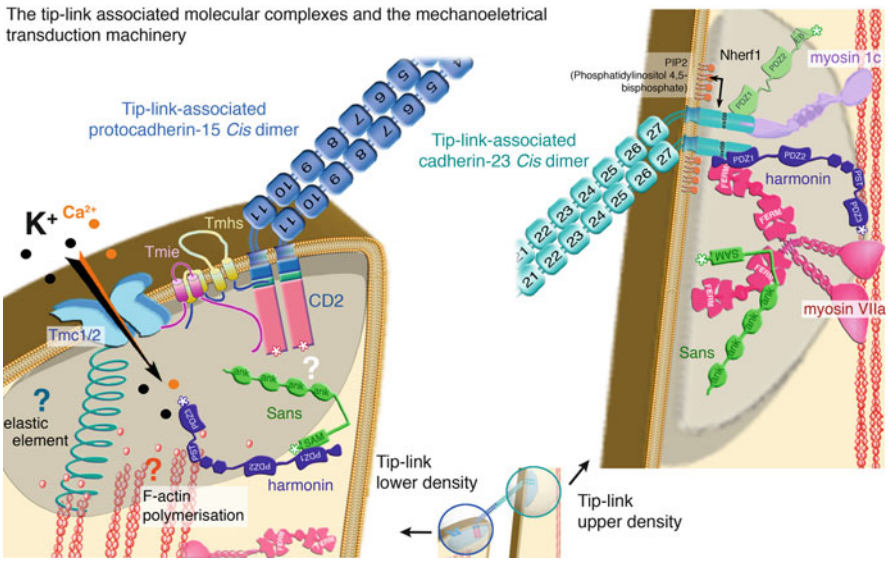

Fig. 13.4 In the mature hair bundle, large extracellular regions of the transmembrane proteins of cadherin-23 and protocadherin-15 CD2 form the tip-link. At the upper extremity of the tip-link, cadherin-23 is connected to the actin core of the stereocilia through interactions with USH1 proteins: harmonin b, and/or myosin VIIa. Myosin 1c and Nhref1 have also been shown to interact with cadherin-23 in vitro. In mature hair cells, the protocadherin-15 CD2 isoform forms the lower extremity of the tip-link. Interactions of this isoform with the transmembrane proteins Tmhs, Tmie, and Tmc1/2 channels have been described in vitro. The identity of the elastic element underlying the gating spring model and the way in which the tip-link and Ca^{2+} influx through the MET channel are coupled to F-actin polymerisation remain unclear

endolymph close to the tip-links is probably crucial for their formation and stability, particularly given the low concentration of Ca^{2+}, about 20–40 μM, in the endolymph (Bosher and Warren 1978).

According to the 'gating spring' model, mechanoelectrical transduction in vertebrate hair cells involves an elastic element (see Fig. 13.4) that pulls on the transducer channels to open these channels upon hair bundle deflection towards the tallest stereocilia (Howard and Hudspeth 1988). Until recently, the tip-link was thought to act as the gating spring and was assumed to have a working extension of 10–20 nm (Corey and Hudspeth 1983; Howard and Hudspeth 1988), to accommodate large sound stimuli (see Sotomayor et al. 2005). Whether cadherin homo- and hetero-dimers, which so far are thought to form a stiff element, also contribute to the gating spring is yet unclear. Other components of the stereocilia may also perform this role in conjunction with the tip-link. The extension and stiffness of large ankyrin-repeat–containing proteins (i.e., TRPA1, TRPN1) are thought to be similar to those predicted by the gating-spring model (Sotomayor et al. 2005), but there is still no evidence to suggest that these proteins are present in the hair bundles of vertebrates. The asymmetric nature of the tip-link suggests that its two ends have different biophysical and biochemical properties. Investigations are currently

underway to determine the role of the various proteins in the functioning of the mechanoelectrical transduction machinery.

13.4.3 Cadherin-23 at the Core of the Upper Tip-Link Molecular Complex

Three classes of cadherin 23 isoforms have been described (Lagziel et al. 2005). The longest splice isoform of cadherin-23 consists of 27 EC domains (Fig. 13.3a, b). In mature mouse auditory hair cells, cadherin-23 is restricted to the upper insertion point of the tip-link (Kazmierczak et al. 2007). Cadherin-23 is required for the correct localisation of harmonin and myosin VIIa to the upper end of the tip-link (Lefevre et al. 2008; Bahloul et al. 2010).

Little is known about the respective roles of the different splice isoforms of cadherin-23 (Lagziel et al. 2005; Lagziel et al. 2009). Molecular dynamics simulations of the composition of the tip-link, based on its crystal structure and tip-link size, combined with the use of antibodies specific for class-a isoforms have led to suggestions that the tip-link contains cadherin-23a (the longest isoform) (Kazmierczak et al. 2007). The roles of the smaller isoforms of cadherin-23, cadherin-23b (smaller extracellular domain), or cadherin-23c (the cytoplasmic splice isoform) are currently unknown.

