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Sensing Sound: Cellular Specializations and Molecular Force-Sensors

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Summary

Organisms of all phyla express mechanosensitive ion channels with a wide range of physiological functions. In recent years, several classes of mechanically gated ion channels have been identified. Some of these ion channels are intrinsically mechanosensitive. Others depend on accessory proteins to regulate their response to mechanical force. The mechanotransduction machinery of cochlear hair cells provides a particularly striking example of a complex force-sensing machine. This molecular ensemble is embedded into a specialized cellular compartment that is crucial for its function. Notably, mechanotransduction channels of cochlear hair cells are not only critical for auditory perception. They also shape their cellular environment and regulate the development of auditory circuitry. Here we summarize recent discoveries that have shed light on the composition of the mechanotransduction machinery of cochlear hair cells and how this machinery contributes to the development and function of the auditory system.

In Brief:

Qiu and Müller review recent progress in the field of auditory mechanotransduction that has revealed that the mechanotransduction machinery of cochlear hair cells is a complex molecular machine. Notably, hair cell mechanotransduction is not only important for sensory perception but this process also shapes hair cell morphology and auditory circuits.

Introduction

Molecules designed to respond to mechanical stimuli are perhaps some of the earliest molecular sensors to emerge during evolution. These molecules are found throughout all phyla. Some of these sensors respond to osmotic pressure to prevent the rupture of the cell membrane during environmental fluctuations. Others are expressed in specialized sense

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Declaration of Interests

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organs and respond to defined stimuli such as touch and sound. In recent years, tremendous progress has been made towards the identification of the molecular components of a variety of force sensors. Two major concepts have emerged. First and perhaps not surprisingly, force-sensors are built from different molecular components reflecting the diversity of their functions to detect a wide range of mechanical stimuli. Second, at the heart of many (but not all) of these force-sensors are mechanically gated ion channels. Some of these ion channels, including the bacterial MscL/MscS proteins and the mammalian PIEZO proteins, are intrinsically mechanically sensitive when expressed in lipid membranes. Others such as the DEG/ENac proteins of C. elegans, the NOMPC protein of Drosophila and the mechanotransduction channel of cochlear hair cells are components of larger molecular assemblies that contain additional proteins critical to transmit force onto the pore-forming subunits of these ion channels (Jin et al., 2020; Kefauver et al., 2020).

Mechanotransduction by sensory hair cells of the inner ear has been an influential model to study mechanisms of mechanosensation. Recent progress in the field has been driven by the study of genes that are linked to deafness. New discoveries challenge some of the earlier concepts that had been developed based on biophysical studies alone. These new studies have revealed that the mechanotransduction machinery of cochlear hair cells is assembled from an astonishing number of diverse proteins. While the lack of a reconstituted system to study ion channel function outside hair cells has been a significant roadblock for the field, the study of genetically modified mice has begun to unravel the function of individual components of the mechanotransduction machinery of hair cells. Surprisingly, these studies have also revealed that mechanotransduction by hair cells is not only essential for auditory perception. Functional transduction channels are necessary for the normal development of hair cells and for the maturation of the afferent neurons that innervate them. We will review here the current status of the field of auditory mechanotransduction and will highlight remaining open questions.

The Organ of Corti: An Exquisitely Organized Cellular Ensemble

The perception of sound is an impressive task. Humans can distinguish sounds from near 0 dB up to 120 dB thus enabling us to hear the rustling of leaves in the wind or the roaring of an airplane engine. Humans perceive frequencies ranging from dozens of Hz up to 20 kHz. This frequency range is dramatically extended in other species. Mice hear sound frequencies of up to 70 kHz, while the frequency range of several bat species extends to an astonishing 200 kHz (Dale et al., 2001; Hopp et al., 1998). These variations in frequency range are adaptations optimized for species-specific tasks. Sound perception at the lower end of the frequency spectrum enables humans to communicate and mice to perceive predators. Mice use ultrasound communication for inter-species communication, while bats use ultrasound vocalization and detection to capture prey.

The end-organ for the perception of sound: a highly organized sensory epithelium—The basic building plan of the end-organ for the perception of sound is remarkably conserved across mammalian species and represents perhaps one of the most elegant examples of a mechanosensory structure dedicated to a specific task. The auditory sensory epithelium of mammals is embedded into the Organ of Corti, which is localized

within the cochlear snail of the inner ear (Fig. 1A). The Organ of Corti contains one row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs) that run along the length of the cochlear duct. These cells are the specialized mechanosensory cells of the inner ear. The function of hair cells depends on supporting cells that assume specific positions within the Organ of Corti (Fig. 1A). Transcriptomics studies have begun to reveal cell-type specific signatures for each cell type within the Organ of Corti, thus enabling new opportunities for a molecular investigation of their functions (https://umgear.org/). The cells of the Organ of Corti are sandwiched between two molecularly distinct extracellular matrix assemblies, the tectorial membrane that overlies the Organ of Corti, and the basilar membrane that runs below it (Fig. 1A) (Fettiplace, 2017; Schwander et al., 2010).

Hair cells derive their names from a bundle of specialized actin-rich microvilli, known as stereocilia, that crown their apical surface. The stereocilia are the mechanically sensitive organelle of a hair cell. Sound waves that reach the ear drum are converted into fluid motions that travel down the cochlear duct, leading to vibrations within the elastic basilar membrane. These vibrations cause deflections of the stereocilia of hair cells against the overlying tectorial membrane, thus leading to the activation of mechanotransduction channels within stereocilia (Fettiplace, 2017; Schwander *et al.*, 2010). To achieve tight coupling of hair cells to the tectorial membrane, the longest stereocilia of OHCs form attachment crowns. Mutations in the genes that affect the formation of attachment crowns cause deafness, highlighting the importance of coupling between hair bundles and the tectorial membrane for sound perception (Avan et al., 2019; Verpy et al., 2011; Zeng et al., 2016). It was long thought that the stereocilia of IHCs are not in contact with the tectorial membrane, but this concept has recently been challenged. Using fluorescence confocal imaging in an acute organ preparation, it was shown that the stereocilia of both IHCs and OHCs appear to be in contact with the tectorial membrane, at least in guinea pig (Hakizimana and Fridberger, 2021). However, studies by others are consistent with the model that stereocilia of IHCs are not directly coupled to the tectorial membrane and instead are activated by fluid motion (Jia et al., 2007; Russell and Sellick, 1983).

Mechanosensation: distinct functions for IHCs and OHCs—The building plan of the cochlea provides first clues to the mechanisms that enable mammals to perceive sound of different frequencies and intensities. Anatomical features of the Organ of Corti vary systematically along the length of the cochlear duct. Because of such changes including a gradual increase in the stiffness and mass of the cochlear partition from the apex to the base, sounds of different frequencies induce maximal vibrations at different positions along the cochlea duct, thus stimulating sensory hair cells at specific so-called tonotopic positions. Highest sound frequencies are perceived by hair cells at the base of the cochlea and lowest frequencies at the apex, with a gradient in-between (Fig. 1B) (Fettiplace, 2017; Schwander *et al.*, 2010).

Both IHCs and OHCs are mechanosensory cells, yet they have distinct functions. IHCs are the bona fide auditory receptors. In contrast, OHCs amplify input sound signals because these cells can change the length of their cell body in response to changes in membrane potential, a process called electromotility (Ashmore, 1987; Brownell et al., 1985; Kachar et al., 1986). The amplification process depends on the molecular motor protein prestin,

which is embedded into the lateral membrane of the cell body of OHCs (Belyantseva et al., 2000; Zheng et al., 2000). Cryo-EM studies have determined the structure of prestin (Bavi et al., 2021; Butan et al., 2022; Ge et al., 2021). The structure of dolphin prestin in six conformations is consistent with a model where structural rearrangements in a voltage sensor domain are coupled to conformational changes at the protein-membrane interface that cause membrane expansion (Bavi *et al.*, 2021). The shape changes in OHCs amplify the vibrations caused by input sound signals in a localized fashion within frequency-specific positions. The vibrations are then sensed by IHCs, leading ultimately to the initiation of action potentials in spiral ganglion neurons (SGNs) that innervate these sensory cells with tonotopic specificity to transmit sound information to the CNS.

Hair-cell stereocilia: form defines function—The stereocilia of a hair cell are an extraordinarily sensitive sensory compartment. Computationally derived estimates indicate that transducer currents in the mammalian cochlea saturate at ~2° angular rotation (He et al., 2004). The core of stereocilia consists of highly crosslinked parallel actin filaments. Near their base, stereocilia form a characteristic taper. During mechanical stimulation, stereocilia act as stiff rods that tilt around their base (Hudspeth, 1983). Within a hair bundle, stereocilia are organized into rows of increasing heights, and only deflection towards the longest stereocilia lead to the activation of mechanotransduction channels in stereocilia (Hudspeth and Corey, 1977; Shotwell et al., 1981). These ion channels are localized at the base of tip links, fine extracellular filaments that connect the stereocilia and are visible in the electron microscope (Fig. 2A) (Beurg et al., 2009; Pickles et al., 1984). Disruption of tip-links affects channel gating, indicating that tip links are critical to transmit force onto mechanotransduction channels (Assad et al., 1991; Zhao et al., 1996). This has perhaps been most directly demonstrated by elegant studies in bullfrog hair cells. Hudspeth and colleagues generated superparamagnetic beads that bind to the tip links of hair cells. Application of a magnetic-field gradient exerts mechanical force on the tip links that evokes mechanotransduction currents (Basu et al., 2016).

While the basic building plan of hair bundles is similar across species, variations between different types of hair cells and species have been observed. In mice each cochlear hair cell contains three rows of stereocilia, but the stereocilia of OHCs form a bundle with a more eccentric V shape compared to the flatter V shape of IHC. Hair cells in other species such as bullfrogs, have more rows of stereocilia per bundle and form a tighter bundle with no obvious V shape (Barr-Gillespie, 2015; Schwander *et al.*, 2010). The different organization of stereocilia likely has significant impact on the way a hair bundle moves in response to mechanical inputs (O'Maoileidigh and Ricci, 2019). Stereocilia across hair bundles in bullfrogs and turtles move as a unit during mechanical stimulation, thus allowing for exquisite sensitivity and coherent gating of mechanotransduction channels across the stereociliary bundle (Crawford and Fettiplace, 1985; Karavitaki and Corey, 2010; Kozlov et al., 2007). It is less clear if this mode of motion applies to the mammalian hair bundle, which has a more extreme shape with less coherence (Nam et al., 2015; Wang et al., 2021b). Variations in the way mechanical stimuli of different intensities deflect the whole bundle or only parts of it could have significant effects on downstream signaling mechanisms.

The Mechanotransduction Process: Channel Activation and Adaptation

At rest, mechanotransduction channels in cochlear hair cells are partially open. In rodents, the resting open probability of OHCs is ~50% and of IHCs ~20% when measured in vitro at the low Ca^{2+} concentration typical for endolymph (Beurg et al., 2010; Corns et al., 2014; Johnson et al., 2011; Peng et al., 2013). This defines the set point of the channel along the activation curve in the absence of a mechanical stimulus and keeps the channels at the position of greatest sensitivity to mechanical stimulation. Sound induced deflections of the stereocilia in the direction of the longest stereocilia activate the mechanotransduction channel followed by channel adaptation. Deflections in the opposite direction close the channels that are open at rest (Fig. 2A) (O'Maoileidigh and Ricci, 2019; Fettiplace, 2017).

