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Usher protein functions in hair cells and photoreceptors

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Abstract

The 10 different genes associated with the deaf/blind disorder, Usher syndrome, encode a number of structurally and functionally distinct proteins, most expressed as multiple isoforms/protein variants. Functional characterization of these proteins suggests a role in stereocilia development in cochlear hair cells, likely owing to adhesive interactions in hair bundles. In mature hair cells, homodimers of the Usher cadherins, cadherin 23 and protocadherin 15, interact to form a structural fiber, the tip link, and the linkages that anchor the taller stereocilia's actin cytoskeleton core to the shorter adjacent stereocilia and the elusive mechanotransduction channels, explaining the deafness phenotype when these molecular interactions are perturbed. The conundrum is that photoreceptors lack a synonymous mechanotransduction apparatus, and so a common theory for Usher protein function in the two neurosensory cell types affected in Usher syndrome is lacking. Recent evidence linking photoreceptor cell dysfunction in the shaker 1 mouse model for Usher syndrome to light-induced protein translocation defects, combined with localization of an Usher protein interactome at the periciliary region of the photoreceptors suggests Usher proteins might regulate protein trafficking between the inner and outer segments of photoreceptors. A distinct Usher protein complex is trafficked to the ribbon synapses of hair cells, and synaptic defects have been reported in Usher mutants in both hair cells and photoreceptors. This review aims to clarify what is known about Usher protein function at the synaptic and apical poles of hair cells and photoreceptors and the prospects for identifying a unifying pathobiological mechanism to explain deaf/blindness in Usher syndrome.

Keywords

Usher syndrome; Cochlear hair cell; Photoreceptor; Mechanotransduction; Ribbon synapse

1. Introduction

Usher syndrome is a genetically heterogeneous disease affecting neurosensory cells in the cochlea, the retina, and for some clinical sub-types, the vestibular system. Ten genes have been associated to the 12 loci thus far identified in families with the syndrome (Adato et al., 2002; Fields et al., 2002; Weil et al., 1995, 2003; Ahmed et al., 2001; Bitner-Glindzicz et al., 2000; Bolz et al., 2001; Bork et al., 2001; Eudy et al., 1998; Verpy et al., 2000; Weston et al., 2004; Riazuddin et al., 2012). These genes encode proteins with a wide range of functions including an actin-binding molecular motor (myosin VIIA, USH1B), complex transmembrane cell adhesion molecules (cadherin 23, USH1D), protocadherin 15, USH1F), (Usherin long and short isoforms, USH2A), scaffold proteins (Harmonin, USH1C), (SANS, USH1G), (whirlin, USH2D), a G-protein coupled receptor (VLGR1, USH2C), a calcium binding protein (CIB2, USH1J) and a tetraspanin transmembrane protein (clarin-1, USH3A).