13.4.4 Protocadherin-15 at the Core of the Lower Tip-Link Molecular Complex

There are about 24 different protocadherin-15 transcripts (Ahmed et al. 2008; Pepermans and Petit 2015) classified as CD1, CD2, CD3, and SI on the basis of the predicted encoded proteins (see Fig. 13.3c). The expression of the secreted forms of protocadherin-15 (SI) by the hair cells and the roles of these forms have yet to be described. The three integral membrane isoforms consist of 11 EC repeats, a single transmembrane domain, and a variable cytoplasmic domain carrying different C-terminal PDZ-binding motifs (PBMs). The various cytoplasmic regions of these protocadherin-15 transmembrane splice isoforms are encoded by different final coding exons: exon 35 for CD1, exon 38 for CD2, and exon 39 for CD3 (Ahmed et al. 2008; Pepermans and Petit 2015). The different cytoplasmic domains probably impart functional specificity by conferring on the protein an ability to interact with different binding partners. Establishment of the distribution of each isoform in the developing and mature hair bundles, and assessments of their contribution to tip-link formation are thus important. Specific mutants of each isoform have been generated to address these issues, and the distribution of each

isoform in the hair bundle has been determined (Alagramam et al. 2011; Webb et al. 2011; Pepermans et al. 2014).

The lower part of the mechanoelectrical transduction machinery has been shown to undergo molecular maturation at about the time of hearing onset in mice. This maturation involves a switch from the initial functional redundancy between the three Pcdh15 isoforms to a situation in which only the protocadherin-15 CD2 isoform is essential for tip-link formation, at mature stages. The CD2 isoform has been localised to the lower insertion point of the tip-link in mature hair bundles, in studies with an antibody specific for this isoform. Moreover, the postnatal deletion of CD2, which preserves the formation and differentiation of the hair bundles, has been shown to abolish tip-links and mechanoelectrical transduction currents. Finally, mutation analysis in patients with an isolated (nonsyndromic) form of profound deafness revealed frameshift mutations in exon 38 of *PCDH15*. This exon is specific to CD2 isoforms (Pepermans et al. 2014). Knockout mice defective for any of the first five USH1 proteins – myosin VIIa, harmonin, cadherin-23, protocadherin-15, and Sans – displayed a loss of the stereocilia in the short and middle rows (Lefevre et al. 2008). Subsequent studies of conditional knockout mice with postnatal inactivation of USH1 proteins (i.e., Sans, cadherin-23, or protocadherin-15 CD2 isoforms; Caberlotto et al. 2011b; Pepermans et al. 2014) provided further evidence for a connection between the mechanoelectrical transduction machinery and the F-actin polymerisation machinery, by showing a progressive regression of the short and middle-row stereocilia following the loss of tip-links. This connection may be constitutive or functional, possibly involving the tip-link tension and/or the magnitude of Ca^{2+} influx through the MET channels as a regulator of F-actin polymerisation (Lefevre et al. 2008; Caberlotto et al. 2011a; Pepermans et al. 2014).

13.5 Constructing the Tip-Link Interactome

In all cells, cadherins have diverse roles, involving interactions with multiple cytoplasmic proteins, including adaptor proteins, actin- and/or microtubule-binding proteins, protein kinases and phosphatases, and transcription regulators (Nelson 2008; Harris and Tepass 2010; Takeichi 2011; Hirano and Takeichi 2012). The tip region of the stereocilia may be considered to be a mechanoresponsive adhesion site, a focal adhesion point at which membrane-associated complexes interact with extracellular components. Studies of the 'integrin adhesome', a key component of focal adhesions, have revealed an associated network of more than 150 linked components potentially modified by 690 identified interactions (Zaidel-Bar et al. 2007). It seems likely that the lower and upper tip-link regions of high electron density also contain a large number of proteins governing the structural and signalling activities of the mechanoelectrical transduction machinery.

At the upper insertion point of the tip-link, cadherin-23 has been shown to form a ternary complex with harmonin and myosin VIIa (Bahloul et al. 2010). The