Mechanotransduction channels of cochlear hair cells are non-selective for cations with a four times higher permeability for Ca^{2+} compared to K^+ and Na^+ (Beurg et al., 2006; Ohmori, 1985). Since the endolymph fluid that surrounds the stereocilia is high in K^+ and low in Ca^{2+}, most of the transduction current in vivo is carried by K^+. However, Ca^{2+} profoundly regulates channel activity (Fettiplace, 2017). This was perhaps most quantitatively evaluated in turtle hair cells, where a reduction in the extracellular Ca^{2+} concentration leads to an increase in single channel conductance, a leftward shift of the activation curve and a reduction in the extent of adaptation (Ricci and Fettiplace, 1998).

Studies in turtle and rodent hair cells have determined a unitary channel conductance of 100–300 pS, depending on species, Ca^{2+} concentration and cochlear localization. In turtle hair cells and rodent OHCs, highest conductance values are observed towards the base of the cochlea and lowest values towards the apex indicative of molecular differences in the transduction complex along the tonotopic axis (Beurg *et al.*, 2006; Ricci et al., 2003). There are no significant tonotopic differences in the conductance of IHCs (Beurg *et al.*, 2006). Changes in conductance as well as differences in the kinetics of channel gating along the tonotopic axis could be critical for the detection and discrimination of a wide range of frequencies (Ricci, 2002).

Recent studies in mice suggest that the unitary conductance of the mechanotransduction channel may be closer to 50 pS. This conclusion is based on the observation that each MET channel complex exhibits multiple conductance states in ~50 pS increments (Beurg et al., 2018). However the lowest conductance states are observed extremely rarely in murine hair cells and have not been observed in turtle (Ricci *et al.*, 2003). Further studies are necessary to define the single channel conductance of the mechanotransduction channel and possible variations between species.

Channel activation: ultrafast signal transmission—The activation of the hair cell mechanotransduction channel is much faster than for most ion channels and cannot be accurately determined with traditional stiff probe stimulation (Doll et al., 2012; Ricci et al., 2005). Early studies from bullfrog hair cells measured field potentials and concluded that channels open in response to mechanical stimulation within tens of μs (Corey and Hudspeth, 1983). Direct measurements of the activation time constant in turtle provided similar evidence for channel opening within μs (Crawford et al., 1989). This has led to the idea that the channel is directly gated by mechanical force (Corey and Hudspeth, 1983).

Recent measurements in mouse cochlear hair cells combined with modeling estimate the value for activation at about 4 μs for basal and 10 μs for apical cochlear hair cells (Beurg et al., 2021a). A similar tonotopic gradient of activation kinetics had been reported in turtle (Ricci et al., 2005).

Studies in bullfrog hair cells have revealed a non-linear displacement of the hair bundle upon force application and a gating compliance, which has led to the influential gating spring model. This model proposes that mechanical force is transmitted to the mechanotransduction channel through an elastic element, or gating spring. The stiffness of the gating spring was determined to be at about 1 pN/nm (Howard and Hudspeth, 1988). More recently, the gating spring stiffness has been analyzed in the rat cochlea. This elegant study revealed that gating spring stiffness increases in hair cells with increasing characteristic frequency (Tobin et al., 2019). This tonotopic variation in gating spring stiffness could be important to optimize the sensitivity of the mechanotransduction channel to forces generated by different sound frequencies. In addition, cAMP affects mechanotransduction by hair cells (Ricci and Fettiplace, 1997), and an increase in cAMP levels leads to a decrease in gating spring stiffness (Mecca et al., 2022). While the mechanisms that regulate cAMP levels in hair cells are not known, this observation suggests that gating spring stiffness might be modulated by cellular signaling mechanisms.

How to adapt: old friends and new models—Following activation, mechanotransduction channels in hair cells adapt, which is reflected by a decrease in transducer currents during a sustained hair bundle displacement (Fig. 2A). Adaptation is thought to be important to extend the dynamic range of a hair cell and to contribute to frequency selectivity and signal amplification. Studies in non-mammalian vertebrates identified two components of adaptation, fast and slow, progressing with a time constant of several milliseconds and tens of milliseconds, respectively. Fast adaptation has been proposed to depend on the binding of Ca^{2+} to the mechanotransduction channel or a side near the channel leading to channel closure. Slow adaptation has been proposed to depend on a motor protein localized at the upper end of tip links (Fettiplace, 2017; Gillespie and Müller, 2009). This motor protein, originally proposed to be MYO1C (Gillespie and Cyr, 2004), climbs up the actin filaments toward the tips of the stereocilia to establish tension in the transduction machinery. The motor is released from F-actin when Ca^{2+} flows into stereocilia and tension on the transduction machinery is reduced thus leading to channel closure (Assad *et al.*, 1991; Howard and Hudspeth, 1988). The motor model also proposes that adaptation is critical to set the resting tension of the mechanotransduction channel, which keeps the channel partially open at rest. Notably, adaptation rates vary tonotopically with higher adaptation rates in hair cells responding to high frequencies (Ricci and Fettiplace, 1997; Ricci *et al.*, 2005; Waguespack et al., 2007), which could yet be another specialization critical for frequency discrimination.

As discussed below, recent observations in mammals suggest that this motor model of adaptation is not universally true and likely does not apply to cochlear hair cells. These studies also suggest that adaptation and resting tension in mammals are regulated by distinct mechanisms.

The Mechanotransduction Complex: A Dazzling Array of Molecules

The mammalian inner ear contains only a small number of hair cells, approximately 3,000 in mice and 15,000 in humans. Due to this sparsity of material, biochemical methods have been largely unsuccessful to provide insights into the molecular composition of the tip link and mechanotransduction channel. Models of mechanotransduction have largely been developed based on biophysical studies without a molecular framework. A breakthrough in the field came with the positional cloning of genes that are linked to deafness. The study of the affected genes has revealed that mechanotransduction by hair cells depends on a molecular machine consisting of many parts (Fig. 2B). Structural biology has provided first insights into the atomic structure of some of the transduction molecules.

Molecular composition of the tip-link: a striking molecular asymmetry—The first molecules that were definitively linked by genetic studies to the mechanotransduction machinery of cochlear hair cells are two members of the cadherin superfamily, PCDH15 and CDH23. Mutations in the genes encoding these proteins cause deafness and defects in mechanotransduction (Kazmierczak and Müller, 2012). CDH23 and PCDH15 have subsequently been shown to be components of tip links (Ahmed et al., 2006; Kazmierczak et al., 2007; Siemens et al., 2004; Sollner et al., 2004). These adhesion receptors form cis-homodimers that interact in trans via their N-termini to form the upper and lower parts of tip links (Fig. 2B) (Kazmierczak et al., 2007). Distinct from classic cadherins that contain 5 extracellular cadherin (EC) repeats, PCDH15 and CDH23 contain 11 and 27 EC repeats, respectively. A complete tip-link thus contains an astonishing 76 EC domains to make up an extracellular filament that is approximately 150 nm in length (Kazmierczak et al., 2007).

Crystal structures of fragments of PCDH15 and CDH23 have confirmed the classic cadherin fold for their EC domains (Araya-Secchi et al., 2016; Choudhary et al., 2020; De-la-Torre et al., 2018; Dionne et al., 2018; Elledge et al., 2010; Jaiganesh et al., 2018; Narui and Sotomayor, 2018; Powers et al., 2017; Sotomayor et al., 2010; 2012). Single particle reconstruction of the entire EC1-EC11 domain of PCDH15 as well as a high-resolution structures of smaller PCDH15 fragments have shown that dimerization is mediated by the EC3 domains of two PCDH15 molecules (Fig. 3) (Dionne et al., 2018). A second dimerization site is localized closer to the cell membrane (Fig. 3) (Ge et al., 2021). Mutations that perturb PCDH15 dimerization affect mechanotransduction, demonstrating the importance of the dimeric tip-link structure for tip-link function (Dionne et al., 2018). Mechanisms that drive dimerization of CDH23 are less clear.

In classical cadherins, the basic adhesive unit consists of two cadherin monomers that interact with each other via their N-termini. Trp residues in the first N-terminal cadherin repeats are buried into complementary binding pockets in the first N-terminal cadherin repeat of the trans binding-partner (Boggon et al., 2002; Haussinger et al., 2004; Patel et al., 2006). Trp residues are not conserved in EC1 of PCDH15 and CDH23, and structures of the N-terminus of CDH23 reveal significant differences to classical cadherins (Elledge et al., 2010; Sotomayor et al., 2010). Instead, the adhesive units of tip links are dimers of PCDH15 and CDH23 that interact via an enlarged binding surface formed by EC1 and EC2 of the two molecules (Fig. 3) (Sotomayor et al., 2012).

The dimeric structure of the tip-link cadherins combined with a new and enlarged binding surface (compared to classical cadherins) are likely evolutionary adaptations to achieve the necessary binding strength that is required to maintain tip-link integrity during mechanical stimulation. This is consistent with single-molecule force spectroscopy experiments that compared the binding strength of monomeric and dimeric N-terminal fragments of tip-link cadherins (Mulhall et al., 2021). The remarkable molecular asymmetry in the tip link is suggestive of distinct functions for the divergent cytoplasmic domains of the two cadherins at the upper and lower ends of tip links.

Tip links were originally proposed as candidates for the gating spring of the mechanotransduction channel (Assad et al., 1991). However, initial structure-based predictions indicated otherwise. Modeling studies of the classical C-cadherin suggest that its extracellular domain is stiff in the presence of Ca^{2+} and shows limited elasticity in the absence of Ca^{2+} (Oroz et al., 2019). Three conserved amino acids within the linker domains between EC domains are required for Ca^{2+} binding and rigidification (Oroz et al., 2019; Sotomayor and Schulten, 2008). Modeling studies for the EC1-EC2 fragment of CDH23 predicted a stiffness of 570 nN/m. When these values were extrapolated to all EC domains of the tip link, a stiffness of 40–60 nN/m was predicted (Sotomayor et al., 2010). These values are too high compared to the measured stiffness of the gating spring (1 pN/nm)(Howard and Hudspeth, 1988). However, not all linkers between EC domains of PCDH15 and CDH23 contain the three conserved amino acids that bind Ca^{2+} and rigidify cadherins (Araya-Secchi et al., 2016; Jaiganesh et al., 2018; Powers et al., 2017). This could significantly affect the elasticity of tip links. In fact, single molecule measurements of the stiffness of a monomeric extracellular domain of PCDH15 using a high-precision optical trap revealed a stiffness lower than the gating spring stiffness. Considering that PCDH15 are dimeric and interact with CDH23, the predicted values for the stiffness of the entire tip link were estimated to be in line with the stiffness of the gating spring (Bartsch et al., 2021). While further studies are warranted, tip links are good candidates to contribute to the gating spring. Several molecules that are in series with tip links could also be part of the gating spring, which might include both proteins and membrane lipids. Recent data suggest that the membrane of stereocilia contributes to calcium-modulated viscoelastic element that can regulate hair cell mechanotransduction (George et al., 2020).