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All of these proteins are expressed in both hair cells and photoreceptors. In mouse models harboring mutations in the various genes associated with Usher syndrome (Table 1), there is a common developmental defect in stereocilia organization. In some cases the three rows are properly oriented within the cuticular plate, but the lengths are variable and the stereocilia have a splayed appearance. In others, the number, length, and orientation within the cuticular plate are affected. The universal observation of stereocilia abnormalities led earlier work to conclude that Usher proteins function in the development of the hair cell stereocilia bundles. This hypothesis was reinforced by the presence of the long isoform of Usherin and VLGR1 (very large G-protein coupled receptor type 1) at the ankle links during stereocilia development in the mouse. These structures are known to be transiently present during maturation of the bundles, and thought to be essential to proper bundle development. Further reinforcement was provided by definitive demonstration that protocadherin 15 and cadherin 23 form the tip links in stereocilia, and that harmonin links cadherin 23 to stereociliary F-actin at the upper tip link density (UTLD) (Grillet et al., 2009). While disruption of harmonin in the deaf circler mouse (*dfcr*) abolishes the UTLD, the tip links remain intact. Mechanotransduction is lost in this mouse, demonstrating an essential role for the harmonin/F-actin linkage in this process. More recently myosin VIIA (USH1B) and SANS (USH1G) were shown to localize to the UTLD in vestibular hair cells of the guinea pig, implicating these Usher proteins as structural components of the mechanotransduction system (Grati and Kachar, 2011). In addition to the protocadherin 15/cadherin 23/harmonin protein interactions, a number of other Usher protein interactions have been inferred through pull-down assays using heterologous cell lines and co-immunolocalization studies in both cochlear hair cells and photoreceptors, inferring the existence of one or more Usher protein interactomes, with the scaffold proteins, harmonin, whirlin and SANS playing key roles as protein linkers (reviewed in: El-Amraoui and Petit, 2005; Reiners et al., 2006; Kremer et al., 2006). In this regard, while harmonin and whirlin interact with Usher and non-Usher proteins mostly through their PDZ domains, SANS interactions take place through the ankyrin repeats present at its N-terminus. Mutations associated to these domains results in loss of specific interactions between the different Usher proteins (Siemens et al., 2002; El-Amraoui and Petit, 2005; Adato et al., 2005; Reiners et al., 2005a,b; Pan et al., 2009; van Wijk et al., 2009; Grillet et al., 2009; Yan et al., 2010; Kersten et al., 2010, 2012; Bahloul et al., 2010; Caberlotto et al., 2011; Wright et al., 2012). Combined, these reports have led to an emergent model for Usher protein function *in vivo* involving a role in the development of stereocilia hair bundles and as proteins comprising key structures of the mechanosentitive apparatus of stereociliary tip links. While this may be true, it is notable that photoreceptors do not have stereocilia or mechanosensitive apparatuses, yet photoreceptors express all of the Usher proteins (Reiners et al., 2006; Maerker et al., 2008) and progressively degenerate in Usher patients, establishing a critical function for these proteins in photoreceptor cell health. Furthermore, all of the Usher proteins (but not all variants) have been localized to the synapses of photoreceptors (Reiners et al., 2006) and most have also been demonstrated to be present at the synapses of cochlear hair cells (Kremer et al., 2006). In photoreceptors, besides at the synapse, Usher proteins localize to the region of the connecting cilia at the juncture of the inner and outer segments. Recently it was shown that harmonin associates with and inhibits Cav1.3 calcium channels at the presynaptic region of inner hair cell ribbon synapses (Gregory et al., 2011). It was recently demonstrated that both clarin1 and protocadherin 15 mutant mice show a delay in synaptic maturation (Zallocchi et al., 2012a). This same paper and several other reports (Reiners et al., 2003, 2005b; Lillo et al., 2006; Lagziel et al., 2009; Zallocchi et al., 2012b) demonstrated the existence of synaptic Usher protein complexes comprised of distinct Usher protein variants as well as the existence of specific synaptic vesicle trafficking machinery that directs the movement of complexes to either apically targeted or basally targeted regions of cochlear hair cells.

Collectively, these studies suggest that Usher protein function in neurosensory cells may be more complex than previously thought. Indeed, numerous studies have demonstrated multiple protein variants for harmonin, cadherin 23, protocadherin 15, Usherin, whirlin, and VLGR1 (Reiners et al., 2003; Lagziel et al., 2005, 2009; Ahmed et al., 2006, 2008; Zallocchi et al., 2012a,b; van Wijk et al., 2006; Adato et al., 2005; Yagi et al., 2005; Wright et al., 2012). It is likely there are many more than currently identified; since they have been explored using a small number of available well qualified specific antibodies. Based on recent work, there is an emergent functional role for unique complexes of Usher proteins at the ribbon synapses as well. In this review, we summarize what is currently known about Usher protein function in stereocilia development, the hair cell mechanotransduction apparatus, in ribbon synaptogenesis and function, and at the periciliary region of the photoreceptor inner segments. The goal is to explain what is established and what is still speculative, with a special emphasis on addressing the consistencies and inconsistencies in our understanding of Usher protein functions in the two neurosensory cells affected by Usher syndrome, the hair cell and the photoreceptor.

2. Usher protein interactions and putative function in stereocilia development

A common feature of all Usher animal models studied to date is some degree of stereociliary dysmorphology, presumably due to defective stereocilia development. A summary of the transient and permanent stereocilia links, and developmental patterns for localization of Usher proteins in the stereocilia is summarized in Fig. 1. In the mouse, stereocilia first emerge along with the kinocilium from developing hair cells at embryonic day 15 (E15) (Nishida et al., 1998; Holme et al., 2002). Between E15 and E18.5, the kinocilium migrates along with the stereocilia establishing the polarity of the stereocilia bundles. This process in perturbed in many of the Usher type I mouse models, where the kinocilium is mis-positioned, and the stereocilia bundles become fragmented, with abnormal numbers, lengths, and orientation (Lefèvre et al., 2008). As early as E16.5 many of the proteins encoded by genes associated with Usher type I are observed in the stereocilia tips with more uniform distribution along the lengths of the stereocilia by E17.5 (Lefèvre et al., 2008). This is the same timepoint when a dense network of transient lateral links are observed by scanning electron microscopy (Goodyear et al., 2005), and immunolocalization studies demonstrate that cadherin 23 and protocadherin 15 contribute to this dense cohesive network (Michel et al., 2005; Lefèvre et al., 2008; Goodyear et al., 2010), although which isoforms of these molecules are present in these electron-dense structures is not known. Between E16.5 and E18.5 stereocilia elongate, first nearest the kinocilium, and sequentially to the stereocilia most distal to the kinocilium, resulting in the “staircase” arrangement of stereocilia in the hair cell bundle (Nayak et al., 2007).