cytoplasmic region of cadherin-23 and harmonin, both independently and as a binary complex, also binds phosphatidylinositol 4,5-bisphosphate (PIP2), which may account for the role of this membrane phospholipid in mechanoelectrical transduction current adaptation (Bahloul et al. 2010). Harmonin-b (the largest harmonin isoform) binds to actin filaments (Boeda et al. 2002), myosin VIIa and cadherin-23 (Boeda et al. 2002; Siemens et al. 2002). Harmonin b has thus been identified as a putative intracellular membrane-cytoskeleton crosslinker, coupling the cadherin-23 cytodomain with the underlying cytoskeleton (Boeda et al. 2002). It is involved in MET current adaptation, probably by recruiting adaptation motors to the upper insertion point of the tip-link (Michalski et al. 2009). It has been suggested that this coupling optimises the sensitivity of the hair bundle to sound-induced motion (Grillet et al. 2009; Michalski et al. 2009). This tip-link connection to the cytoskeleton may also be mediated by myosin 1c, an adaptation motor protein in the hair bundle (Stauffer et al. 2005) that has been shown to coimmunoprecipitate with cadherin-23 (Siemens et al. 2004). Its levels in the stereocilia are decreased in the absence of cadherin-23 (Phillips et al. 2006). The PDZ-domain-containing protein Nherf1 (Kamiya et al. 2014), and Magi-1, a membrane-associated guanylate kinase (Xu et al. 2008), have both been found to bind cadherin-23 in vitro, and may also be considered possible components of the upper mechanoelectrical transduction machinery. Finally, the USH1G protein, Sans, has been identified as a critical component of the tip-link complex (Caberlotto et al. 2011b; Grati and Kachar 2011), because postnatal depletion of this protein leads to a loss of the tip-links, followed by a progressive decrease in the length of the short and middle rows of stereocilia, until their complete disappearance (Caberlotto et al. 2011b).

At the lower insertion point, the Tmc1 (transmembrane channel protein 1), and Tmc2 proteins have been identified as possible subunits of the mechanoelectrical transduction channel (Kawashima et al. 2011). However, it remains unclear whether Tmc1, which persists in the mature hair bundle, unlike Tmc2, is a component of the pore channel (see Pepermans and Petit 2015). Two other transmembrane proteins located in the apical region of the stereocilia, and the absence of which leads to deafness, have been identified as key proteins of the MET machinery: Tmhs (tetraspan membrane protein of hair cell stereocilia; Xiong et al. 2012) and Tmie (transmembrane protein of inner ear hair cells; Zhao et al. 2014). It remains unknown how the mechanoelectrical transduction channel is connected to the lower part of the tip-link. Interactions between the tip-link protein protocadherin-15 and the other components of the lower MET machinery have been investigated (see Fig. 13.4). Yeast two-hybrid and coimmunoprecipitation experiments have shown that the CD1 and CD3 isoforms of protocadherin-15 interact directly with Tmc1 and Tmie (Maeda et al. 2014). All three isoforms of protocadherin-15 were found to coimmunoprecipitate with both TMC1 and TMC2 (Beurg et al. 2015). In another study, Zhao and colleagues showed that the CD2 isoform bound Tmie directly, but that the CD1 and CD3 isoforms bound Tmie only indirectly, via Tmhs (Zhao et al. 2014; see Fig. 13.4). These interactions among multiple splice isoforms suggest that there may be flexibility in the potential interactions between protocadherin-15 isoforms and Tmc1/2 channels. They also highlight the need to

determine precisely the temporal distribution of the components of the mechanoelectrical transduction machinery, in both developing and mature hair cells, to ensure the correct interpretation of their potential interactions in situ. The possibility that these complexes differ along the tonotopic axis of the cochlea cannot be excluded.

Two recent studies based on multi-isotope imaging mass spectrometry and live imaging of single differentiating hair cells to capture stereociliogenesis (Drummond et al. 2015), and on the monitoring of β-actin-GFP incorporation into the stereocilia of adult mouse hair cells in vivo and ex vivo (Narayanan et al. 2015), demonstrated that the actin cores of the stereocilia were stable structures and that new F-actin incorporation occurred very slowly, and almost exclusively at the barbed ends of the filament near the distal tips of the stereocilia. In mutant mice either lacking ADF (actin depolymerising factor) or expressing a mutated form of AIP1 (actin interacting protein 1), two proteins involved in the severing and disassembly of F-actin, the stereocilia of the short and middle rows were found to be shortened, and some were missing (Narayanan et al. 2015), although they did not disappear entirely as they do in USH1 mutant mice (Caberlotto et al. 2011a; Lefevre et al. 2008; Pepermans et al. 2014). This suggests that two as yet uncharacterised mechanisms control the length of stereocilia. These processes probably involve various classes of actin-binding proteins, including nucleators of actin polymerisation, actin-capping proteins, actin-severing proteins, actin-crosslinking proteins, and molecular motors (reviewed by Caberlotto et al. 2011a; Michalski and Petit 2015).

13.6 Concluding Remarks

As described above, many questions about the roles played by cadherin and cadherin-related molecules in the hair cells remain unresolved. In particular, further studies are required to determine how the two extremities of the tip-link are coupled to the F-actin cytoskeleton at the tips of the stereocilia. The diversity of cochlear phenotypes observed in mutant mice lacking the various protocadherin-15 splice isoforms suggests that these isoforms contribute to the formation of different types of links associated with different molecular complexes with temporally and spatially specific functions. It has been suggested that cadherin-23 and protocadherin-15 connect the light-sensitive outer segment to the inner segment of photoreceptor cells and the adjacent calyceal processes, microvillus-like structures similar to the stereocilia of the hair cells (Sahly et al. 2012). Parallel studies of cadherin-mediated pathways in the photoreceptor cells will probably shed light on possible associations between the USH1-associated mechanosensory functions and the calyceal processes.

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