The tetraspan LHFPL5: tight coupling to the lower end of tip links—In the search for additional components of the mechanotransduction complex of hair cells, a large-scale forward genetic screen was carried out to identify mice that are deaf. This screen also analyzed mouse mutants generated by other researchers (Schwander et al., 2007; Xiong et al., 2012). One of the first mouse lines to be identified from the screen carries a mutation in *Cdh23* that affects tip link function, thus providing evidence for a role of CDH23 in mechanotransduction (Schwander et al., 2009). A second affected mouse line carries a mutation in the gene encoding the tetraspan LHFPL5/TMHS (Longo-Guess et al., 2005; Xiong et al., 2012). Mutations in the *LHFPL5* gene also cause deafness in humans (Shabbir et al., 2006). LHFPL5 binds to PCDH15 and co-localizes with PCDH15 to the lower end of tip links, thus reinforcing the concept of molecular asymmetry in the mechanotransduction machinery (Fig. 2B) (Li et al., 2019; Mahendrasingam et al., 2017; Xiong et al., 2012)

Structural studies have shown that a dimer of LHFPL5 binds to a dimer of PCDH15 where extensive contact between the two molecules is mediated by transmembrane helices. In addition, an extracellular loop of LHFPL5 is in contact with the membrane proximal extracellular domain of PCDH15 that follows EC11 and forms a collar above the membrane (Fig. 3) (Ge et al., 2018). This domain was dubbed as the PCDH15 interacting-channel associated (PICA) domain because it could be involved in mediating interactions with the mechanotransduction channel (Dionne et al., 2018). Cryo-EM studies suggest that the collar has structural flexibility (Ge et al., 2018), which could be important to regulate force-transfer from the tip link to the transduction channel.

In mice carrying an *Lhfpl5* null mutation, localization of PCDH15 to stereocilia is affected, thus leading to reduced numbers of tip links and a concomitant reduction of transducer currents by 70–90% (Xiong *et al.*, 2012). Some tip links remain in the mutant mice, allowing for the study of mechanotransduction currents carried by channels in the absence of LHFPL5. The properties of these currents are strikingly altered, including a delay in channel activation and lack of fast adaptation (Xiong et al., 2012). These findings provide evidence that LHFPL5 is an essential component of the MET channel complex that forms a tight unit with the tip link. Since mechanotransduction is not completely abolished in LHFPL5-deficient hair cells, LHFPL5 is unlikely to be an essential pore-forming subunit of the MET channels. Both in overall structure and function LHFPL5 resembles TARP subunits of glutamatergic AMPA receptors (Xiong et al., 2012). These ion channel accessory subunits regulate the localization of AMPA receptors to synaptic sites, and they allosterically affect the pore properties of these ion channels (Jackson and Nicoll, 2011). LHFPL5 is thus most likely a TARP-like molecule for the mechanotransduction channel of cochlear hair cells (Xiong *et al.*, 2012).

The mechanism by which LHFPL5 contributes to the gating of the mechanotransduction channel in hair cells is unclear. Based on the fast activation time constant for the mechanotransduction channel, it seems reasonable to hypothesize that this ion channel binds to the tip link. Data summarized in the next section support this view. Alternatively, the tip-link complex might pull on the membrane to change membrane curvature, thus leading to channel activation. In support of the latter model, the membrane at the lower end of tip links pulls away from the underlying cytoskeleton, suggesting that the tip link applies tension to the membrane (Assad *et al.*, 1991; Kachar et al., 2000). However, it cannot be excluded that this membrane tenting is caused by shrinkage of the stereocilia during fixation. Nevertheless, membrane components such as PIP_2 are concentrated at stereociliary tips and have dramatic effects on channel gating and function (Effertz et al., 2017; Hirono et al., 2004). LHFPL5 is a distant member of the tetraspanin family, which form multimers and are components of lipid microdomains (van Deventer et al., 2017). It would be interesting to determine whether LHFPL5 can affect PIP_2 distribution and membrane properties at the tips of stereocilia.

The mechanotransduction channel revealed: TMC1 and TMC2 as pore-forming subunits—Experiments by the Ricci and Fettiplace laboratories demonstrated that mechanotransduction channels in hair cells are only present at lower ends of tip links (Fig. 2B). Without knowledge of the molecular identity of the transduction channel, these

laboratories took advantage of the observation that transduction channels are non-selective cation channels with a preference for Ca^{2+}. Imaging of Ca^{2+} entry into stereocilia during mechanical stimulation revealed that mechanotransduction channels are only present at the tips of shorter stereocilia near PCDH15 (Beurg *et al.*, 2009), thus reinforcing the concept of molecular asymmetry at tip links.

Genetic studies in mice were once again instrumental for the identification of subunits of the mechanotransduction channel of hair cells. Mutations in the gene encoding the multi-transmembrane protein TMC1 were linked to dominant and recessive forms of deafness (Kurima et al., 2002). TMC1 and its close relative TMC2 are expressed in mechanosensory hair cells of the cochlea, where TMC2 is the predominant isoform in the first few postnatal days before it becomes progressively replaced by TMC1 (Kawashima et al., 2011; Kurima et al., 2015). Mechanotransduction currents are abolished in hair cells from mice lacking both TMC1 and TMC2, while some transducer currents are maintained in *Tmc1* mutant mice in the first few days after birth (Kawashima et al., 2011; Kim and Fettiplace, 2013). The reason for the developmentally regulated expression patterns of TMC1 and TMC2 is not known, but both molecules confer distinct properties to hair cells. Hair cells expressing only TMC1 have reduced Ca^{2+} permeability and larger single channel conductance compared to hair cells expressing only TMC2 (Corns et al., 2017; Kim and Fettiplace, 2013; Pan et al., 2013). In addition, Ca^{2+} permeability is affected in a mouse line expressing a dominant p.M412K mutation in *Tmc1* that is linked to deafness in humans (Corns et al., 2017; Kim and Fettiplace, 2013; Pan et al., 2013). One study reported reduced single channel conductance caused by the p.M412K mutation (Pan et al., 2013), although a second study could not repeat these findings (Beurg et al., 2015a). These studies suggested that TMC1 and TMC2 affect pore-properties of the mechanotransduction channel of cochlear hair cells.

TMC1 and TMC2 are localized in hair cells near the lower end of tip links (Kurima et al., 2015), although the vast amount of TMC1 is found in the ER (Cunningham et al., 2020). In addition, TMC1 and TMC2 can bind to PCDH15 and to LHFPL5 (Beurg et al., 2015b; Maeda et al., 2014; Yu et al., 2020), consistent with the model that transduction channels might be coupled to the tip link. The localization of PCDH15 and TMC1 to stereocilia is perturbed in *Lhfpl5* mutant mice (Beurg et al., 2015b; Xiong et al., 2012), reinforcing the concept that these proteins are part of a larger protein complex near tip links.

Sequence alignments suggest that TMC1 and TMC2 are evolutionarily related to anoctamins, a family of 10 genes that encode Ca^{2+} activated Cl^- channels and lipid scramblases (Hahn et al., 2009). The structure of TMEM16A, one of the anoctamins, was solved and used as a template for homology modelling, predicting that TMC1 has a similar 10 membrane topography as TMEM16A (Fig. 4A,B). The model also revealed a putative ion conducting pathway lined by TM4-TM7 and predicted that TMC1 forms dimers indicative of two pores within a functional ion channel complex (Ballesteros et al., 2018; Pan et al., 2018). Two laboratories interrogated the model that TMCs are pore-forming subunits of the mechanotransduction channel.

The Holt laboratory substituted single amino acids within the putative pore region of TMC1 with cysteines (Pan et al., 2018). These mutations might change the properties of the

channel pore by themselves, or by reacting with bulky cysteine modification agents such as MTSET to form covalent disulfide bonds leading to obstruction of the channel pore. The mutant proteins were expressed in hair cells from *Tmc1/Tmc2* double mutant animals using AAV vectors. Out of 17 cysteine substitutions tested, 11 showed reduced Ca^{2+} permeability (compared to wild-type) that was caused by the mutation itself and/or following the addition of MTSET. Three mutations also caused reduction of single channel conductance. For those three mutants, effects of MTSET were reduced by channel blockers that enter the open pore of the channel, consistent with the model that the amino acids are within the pore (Pan et al., 2018).

The Holt and Fettiplace laboratories investigated effects of *Tmc1* mutations that are linked to deafness for their effects on TMC1-dependent MET currents (Beurg et al., 2019; Beurg et al., 2015a; Beurg et al., 2021b; Goldring et al., 2019; Pan et al., 2013). These findings similarly provide evidence that amino acid residues in the predicted TM4-TM7 region of TMC1 affect the Ca^{2+} permeability of the mechanotransduction channel. A p.D528N mutation had drastic effects on channel conductance, suggesting that this amino acid is in a particular narrow region of the TMC1 pore (Beurg et al., 2021b), which is consistent with data obtained by cysteine scanning mutagenesis (Pan et al., 2018).

Taken together, the studies provide strong evidence that TMC1 and TMC2 are pore-forming subunits of the mechanotransduction channel in hair cells.

The curious case of TMIE: an additional essential ion channel subunit affecting the pore—Surprisingly, parallel studies demonstrated that TMC1 and TMC2 cannot form functional ion channels in hair cells without a small transmembrane protein termed TMIE (Cunningham et al., 2020). TMIE has two predicted transmembrane domains (Fig. 4A,B) (Mitchem et al., 2002; Naz et al., 2002), although the first transmembrane domain might be a signal sequence that is cleaved off during transport of the protein to the cell surface (Gu et al., 2020; Naz *et al.*, 2002). TMIE binds to LHFPL5, PCDH15 and TMC1, and it co-localizes with LHFPL5 and TMC1 at the lower end of tip links (Cunningham et al., 2020; Zhao et al., 2014). Mechanotransduction is abolished in hair cells from mice with a *Tmie* null mutation (Zhao et al., 2014). This is due at least in part because TMC1 transport or retention in stereocilia is affected by TMIE (Cunningham et al., 2020). TMIE similarly regulates the localization of TMC proteins in zebrafish (Pacentine and Nicolson, 2019). However, overexpression of TMC1/2 in the hair cells from *Tmie* mutant mice drives TMC1/2 back into stereocilia without rescuing mechanotransduction currents. Conversely, mechanotransduction is not observed in stereocilia containing TMIE but not TMC1/2. Finally, TMIE binds to TMC1 through its C-terminal cytoplasmic domain, and this interaction is crucial for mechanotransduction (Cunningham et al., 2020). A functional mechanotransduction channel in hair cells thus consists of TMC1 (or TMC2) and TMIE.

The model that TMIE is an essential ion channel subunit is further supported by studies motivated by human genetics. Point mutations in TMIE linked to deafness have been identified in the C-terminal cytoplasmic domain of TMIE (Naz et al., 2002). These mutations are within a polar region of TMIE that binds to TMC1 and to phospholipids, including PIP_2 (Cunningham et al., 2020). The mutations affect PIP_2 binding and pore

properties of the mechanotransduction channel as revealed by a reduction in single channel conductance and a change in the Ca^{2+} selectivity (Cunningham et al., 2020).

It is currently unclear by which mechanism TMIE regulates the pore properties of the mechanotransduction channel of cochlear hair cells. The TMEM16A-based model structure of TMC1 reveals an unusually large cavity for the pore that is open to the lipid bilayer (Ballesteros et al., 2018; Pan et al., 2018). Perhaps, the transmembrane domains of TMIE shield the channel pore from the lipid environment, while the C-terminal cytoplasmic domain, which binds to TMC1 and PIP2, is close to the pore region and thus regulates pore properties.