By post-natal day 0 (P0) the ankle links are formed (Goodyear et al., 2005). These are transient structures located near the base of the stereocilia that disappear by P10. VLGR1 and the long isoforms of Usherin (isoforms B) have been shown to be structural components of these links (McGee et al., 2006; Michalski et al., 2007), and their expression patterns are temporally and spatially consistent with this notion. Recently, it has been shown that the Usher type 2 genetic modifier, PDZD7, co-localizes with Usherin and VLGR1 at the ankle link region, suggesting its possible involvement in stereocilia development (Ebermann et al., 2010; Grati et al., 2012). Mutations in the Usher type 2 genes result in a much milder stereocilia dysmorphic phenotype, and consistent with this, USH2A patients are not completely deaf, and can often function well with hearing aids. The long isoform of the protein encoded by the USH2D gene, whirlin, has been shown to co-localize with Usherin and VLGR1 at the ankle links and interact with them (van Wijk et al., 2006). A knockout mouse for whirlin shows whirlin dependency for localization of Usherin and VLGR1 at the

ankle links, which could be interpreted as either interdependency for stable integration in the stereocilia or for directed transport to the apical domains of the hair cells (Yang et al., 2010). Earlier studies of *whirler* mice showed that the stereocilia were shorter and thicker, consistent with the knockout mouse, but curiously not consistent with the phenotype of VLGR1 knockout mice (Holme et al., 2002; Yagi et al., 2007). This discrepancy in phenotypes might be due to the recent finding that whirlin interacts with the actin bundling protein espin (Wang et al., 2012), and the functional role for this interaction might explain the blunted elongation and thickening of stereocilia in *whirler* and whirlin knockout mice. In adult mice, Usher proteins play a role in the molecular architecture of the tip links and the mechanotransduction apparatus which are discussed in detail below.

A large number of specific interactions between Usher type 1 and type 2 proteins have been inferred, primarily through a combination of co-localization by immunohistochemistry combined with GST pull down assays. In the latter, domains of Usher proteins are expressed as GST fusion peptides in a heterologous (non-neurosensory) cell culture system. This combination of approaches established a number of potential specific interactions that have been summarized in detail in earlier reviews (Kremer et al., 2006; Reiners et al., 2006). The interaction of harmonin with the actin cytoskeleton, where it functions in actin bundling, and the cytoplasmic tail of cadherin 23 has been confirmed *in vivo*, where harmonin is a critical component of the UTLD. Tip links form in the absence of harmonin interaction with cadherin 23, as demonstrated in the *dfcr* mouse model, but the animals are deaf, demonstrating that this complex is essential to mechanotransduction (Grillet et al., 2009). Likewise, the specific interaction between recombinant homodimers of cadherin 23 and protocadherin 15 as the structural components of the tip links has been firmly established (Kazmierczak et al., 2007; Lelli et al., 2010). More recently the functional role of the cytoplasmic domain 2 (CD2) of protocadherin 15 in cell polarity and stereocilia development was explored using a transgenic approach. Among the many variants of protocadherin 15 are those produced through alternative splicing of three distinct cytoplasmic domains, CD1, CD2, and CD3. Using a gene targeting approach, Webb et al. (2011) produced mutants that lacked either CD1, CD2, or CD3 domains. The CD1 and CD3 deletion mutants showed no developmental or functional defects in hair cell stereocilia. The CD2 deletion mutant showed defects in kinociliary links and hair bundle orientation, however all three mutants formed functionally normal tip links. In contrast, the *av3J* protocadherin 15 mouse mutant (a null allele) shows severe defects in stereocilia development. This study underscores the limitations of our current understanding of Usher protein function in stereocilia development. There are clearly additional protocadherin 15 isoforms that contribute to earlier mechanisms of stereocilia development. Recent biochemical analysis documents a number of protein variants for VLGR1, cadherin 23, and protocadherin 15 in the cochlea and the retina (Lagziel et al., 2009; Zallocchi et al., 2012a). Importantly, most of the variants in the two organs are the same molecular weights, which portends to similar functional roles for these proteins in the cochlea and the retina. A related study showed that specific protein variants of VLGR1 and protocadherin 15 are selectively and differentially trafficked to either the apical or basal aspect of cochlear hair cells (Zallocchi et al., 2012b). In this study, antibody preparations against two different regions of each protein were used for immunoblotting analysis using isolated P1 organs of Corti from the mouse, demonstrating variants recognized by both antibodies as well as variants recognized specifically by one or the other. While this work provides good information as to which protein variants are likely to be important to stereocilia development and/or maintenance, it underscores another significant limitation of our understanding of Usher protein function; the limited number of antibody preparations against these proteins are not likely to be comprehensive enough to identify all protein variants present in hair cells and photoreceptors. Thus we may miss functionally important Usher protein variants due to the limited number of antibodies available. An example of this is the presence of a VLGR1

variant recognized by an antibody against the EAR domain in the middle of the VLGR1 protein, in the stereocilia tips of mature (P30) hair cells (Zallocchi et al., 2012a, supplemental Figure 2). As mentioned, prior reports documented the presence of VLGR1 only in the ankle links of hair cell stereocilia and only from P2 to P10 (McGee et al., 2006; Michalski et al., 2007). The function of the variant found in the mature stereocilia remains unknown.

The differential Usher protein trafficking previously described (Lagziel et al., 2009; Zallocchi et al., 2012b) raises another important issue. Several examples, based on immunofluorescence analysis, have documented where the absence of one Usher protein from the stereocilia is associated with the absence of other Usher proteins (Lefèvre et al., 2008; Caberlotto et al., 2011; Yang et al., 2010). While this data is most often interpreted to mean that the protein interaction is required for stable integration of both proteins in stereocilia (or the periciliary region of the connecting cilia of photoreceptors), it might also indicate that the interaction is essential for proper directional transport of the complex to the stereocilia. Along these lines, recent data showed that the tetraspanin TMHS, which is an integral component of the mechanotransduction apparatus, must associate with protocadherin 15 to be properly targeted to the plasma membrane in cultured cells (Xiong et al., 2012). The data linking Usher protein function to the mechanotransduction apparatus is remarkably detailed and solid (described below). In contrast, data supporting the role of Usher proteins in cohesion of the stereocilia as the mechanism underlying the severe stereocilia phenotypes observed in most Usher type I mouse models is less rigorous. It is interesting that two different missense mutations in protocadherin 15 that affect the stability of tip link formation resulted in two very different phenotypes. The *noddy* mutation in the PCDH15 gene is in the region of interaction between the amino terminal domains of protocadherin 15 and cadherin 23 and disrupts the *formation* of tip links. This mouse has significant stereocilia dysmorphology (Geng et al., 2013). Another missense mutation in a calcium binding motif of the CDH23 gene of the *salsa* mouse model is the same mutation associated with DFNB12 (non syndromic hearing loss). Here the tip links form, but are gradually lost (Schwander et al., 2009). Both models show significant staining for their respective cadherins at a time point when interciliary links should be present, which have been shown to be comprised of cadherin 23 and protocadherin 15 in the chick (Goodyear et al., 2010). It would be of interest to know whether interciliary links are intact in either model (which was not reported) since it has been proposed that such links might regulate appropriate maturation of the hair bundle. Alternatively, hair bundle dysmorphology in the *noddy* mouse might be due to other protein interactions essential for proper bundle maturation. In either respect, the *noddy* mouse could provide a fruitful medium for exploring mechanisms underlying abnormal bundle development.

The example of the CD2 deletion mouse model for protocadherin 15, while supporting this cytoplasmic domain module in an important adhesive interaction interface between the kinocilium and the tallest stereocilia (Webb et al., 2011) demonstrates that the many isoforms of this protein, as well as the other Usher proteins, may have distinct functions, most of which are not yet known. Ultrastructural studies of hair cell development in the *Ames Waltzer* mouse model noted considerable disruption of the apical cytoskeleton as early as E16, a stage where the stereocilia are just beginning to emerge from the cuticular plate (Kikkawa et al., 2008). Thus it is possible that the severe stereocilia phenotypes reflect functional defects upstream of the cohesive interactions. There are a large number of endogenously expressed Usher protein variants that are selectively transported to the apical domain of cochlear hair cells that remain functionally uncharacterized (Zallocchi et al., 2012b).