The structure of the mammalian mechanotransduction channel has not been reported yet, but a recent study in bioRxiv reported the structure of a TMC1 protein complex purified from C. elegans (Jeong et al., 2022). The native complex contains both TMC1 and TMIE, confirming that the two proteins form a complex. The TMC1 structure is in good agreement with the model structure for mammalian TMC1 revealing a dimeric architecture and 10 transmembrane domains. Unlike the prediction from the mammalian channel, the structural data indicate that the pore of the channel is formed by TM6–8. TMIE is directly adjacent to the pore region. Interestingly, the TM domain of TMIE does not directly interact with the TM domains of TMC1 but instead forms a striking intramembranous cavity that is occupied by lipid molecules within the putative pore-forming TMC-1 helices TM6 and TM8. A cysteine at the cytosolic boundary of the TM domain in TMIE is palmitoylated and close to the TMC1 pore region, suggesting a possible role of TMIE and lipids in regulating MET channel gating (Jeong et al., 2022). The structure of the C. elegans TMC1 complex appears to reveal the closed conformation of the ion channel, which could undergo substantial structural rearrangements that alter the relative position of TMC1 and TMIE.

There are several unresolved issues. The mammalian MET channel contains a large permeation pathway permeable for cations and large organic molecules. The charge of the predicted pore of the nematode TMC1 channel suggests that it might not be selective for cations (Jeong et al., 2022). Nematode TMC1 might have similar permeation properties as the OSCA channel, a mechanically gated ion channel that displays non-selective cation currents with 17–21% Cl^- permeability (Murthy et al., 2018). In addition, TMC1 in nematodes is required for mechanosensation by OLQ neurons, acts as a pH sensor in ASH neurons, and as a leak channel in HSN neurons and vulval muscles (Tang et al., 2020; Wang et al., 2016; Yue et al., 2018). Interestingly, in hair cells TMC1 is also required to establish a background Na^+ leak current (Liu et al., 2019). It is not clear which channel complex is represented by the available nematode structure. Finally, nematodes do not have orthologs of CDH23 and PCDH15 and thus lack tip links. The structural relationship of the nematode channel complex to the mammalian mechanotransduction channel complex at tip links remains to be established.

CIB2 and CIB3: resemblance to accessory subunits of voltage gated K^+ channels—The study of families afflicted with a recessive form of deafness led to the identification of causative mutations in *CIB2*, a member of the calcium and integrin binding protein family, which consists in mammals of four members (CIB1–4) (Gentry et al., 2005).

Studies in mouse models have demonstrated that null mutations in *Cib2* or a point mutation linked to deafness impair mechanotransduction and lead to degenerative changes in the hair bundle of cochlear hair cells (Giese et al., 2017). CIB2 interacts with TMC1 and TMC2 and is essential for mechanotransduction by cochlear hair cells (Giese et al., 2017; Liang et al., 2021; Michel et al., 2017). Vestibular function is not affected by *Cib2* mutations, which is likely explained by the fact that vestibular hair cells co-express CIB2 and its close homologue CIB3 (Giese et al., 2017; Michel et al., 2017). In support of redundancy between the two proteins, mechanotransduction defects are rescued in cochlear hair cells from *Cib2* mutant mice by overexpression of CIB3 (Liang et al., 2021).

CIB2 and its relatives contain four EF-hand motifs, which are helix-loop-helix structural elements commonly found in Ca^{2+} binding proteins. CIB2 family proteins share this domain organization with KChIP proteins, which are members of the large family of neuronal Ca^{2+} sensor (NCS) proteins. Member of the NCS family have important functions in the nervous system including in phototransduction, neurotransmitter release, and in the regulation of ion channel trafficking and function (Burgoyne et al., 2019). KChIP proteins bind to the N-terminal cytoplasmic tail of K_V4 and regulate channel assembly, cell surface expression, and function (An et al., 2000; Wang, 2008). The structure of KChIP1 has been determined and identified a hydrophobic groove that mediates interactions with an alpha-helix formed by a protein domain within the N-terminal cytoplasmic domain of K_V4 (Fig. 4C) (Kise et al., 2021; Pioletti et al., 2006). CIB2 and its relative CIB3 bind to TMC1 and TMC2 through the N-terminal cytoplasmic domain and through a second stronger binding site in the first cytoplasmic loop between TM domains 2 and 3 (Giese et al., 2017; Liang et al., 2021). The co-crystal structure of a fragment of TMC1 encompassing the first cytoplasmic loop in complex with CIB3 revealed that the cytoplasmic loop of TMC1 folds into a long alpha helix connected by a linker to a shorter alpha helix that binds into a groove in CIB3 resembling the groove recognized by Kv4 in KChIP1 (Fig. 4C) (Liang et al., 2021). Molecular modelling studies predict that TMC1 and TMC2 bind to CIB2 in similar ways (Liang et al., 2021). When TMC1 was purified from *C. elegans*, it was bound not only by TMIE but also by CALM1, the *C. elegans* ortholog of CIB2/3 (Jeong et al., 2022). CALM1 forms a cap on the cytoplasmic surface of the ion channel complex and the structure revealed a similar longer and shorter alpha helix within the first cytoplasmic loop of TMC1 that is buried into a groove of CALM1 (Jeong et al., 2022).

In mouse hair cells, CIB2 is required for TMC1 transport to or retention within stereocilia (Liang et al., 2021). CIB2 resemble in this regard KChIP proteins, which also regulate the localization of K^+ channels (Burgoyne et al., 2019). In addition, CIB2 and KChIP regulate ion channel function directly, although in distinct ways. While KChIP proteins regulate channel inactivation kinetics, CIB2 affects the open probability of the mechanotransduction channels at rest prior to mechanical stimulation (Liang et al., 2021). The latter conclusion was reached by the study of a point mutation in *Cib2* (p.R186W) that causes deafness. The p.R186W mutation reduces the binding affinity of CIB2 for TMC1, indicative of structural changes that affect the protein-protein interaction surface between the two proteins (Liang et al., 2021).

The mechanism by which CIB2 affects ion channel function needs further study. The activity of mechanotransduction channels in cochlear hair cells is regulated by the extracellular and intracellular Ca^{2+} concentration (Fettiplace, 2017). NMR studies suggest that the structure of CIB2 is affected by Ca^{2+} (Vallone et al., 2018). Ca^{2+} ions that enter hair cells through transduction channels could affect the conformation of CIB2 and its interaction with TMC1. The p.R186W mutation in *Cib2* slightly affects the unitary conductance of the mechanotransduction channel in OHCs (Liang et al., 2021). Perhaps this can be explained by allosteric effects of CIB2 on the conformation of the TMC1 protein. The p.R186W mutation is located in the extreme C-terminus of CIB2 that is not well structured in crystals, indicative of flexibility (Liang et al., 2021). The point mutation converts a charged amino acid to one that is hydrophobic. Perhaps the N-terminus of CIB2 is close enough to the ion conductance pathway to affect ion permeability. The analysis of additional mutations in *Cib2* that affect Ca^{2+} binding might be informative to define the extent to which CIB2 mediates effects of Ca^{2+} on the mechanotransduction channel. However, the affinity of CIB2 for Ca^{2+} is low and much higher for Mg^{2+}, suggesting that CIB2 may not work as Ca^{2+} sensor under physiological conditions (Vallone *et al.*, 2018).

Recent studies have proposed a different function for CIB2 that might not be related to its function as a Ca^{2+} binding protein. These studies suggest that CIB2 might serve as a linker to anchor the mechanotransduction channel to the cytoskeleton. CIB2 binds to the adapter protein whirlin (WHRN) and to the motor protein MYO7A (Riazuddin et al., 2012). WHRN and its binding partner MYO15A are localized to tips of stereocilia and are required for the development of stereocilia of normal heights (Belyantseva et al., 2005). Interactions of CIB2 with the WHRN/MYO15A could mediate interactions between the mechanotransduction channel complex and the actin cytoskeleton.

Studies in nematodes have provided evidence that CALM1, the C. elegans ortholog of CIB2, binds to the ankyrin domain protein UNC-44, which could be yet another way to connect the mechanotransduction channel to the cytoskeleton (Tang et al., 2020). Earlier measurements indicated spring-like properties of ankyrin repeats (Lee et al., 2006; Sotomayor et al., 2005). Recent cryo-EM data and functional studies indicate that ankyrin repeats function as gating springs for the mechanically activated NOMPC ion channel (Jin et al., 2017; Wang et al., 2021a; Zhang et al., 2015). UNC-44 might fulfill a similar role for the mechanotransduction channel in hair cells, but UNC-44 was not present in the purified mechanotransduction complex from C. elegans used for structural studies (Jeong et al., 2022). The role of mammalian ankyrins in cochlear hair cells has not been analyzed. Thus, further studies will thus be necessary to define the extent to which UNC-44 is required to anchor the mechanotransduction channel to the cytoskeleton or whether the channel is in fact anchored to the cytoskeleton at all.

Model of the mechanotransduction-channel complex of hair cells: two-fold symmetry and multisubunit composition—The findings summarized above suggest a striking architecture for the mechanotransduction channel of cochlear hair cells. In this model, the basic channel unit is a symmetrical structure that contains two pores and consists of dimers of TMC1, and two TMIE and CIB2 molecules (Fig. 4A). Symmetry and dimeric structure are also defining features of the tip-link complex including PCDH15 and LHFPL5

(Fig. 3). It is tempting to speculate that the dimeric ion channel complex is tethered to the dimeric tip link via LHFPL5, although no structural data are available to support this model. CIB2 might provide a connection to the cytoskeleton by binding to WHRN, UNC44 or perhaps LOXHD1, which has recently been linked to mechanotransduction by hair cells (Trouillet et al., 2021).

Alternatively, the mechanotransduction channel might be gated by changes in membrane curvature. In support of the latter model, truncated TMC1 and TMC2 proteins from turtle and budgerigar have been reconstituted into liposomes and appear to be activated by negative pressure on the membrane (Jia et al., 2020). A recent report also suggests a tonotopic gradient in the number of TMC1 proteins with up to 20 copies per tip link (Beurg et al., 2018). This observation would suggest that direct coupling of the mechanotransduction channel to the tip link is not essential for channel gating.

Perhaps, the activation mechanism of the mechanotransduction channel of hair cells is not solely explained by either the tethered channel or membrane curvature model. Both mechanisms might cooperate to establish the exquisite force-sensitivity of this ion channel.

Adaptation: Quo Vadis?

Much attention has recently focused on the identification of the molecular components of the mechanotransduction channel at the lower end of tip links. In addition, proteins have been identified that are localized to the upper end of tip links. These proteins are thought to regulate resting tension in the mechanotransduction channel and perhaps adaptation.

The upper end of tip links: adapters and molecular motor proteins—The classical motor model of adaptation predicts that the upper end of tip links is connected to a protein complex that includes myosin motors that establish resting tension in the mechanotransduction complex and regulate slow adaptation (Markin and Hudspeth, 1995). MYO1C was proposed to be the adaptation motor (Holt et al., 2002). The motor model also predicts that Ca^{2+} entering through the mechanotransduction channel regulates myosin motor function. In its original form, the model cannot apply to cochlear hair cells since mechanotransduction channels are only localized at the lower end of tip links (Beurg *et al.*, 2009). In addition, MYO1C is not concentrated at the upper end of tip links of mammalian hair cells (Schneider et al., 2006).