3. Usher proteins, mechanotransduction, and the upper and lower tip link densities

Cochlear hair cells are unique in their ability to translate noise-initiated mechanical vibrations propagated across the basilar membrane into neurosensory input that is decoded in the cochlear nucleus into what we perceive as sound. This process is mediated through an apparatus comprised of stereociliary tip links that are anchored to the actin cytoskeleton of the taller stereocilia at one end, and to the mechanotransduction channel in the shorter adjacent stereocilia at the other. In the last seven years it has become clear that many of the proteins that comprise the mechanotransduction apparatus are associated with Usher syndrome (Sakaguchi et al., 2009). Biochemical analysis showed that the actual tip link itself is comprised of homodimers of cadherin 23, bound at its carboxy-terminal ends to the upper tip link density (UTLD) and protocadherin 15, bound at its carboxy-terminal ends to the lower tip link density (LTLD). The two homodimers interact at their amino-terminal ends forming the structural link between adjacent stereocilia and between the tallest stereocilia and the kinocilium (Kazmierczak et al., 2007; Indzhykulian et al., 2013). Myosin VIIA, SANS, and harmonin localize to the UTLD of mature cochlear hair cells, and mutations in the USH1C gene encoding harmonin abolish the UTLD and reduce the sensitivity of hair bundles to mechanical stimulation (Grillet et al., 2009; Grati and Kachar, 2011). *In vitro* studies show an interaction between myosin VIIA and myosin IC cytoplasmic tails and PHR1, suggesting a role for myosin VIIa in mechanotransduction slow adaptation (Etournay et al., 2010). The cytoplasmic domains of the cadherin 23 homodimer interact with harmonin-b and harmonin-b interacts with SANS which interacts with the MyTH4 domain and FERM domain of myosin VIIA. These specific interactions have been resolved by X-ray crystallography (Pan et al., 2009; Yan et al., 2010; Wu et al., 2011), and the ultra-high affinity of the harmonin-b multidomain interaction with cadherin 23 has been determined by surface plasmon resonance assay (Bahloul et al., 2010). Harmonin anchors the complex to the actin cytoskeleton (for a review, see Schwander et al., 2010). Collectively, these data provide a compelling model for the role of this Usher protein complex UTLD that anchors the cadherin 23 homodimer to the actin cytoskeleton.

The molecular composition of the lower tip link density is much less well characterized. There is an electron dense LTLD region that is thought to contain as of yet unidentified elastic filaments that allow fast adaptation to occur following the mechanical deflection of stereocilia in the direction of the tallest stereocilia (Eatock, 2000). The tip link itself is a rigid structure that does not have the elastic properties to confer the fast adaptation (Sotomayor et al., 2010). The cytoplasmic domain of protocadherin 15 is anchored to the LTLD, where it interacts with the tetraspanin TMHS. TMHS mutant mice are deaf, and fast adaptation is abolished (Longo-Guess et al., 2005; Xiong et al., 2012). It has been proposed that TMHS may facilitate both the transport and assembly of the, as of yet unidentified mechanotransduction channel which would be functionally coupled to the tip link apparatus *via* protocadherin 15 interaction (Xiong et al., 2012).

The well characterized molecular assemblage of the tip link, its connection to the actin cytoskeleton, and its clear role in mechanotransduction (summarized in Fig. 2) begs an important question: since mechanotransduction does not occur in photoreceptors, what is the cause of deafness for syndromic Usher missense mutations that specifically disrupt the mechanotransduction apparatus? Do these same mutations disrupt one function in cochlear hair cells, and another unrelated function in photoreceptors?

4. Usher proteins in synaptogenesis/maintenance

Even though early immunolocalization studies identified expression of Usher proteins at the synapse of both cochlear hair cells and photoreceptors, the function of the Usher proteins at these synapses has remained relatively unexplored, perhaps because of the aberrant morphology of the hair cell bundle, that is easily observed in the different Usher models by common microscopy techniques (Lefèvre et al., 2008; Geng et al., 2012; Riazuddin et al., 2012; Webb et al., 2011; Mogensen et al., 2007; Michalski et al., 2007; Ernest et al., 2000; Phillips et al., 2011; Seiler et al., 2004; Söllner et al., 2004). These early observations created a paradigm in which only the full length Usher variants were the ones associated with this syndrome and by transitive properties the focus of study at the hair cell stereocilia and at the connecting cilium of photoreceptor cells (Michalski et al., 2007; Maerker et al., 2008). However the emergence of a more complex picture with a diversity of Usher variants playing roles at both the apical and basal aspects of hair cells and photoreceptors is challenging this paradigm.