Motivated by the study of genes linked to deafness, a different protein complex has been identified at the upper end of tip links that consists of the adapter protein HARM-B, the ankyrin-domain containing protein SANS and the molecular motor protein MYO7A (Grati and Kachar, 2011; Grillet et al., 2009). These proteins bind to each other and to the cytoplasmic domain of CDH23, and the crystal structures highlighting some of these interactions have been determined (Fig. 5) (Kazmierczak and Müller, 2012). SANS, MYO7A and HARM-B also bind to F-actin thus establishing a potential link between the tip link and the cytoskeleton of hair cells (Kazmierczak and Muller, 2012). It is tempting to speculate that each strand of CDH23 assembles a similar protein complex thus extending the concept of a symmetric dimeric structure throughout the entire tip-link structure.

The importance of SANS, MYO7A and HARM-B for mechanotransduction has been investigated with the help of genetically modified mice. Deaf circler (*dfcr*) mice carry a mutation that prevents interaction of HARM-B with F-actin (Grillet *et al.*, 2009; Johnson et al., 2003). These mice preserve their tip links but HARM-B is displaced from the upper end of tip links. Gating of transduction channels throughout the hair bundle is less well coordinated in the mutants, and transducer current activation and adaptation are slowed (Grillet et al., 2009). Similar observations have been made for *dfcr2J* mice, which entirely lack HARM-B (Michalski et al., 2009). The analysis of the function of SANS and MYO7A in mechanotransduction has been complex because null mutations in the two genes lead to defects in hair bundle development (Caberlotto et al., 2011; Self et al., 1998). However, MYO7A is expressed in several isoforms. The longest isoform MYO7A-C contains an extension of 11 amino acids at the N-terminus of a shorter MYO7A-S isoform (Li et al., 2020). Hair bundles maintain their normal shape in genetically modified mice expressing only the shorter MYO7A-S isoform, but MYO7A levels in IHC are diminished. Resting tension is reduced and there is a significant delay in current onset following mechanical stimulation (Li et al., 2020). These findings are consistent with a model where the protein complex at the upper end of tip links might provide a stable anchor point that keeps the tip link and transduction complex under tension.

The classical motor model of adaptation was developed based on studies in hair cells tuned to low frequencies (i.e bullfrogs and turtles). Although the activity of mechanotransduction channels in both mammalian and non-mammalian species is dependent on the intracellular and extracellular Ca^{2+} concentration, cochlear hair cells respond to much higher frequencies and recent findings suggest that fast adaptation predominates in cochlear hair cells and may be independent of Ca^{2+} (Caprara et al., 2019; Peng et al., 2013). While data by others do not necessarily support this conclusion (Beurg *et al.*, 2015a; Corns *et al.*, 2014), a recent study also has provided evidence that there is no strict correlation between Ca^{2+} entry into stereocilia and adaptation rate (Goldring *et al.*, 2019).

Slow adaptation in cochlear hair cells is still observed and requires Ca^{2+} entry and myosin motor function, although the specific myosin appears not to be MYO1C (Caprara et al., 2020). In addition, channel open probability at rest and adaptation are regulated in cochlear hair cells by distinct mechanisms (Caprara et al., 2020; Liang et al., 2021; Peng et al., 2016). Peng and colleagues therefore proposed a revised motor model, which suggests that different myosin motors located at upper and lower tip-link insertions are required for tension generation and slow adaptation, respectively (Caprara et al., 2020). In this model, slow adaptation is not related to the modulation of tip-link tension, requires activity of myosin motors other than MYO1C and other modulators, such as PIP_2. It is still unclear whether Ca^{2+} entry through the mechanotransduction channel at the lower end of the tip link can influence the proteins at the upper end of the tip link. Further studies will be necessary to test this model and to identify the molecules that regulate adaptation in cochlear hair cells.

Mechanotransduction: Beyond Processing Input Sound Stimuli

Exciting new evidence has revealed important functions for mechanotransduction channels in hair cells that go beyond roles in sensory transduction. New evidence suggests that active

mechanotransduction channels in hair cells affect the fine structure of the hair bundle and the assembly of auditory circuits.

Mechanotransduction and hair bundle morphogenesis—The morphogenesis of the hair bundles is a complex process. Studies in chickens have defined four phases of bundle development. In stage I, postmitotic hair cells start to develop morphological specializations at their apical surface. In stage II, stereocilia start to form, elongate and develop a rudimentary staircase. In stage III, stereocilia widen, while in stage IV, stereocilia elongate to reach their mature length (Tilney et al., 1992). The development of hair bundles in rodents progresses through four stages that are very similar to those in chickens (Krey et al., 2020). Studies in mice have provided insights into the molecular machinery that shape the hair bundle. In rodents, mature hair bundles of cochlear hair cells contain three rows of stereocilia. A protein complex consisting of the short isoform of the motor protein MYO15A-S, the adapter protein WHRN, the actin regulatory proteins EPS8 and the planar cell polarity proteins GPSM2 and GNAI3 becomes stabilized at the tips of row 1 stereocilia, the longest stereocilia (Fig. 6A). Mutations in the murine genes encoding these proteins lead to abnormally short stereocilia indicative of a function for this protein complex in regulating elongation of the actin core of stereocilia (Belyantseva et al., 2005; Fang et al., 2015; Manor et al., 2011; Mauriac et al., 2017; Tadenev et al., 2019; Zampini et al., 2011). A different set of proteins including the long isoform of the molecular motor MYO15A-L, as well as the actin capping proteins CAPZB, TWF2 and EPS8L2 are concentrated in row 2 stereocilia (Fig. 6A) (Avenarius et al., 2017; Fang et al., 2015; Furness et al., 2013).

The mechanotransduction machinery of cochlear hair cells in rodents matures in the early postnatal phase coincident with the development of the stereociliary bundle (Lelli et al., 2009; Waguespack et al., 2007). These findings suggested a potential role for mechanotransduction in determining bundle shape, which could explain why hair bundles in mutant mice that affect the mechanotransduction machinery lose their typical V shape (Beurg *et al.*, 2018; Giese *et al.*, 2017). Tilney had already suggested that Ca^{2+} might be a driving force in regulating the morphogenesis of stereocilia (Tilney et al., 1992). This is a compelling concept, especially because the mechanotransduction channel of cochlear hair cells is permeant for Ca^{2+}, which could regulate actin polymerization at stereocilia tips. Consistent with a role for mechanotransduction in hair bundle morphogenesis, mutations in the gene encoding SANS affect tip-link maintenance and mechanotransduction, as well as the length of stereocilia in rows 2 and 3, the transducing stereocilia (Caberlotto et al., 2011). Similarly, within 5 hours of treatment of inner ear organ cultures with mechanotransduction channel blockers, the tips of transducing stereocilia start to thin and they shorten over the next 24 hours. Shrinkage is reversible when the blockers are removed (Velez-Ortega et al., 2017).

To gain insights into the mechanisms by which mechanotransduction affects the shape of the hair bundle, a recent study has investigated how mechanotransduction affects distinct stages of stereocilia morphogenesis across the hair bundle. The findings demonstrated that mutations that affect mechanotransduction channels lead to changes in the widening of stereocilia, especially in row 2. In addition, while a stereocilia staircase still develops, the difference in stereocilia length across rows is less pronounced (Fig. 6B). These

morphological changes are accompanied by molecular changes where stabilization of protein complexes typical for row 1 and row 2 no longer occurs. MYO15A-S, MYO15A-L as well as EPS8 and EPS8L2 are found at all stereociliary tips, rather than segregated to either row 1 or row 2 tips. By the third postnatal week, GNAI3, GPSM2, CABZB2 and TWF2 are no longer concentrated at stereociliary tips (Krey et al., 2020).

The mechanisms that link mechanotransduction defects to altered hair bundle morphology need further study. Hair bundle morphogenesis in mice progresses in the first week after birth, but mice only hear sound by about P12-P14 when the ear canal opens (Mikaelian and Ruben, 1965). Thus, mechanotransduction channels are not activated by incoming sound signals while hair bundles develop. However, mechanotransduction channels are not closed at rest leading to an influx of Ca^{2+} into the cells even in the absence of a sound stimulus (Beurg et al., 2010; Corns et al., 2014; Johnson et al., 2011; Peng et al., 2013). Influx of Ca^{2+} may locally regulates the function of proteins that are important for stereocilia growth. To explore the mechanisms by which mechanotransduction affects stereocilia morphology, it would be interesting to analyze in real time how acute block of mechanotransduction channels affects the distribution and movement of proteins within stereocilia. Some proteins of the mechanotransduction complex, such as the Ca^{2+} binding proteins CIB2, might also have direct roles in morphogenesis. This is consistent with the findings that hair bundles in *Cib2* mutant mice develop significant morphological defects (Giese et al., 2017).

Mechanotransduction and auditory circuit development—SGNs are the sensory afferent neurons of the auditory systems. Type I SGNs constitute 95% of the total pool of SGNs and mono-synaptically innervate IHCs, where each IHC is contacted by 5–30 type I SGNs. Type II SGNs constitute the remaining 5% of the total pool of SGNs and innervate OHCs, where each type II SGNs branches out onto dozens of OHCs (Fig. 7A). Type I SGNs transmit sound information to the brain, while type II SGNs are thought to have a role in damage perception or pain signaling. Similar to hair cells, type I SGNs are tonotopically organized, where each SGN is most sensitive to a particular frequency that coincides with its innervation specificity on IHCs along the tonotopic axis of the cochlea (Appler and Goodrich, 2011; Sun et al., 2021).

Type I SGNs innervating a single IHCs vary in their properties and innervation specificity onto IHCs. Type I SGNs fire spontaneous action potentials at varying rates. Based on differences in spontaneous rates (SRs) and sensitivity to sound, these neurons have been divided into high-SR, medium-SR and low-SR fibers, although in many species SR rates seem to show a more gradual increase from low to high that cannot so easily be fit into three groups (Heil and Peterson, 2015). In cats, low and high-SR fibers preferentially contact the modiolar and pillar side of IHCs, respectively (Liberman, 1982). Single rodent IHCs also appear to be innervated by SGNs with different SRs (Fig. 7B) (Liberman et al., 2011; Wu et al., 2016).

Single cell RNA sequencing studies have shown that type I SGNs are at birth molecularly diverse and that they further refine their molecular phenotype over the next few postnatal weeks to define three subclasses of type I SGNs (Petitpre et al., 2018; Shrestha et al., 2018; Sun et al., 2018). This refinement process is severely disrupted in mice carrying mutations

in genes that encode components of the mechanotransduction machinery including *Tmie*, *Lhfpl5* and *Pcdh15* (Sun et al., 2018). The molecular refinement process coincides with the time frame when mechanotransduction currents can first be recorded from hair cells and when type I SGNs establish their innervation specificity by a process that involves the pruning of excessive nerve sprouts (Fig. 7C) (Sun et al., 2018). The findings suggest that signals from hair cells that depend on active mechanotransduction channels are critical to direct hair cell innervation by type I SGNs. The formation or maintenance of ribbon synapses is affected in *Tmc1/2* mutant mice (Lee et al., 2021), further demonstrating the importance of mechanotransduction channels for patterning auditory afferents.

The mechanisms by which mechanotransduction channels affect the differentiation of SGNs is unclear. Hair cells do not receive sound-driven sensory input prior to P12-P14 when the ear canal opens (Mikaelian and Ruben, 1965). However, prior to the onset of hearing, waves of spontaneous activity that are initiated by supporting cells in the auditory sensory epithelium are propagated to SGNs and from there along the auditory pathway to the CNS (Fig. 7C). These waves of spontaneous activity depend on glutamate release from IHCs (Wang and Bergles, 2015). This release of glutamate depends on the vesicular glutamate transporter VGLUT3 that is expressed in IHCs (Seal et al., 2008). In mice with mutations in *Vglut3*, spontaneous activity can still be observed, but its pattern is changed (Babola et al., 2018). The spontaneous activity patterns of SGNs and the molecular diversification of type I SGN are also perturbed in *Vglut3* and *Tmie* mutant mice (Sun et al., 2018), revealing an interesting interplay between mechanotransduction, spontaneous activity, and neuronal subtype specification. How mechanotransduction participate in this regulatory circuit remains to be established, but some hints have emerged. Prior to the onset of hearing, spontaneous activity is initiated by release of ATP from inner supporting cells. ATP release induces crenations in supporting cells (Wang and Bergles, 2015). Perhaps, these crenations activate mechanotransduction channels in hair cells, thus triggering glutamate release from hair cells to contribute to the propagation of spontaneous activity patterns to SGNs.

Challenges Ahead

The field of auditory mechanotransduction has made a leap forward. Core components of the mechanotransduction machinery have been identified, but we still have a limited understanding of their role within this protein complex. A significant stumbling block for the field has been the lack of a reconstituted system that allows to study ion channel function. Properties of the mechanotransduction channel have been inferred from the study of hair cells where single channel features are evaluated in the whole-cell patch configuration and not by directly patching on membrane segments containing the ion channel. These studies have led to the model that TMC1 and TMIE affect pore properties of the mechanotransduction channel. How Ca^{2+} regulates channel function is unclear, but CIB2 is a good candidate to mediate some effect of Ca^{2+} on this ion channel. The atomic structure of a TMC1-containing ion channel complex has been revealed in *C. elegans*, but the structure of the mammalian channel complex and how it couples to the tip link are not known. While candidate gating spring molecules have been identified, further studies are necessary to study the function of these molecules. Mechanisms of adaptation appear to differ between hair cells in different species and the molecular mechanisms of

adaptation remain to be clarified. Finally, we lack an understanding of the mechanisms by which mechanical force leads to structural changes in the mechanotransduction channel that allow ion flux through the channel pore. The identification of core components of the mechanotransduction machinery of hair cells opens the door to resolve these questions. The demonstration that active mechanotransduction channels are required for the normal development of hair cells and SGNs opens up entirely new areas for investigation.

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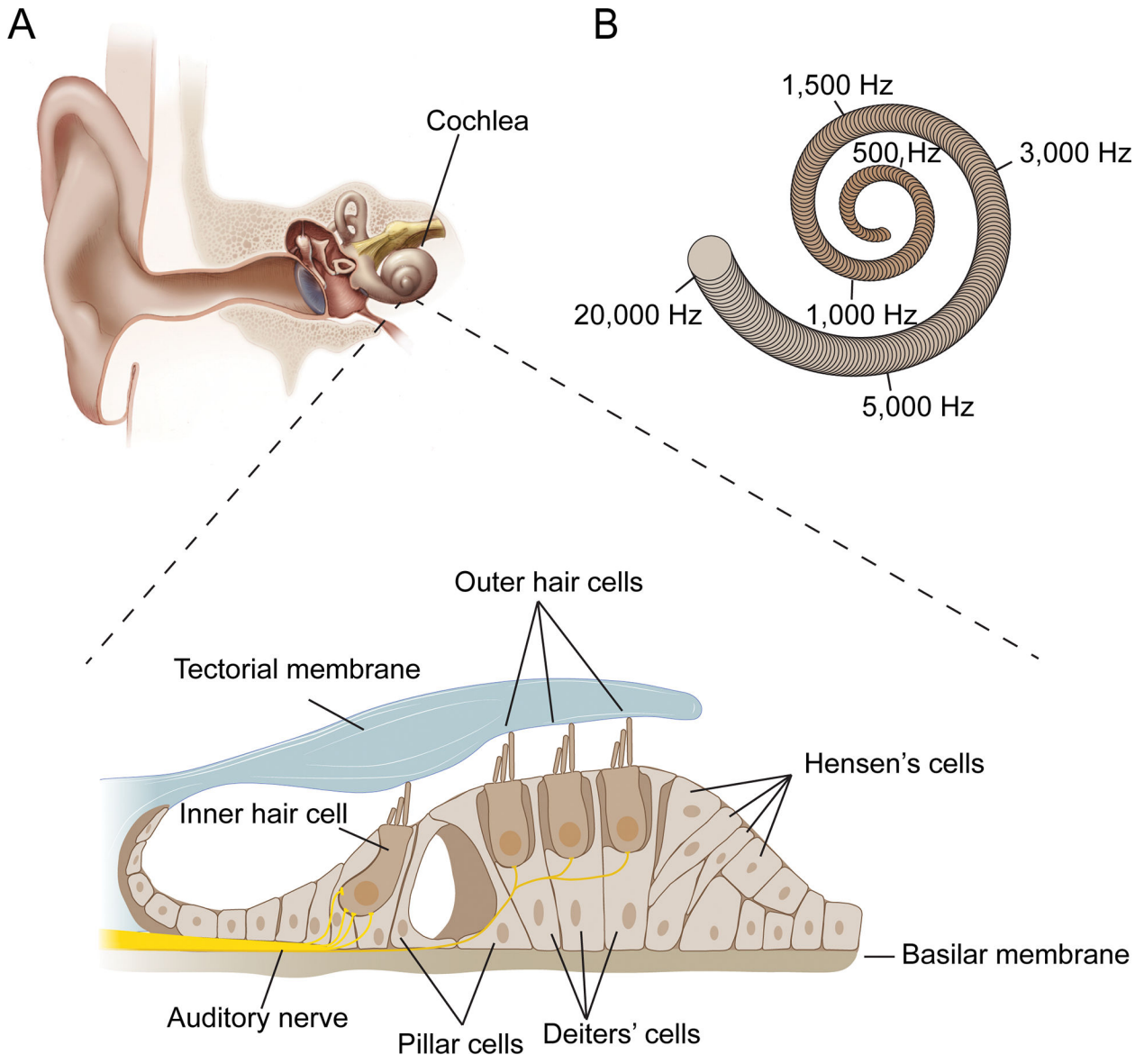

Figure 1. The auditory sense organ.

(A) Diagram of the outer, middle and inner ear. The bottom shows a cross section of the Organ of Corti that is situated within the snail-shaped cochlea of the inner ear. Sensory hair cells, including one row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs), are indicated. Supporting cells such as Pillar cells, Deiters' cells and Hensen's cells separate and support hair cells. The cells of the Organ of Corti are sandwiched between two extracellular matrix assemblies, the tectorial membrane and the basilar membrane. (B) Schematic of the snail-shaped cochlea indicating that different sound frequencies are encoded by hair cells at different tonotopic positions along the cochlear duct. Frequency positions are for the human cochlea.

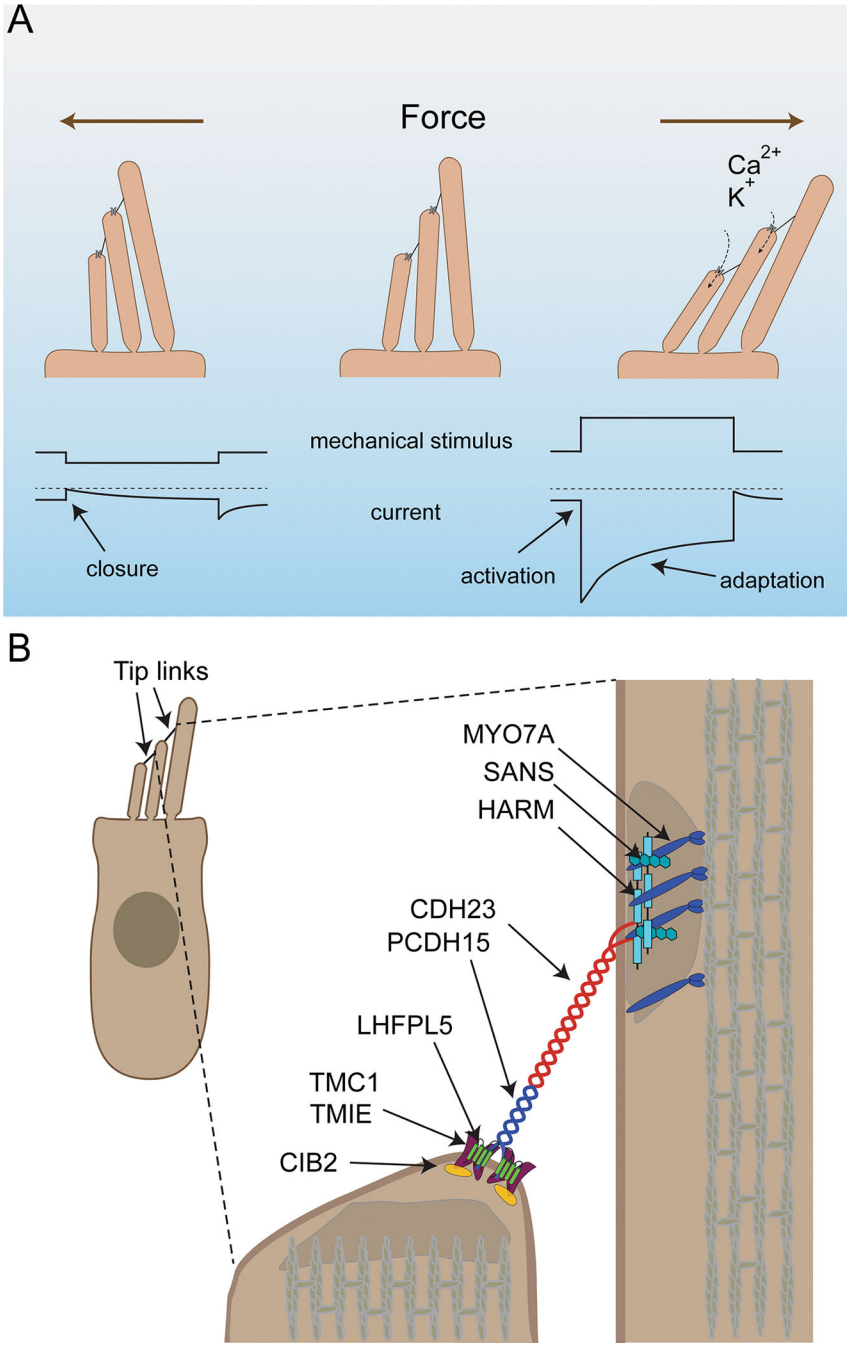

Figure 2. The mechanotransduction machinery of cochlear hair cells.

(A) Diagram of the mechanotransduction process. On top the direction of stereocilia deflection is shown, on the bottom idealized traces from recordings. Upper traces show mechanical deflections of the stereocilia: downward shift (left) indicates a deflection towards the shortest stereocilia, an upward shift (right) deflection towards the longest stereocilia. Deflection towards the shortest stereocilia (left) leads to a closing of mechanotransduction channels that are open at rest followed by slow channel re-opening. Deflection towards the longest stereocilia (right) leads to an increased influx of Ca^{2+} and

K^+ into stereocilia. This is reflected in the lower traces by a downward current, which then adapts due to channel closure even during a maintained stimulus. (B) Diagram of a hair cells indicating proteins important for mechanotransduction and their asymmetric localization at the upper and lower ends of the tip link.

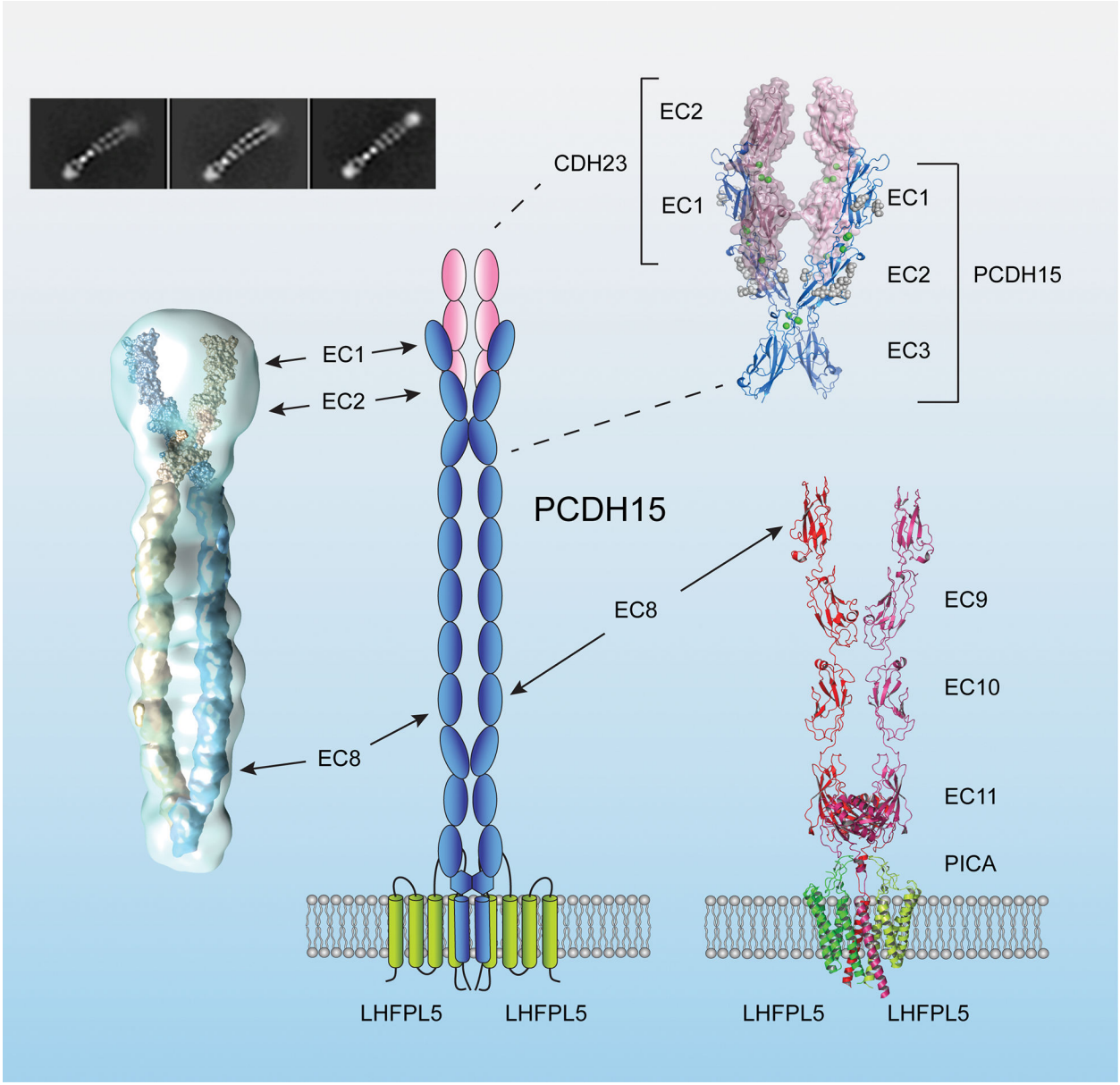

Figure 3. Structure of the PCDH15-LHFPL5 complex and the PCDH15-CDH23 binding site.

A diagram of the lower end of tip links. The interaction between PCDH15 and CDH23 is mediated by N-terminal EC1 and EC2 domains (upper right, PDB 4APX and 6CV7) (Sotomayor et al., 2012). Dimerization of PCDH15 is mediated by two sites. The first dimerization site is at EC3 and was observed by single particle reconstruction of the entire 11 EC domains (left) and a high-resolution X-ray crystallographic structure of an EC1–3 fragment (upper right: PDB:6CV7) (Dionne et al., 2018). The second dimerization sited is close to the membrane proximal domain observed in crystals of a C-terminal fragment of PCDH15 (Ge et al., 2018). A PCDH15 dimer interacts with an LHFPL5 dimer (bottom right). The PICA domain of PCDH15 forms a collar above the membrane that might have structural flexibility (PDB: 6C13 and 6C14) (Ge et al., 2018).

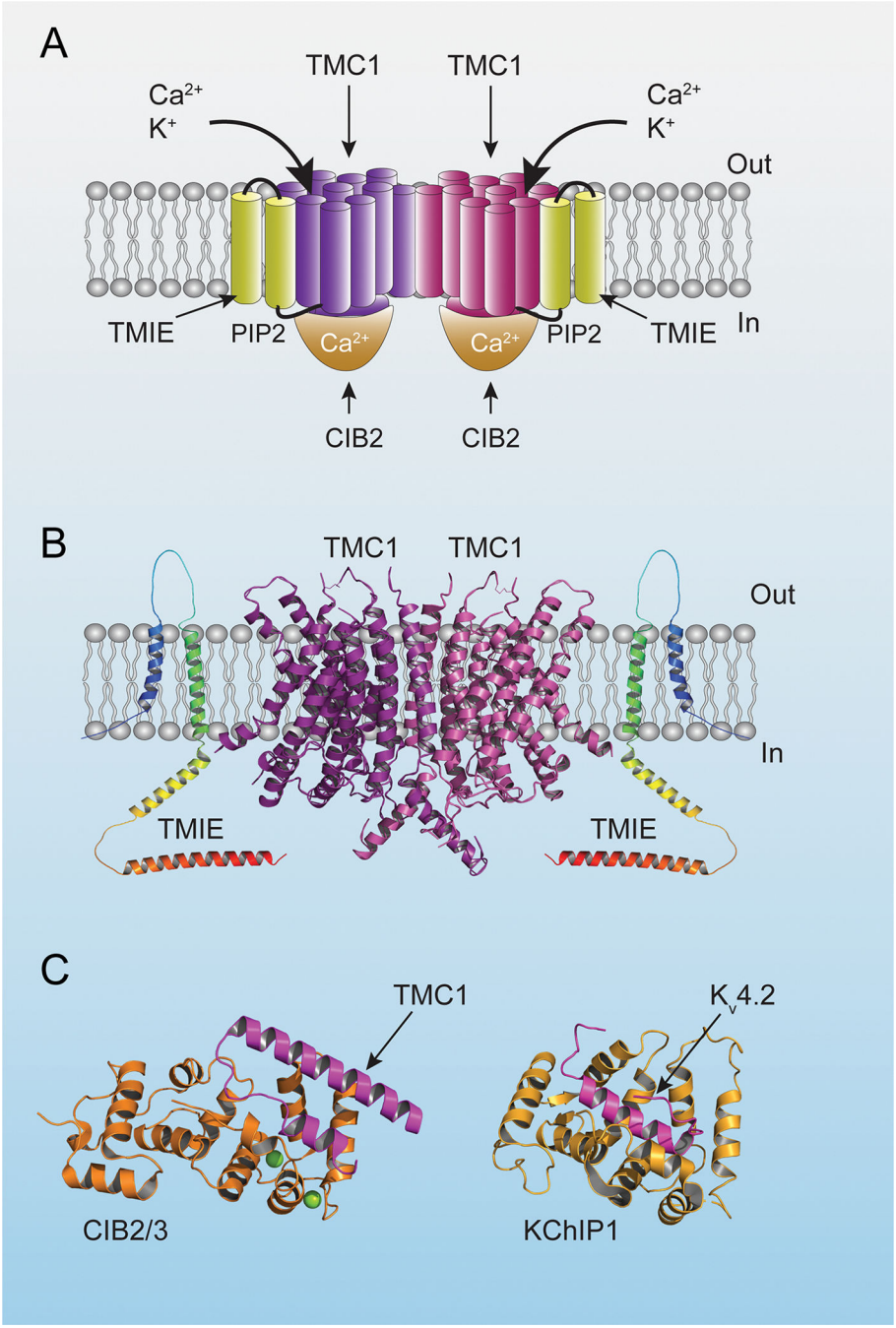

Figure 4. Diagram of the ion channel complex.

(A) Diagram of the mechanotransduction-channel complex based on structural data from *C. elegans* (Jeong et al., 2022). Transmembrane domains of TMC1 are in purple and burgundy to highlight the dimeric nature of the complex. (B) Structural model of TMC1 based on the structure of TMEM16A (Ballesteros et al., 2018). The structure of TMIE was predicted by alpha-fold (https://alphafold.ebi.ac.uk/). (C) Crystal structure of CIB2/3 and KChiP1 in complex with the first cytoplasmic loop of TMC1 and the N-terminal cytoplasmic domain of $K_V4.2$, respectively (PDB: 6WUD and 7E84). Note the similar fold for CIB2/3 and KChiP1

with a hydrophobic groove occupied by α-helices of the TMC1/K_V4.2 binding partners (Liang et al. 2021).

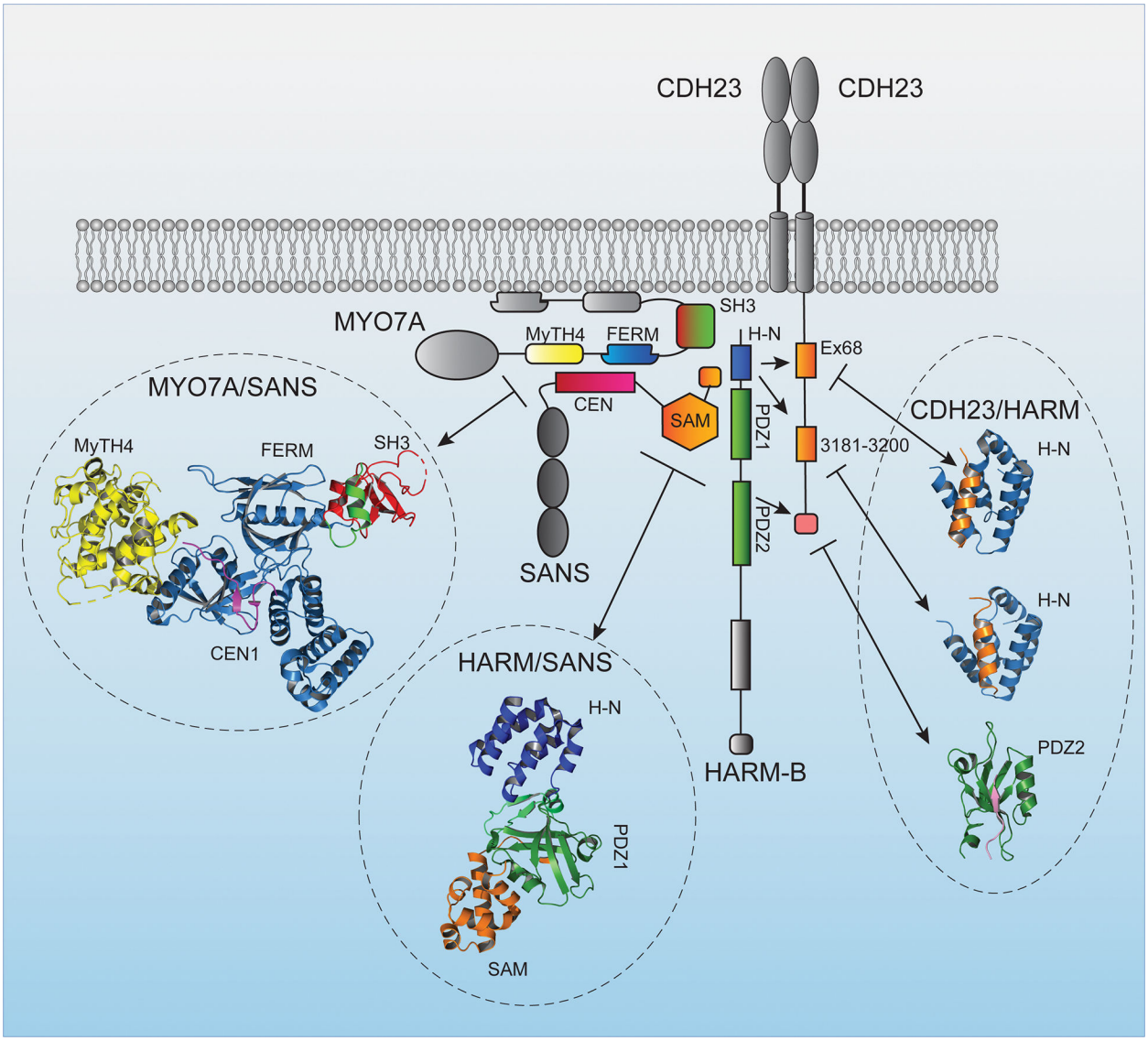

Figure 5. Diagram of the protein complex at the upper end of the tip link.

Diagram of proteins that are localized to the upper end of the tip link consisting of the adapter protein HARM-B, the ankyrin-domain containing protein SANS, and the motor protein MYO7A. Known protein-protein interaction domains are indicated. Protein domains in the diagram for which structures have been determined are color coded and the structures are shown (PDB:3PVL, 3K1R, 2LSR, 2KBR, 2KBS) (Pan et al., 2009; Wu et al., 2011; Wu et al., 2012; Yan et al., 2010). The structure of the interaction site between the N-terminus of CDH23 and HARM-B was omitted.

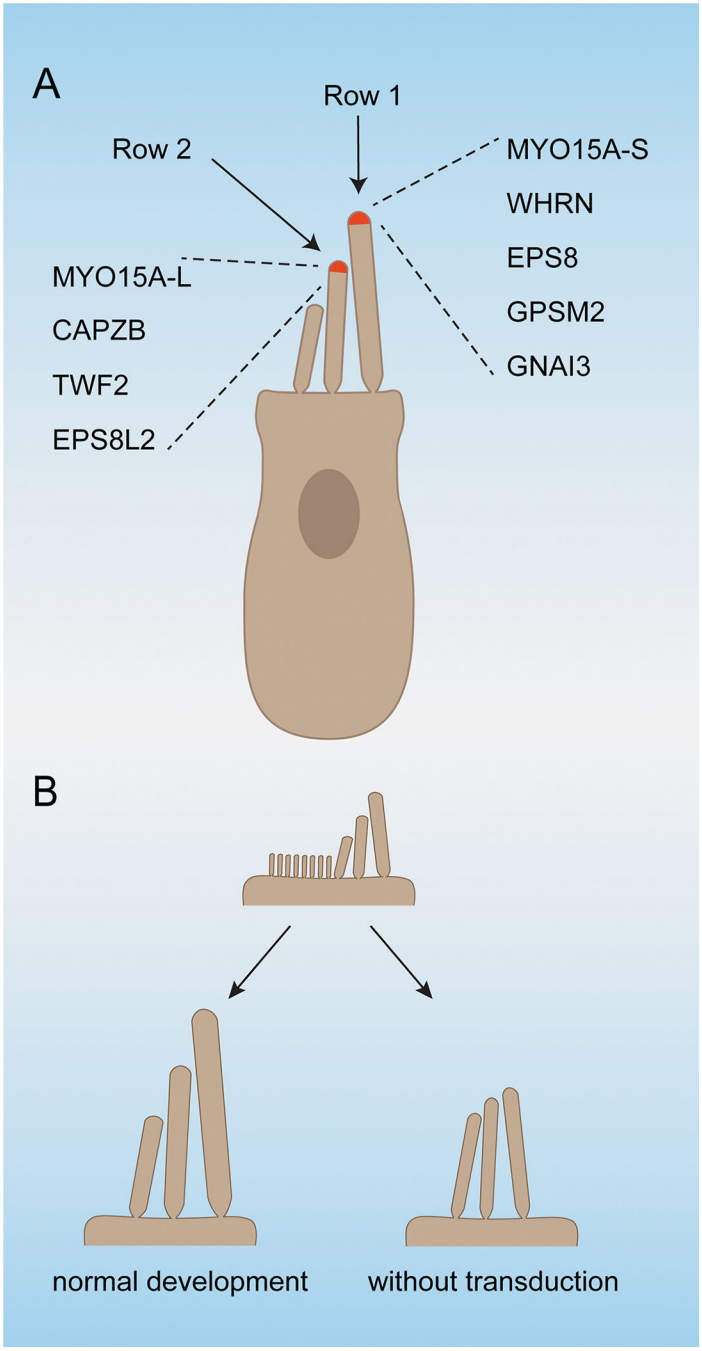

Figure 6. Mechanotransduction and hair cell development.

(A) Diagram of a hair cell indicating the protein complexes that are stabilized in row 1 and row 2 stereocilia and that are required for normal hair bundle development and/or maintenance. (B) Diagram of the shape of an immature hair bundle and how it matures in wild-type mice, and in mice with defects in mechanotransduction. In the absence of mechanotransduction, a hair bundles containing stereocilia of graded heights still develop, but the dimension of stereocilia is changes and the heights-gradient between stereocilia is reduced.

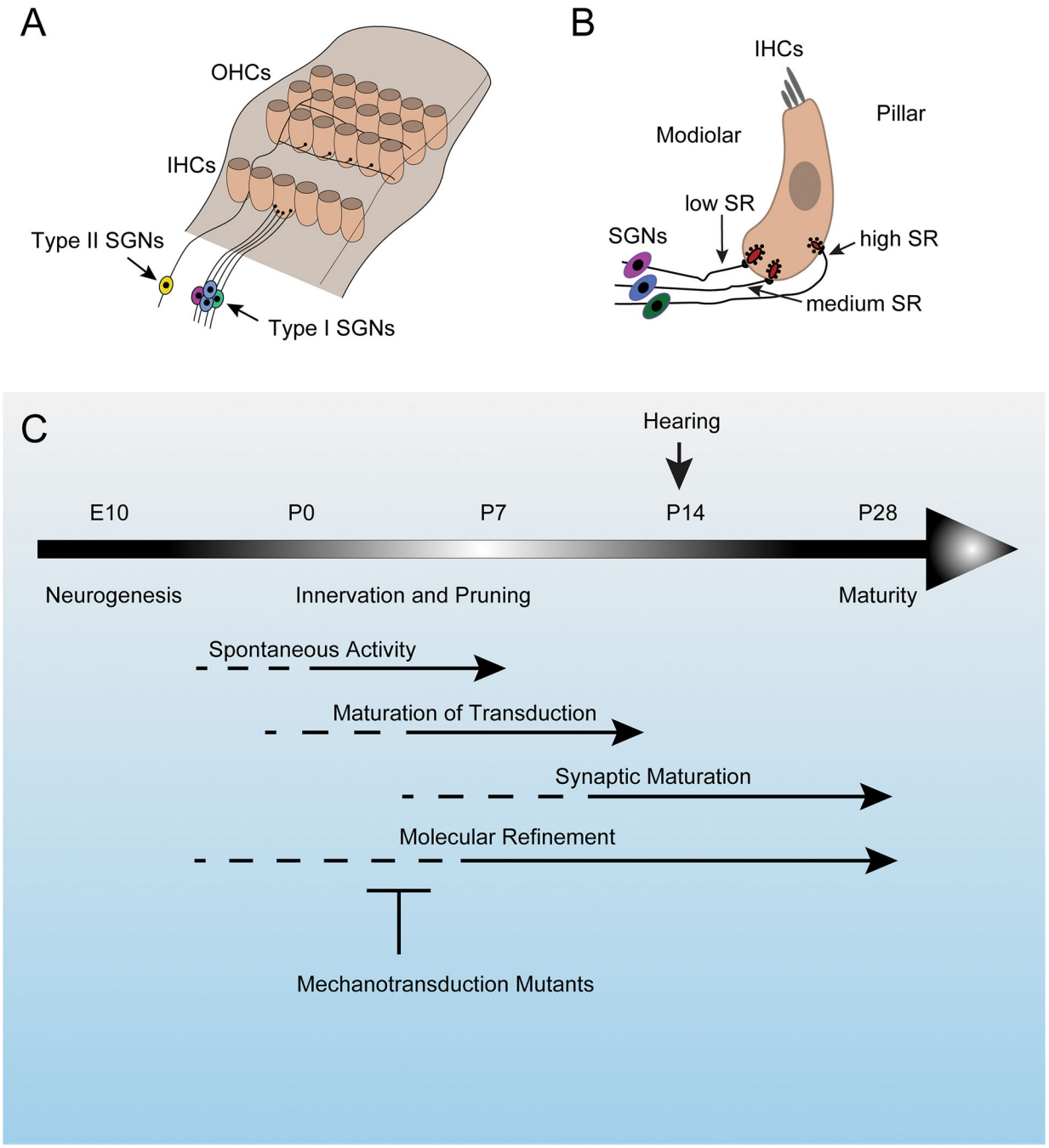

Figure 7. Development of spiral ganglion neurons.

(A) Diagram of the auditory sensory epithelium highlighting type I SGNs and type II SGNs and their innervation specificity onto IHC and OHCs, respectively. (B) Diagram of an IHC showing the innervation specificity of type I SGNs with different spontaneous rates (SRs) along the modiolar-pillar axis of the hair cell. (C) Timeline of the development of SGNs highlighting key events. Mechanotransduction defects affect synaptic maturation and molecular refinement.