Hair cells and photoreceptors are sensory neurons that can transmit a broad range of information for long periods of time due to a specialized organelle termed the synaptic ribbon (reviewed in Zanazzi and Matthews, 2009). The function of the synaptic ribbon is to tether a large number of synaptic vesicles to the active zone where the neurotransmitter, mainly glutamate, can be released in response to calcium influx. Except perhaps for CIB2 (USH1J) distinct variants for all the known Usher associated proteins are present at the hair cell and photoreceptor synapses and at the neuronal terminals that innervate the base of cochlear and vestibular hair cells. This has been demonstrated by different groups (Lagziel et al., 2009; Kersten et al., 2010; Reiners et al., 2003, 2005a,b; Gregory et al., 2011; Zallocchi et al., 2009, 2012a,b; Williams et al., 2009; Phillips et al., 2011; Adato et al., 2002; Overlack et al., 2008; van Wijk et al., 2006; Yagi et al., 2007). The full length and small variants of cadherin 23, protocadherin 15 and VLGR1 can be detected in synaptosome preparations from mouse organ of Corti as well as neuroretina and by the use of antibodies recognizing specific domains within these molecules (Lagziel et al., 2009; Zallocchi et al., 2012a,b). The variant V3 of cadherin 23 (~30 kDa) is present at the synapses and neuronal terminals, where it co-localizes with SNAP25, a component of the SNARE complex (Lagziel et al., 2009; Zallocchi et al., 2012b). The small variants of protocadherin 15-CD1 and VLGR1 (~30 kDa) along with isoform 2 of clarin-1 are present at the hair cell and photoreceptor synapses and afferent neuronal fibers where they interact with each other and with cadherin 23-V3 forming a small synaptic complex. This complex associates with SNAP25 through VLGR1 suggesting their involvement in vesicle docking and fusion (Lagziel et al., 2009; Zallocchi et al., 2012a,b). The absence or loss of function of one of the components of the complex, *i.e*. clarin-1 or protocadherin 15 respectively, produces a delay in synaptic maturation and in type I afferent remodeling at the base of the outer hair cells (Zallocchi et al., 2012a), reinforcing the notion that some of these variants play a role in synaptogenesis. Because clarin-1 is a member of the hyperfamily of small tetraspanin proteins with a significantly degree of homology to stargazin, a protein that controls the expression and mobilization of the AMPA glutamate receptors to the synaptic cleft, a similar function for clarin-1 has been suggested at the hair cell and photoreceptor synapses (Adato et al., 2002).

In the case of harmonin (USH1C), isoform “a” is expressed at the base of hair cells and photoreceptors (Reiners et al., 2003; Gregory et al., 2011). Recent work by Gregory et al. (2011) demonstrates a presynaptic association between harmonin-a and Cav1.3 Ca^{2+} channels in mouse inner ear. This association (which increases with the maturation state of the hair cells) reduces the availability of the channels and therefore their functional levels at the cell surface, revealing a novel function for harmonin that goes beyond the scaffolding

properties to a regulator of the electrical and calcium signals in hair cells. In zebrafish *ush1c* morphants (harmonin knockdowns) floating ribbon synapses are observed, which suggests a key role in retinal synaptogenesis and maintenance (Phillips et al., 2011). In mouse retina, whirlin (USH2D) shows an association with the Cav1.3 Ca^{2+} channels which suggests a potential role in calcium channel organization and membrane fusion (Kersten et al., 2010). Collectively, these studies not only demonstrate the importance of Usher protein synaptic function *per se* but also underscore the complexity of the syndrome where different variants are differentially trafficked to the apical or basal aspect of the cell to exert their specific functions through Usher and non-Usher protein interactions.

5. Usher protein function in photoreceptors

Usher protein function in retinal photoreceptors has been more difficult to explore, largely due to the mild or absent photoreceptor degeneration phenotype in the Usher mouse models (see Williams, 2008 review). Usher proteins localize to the periciliary region and basal bodies near the connecting cilia and to the synaptic region of the photoreceptors (Williams et al., 2009; Reiners et al., 2006; Maerker et al., 2008). Many of the same Usher protein variants for protocadherin 15, cadherin 23 and VLGR1 found in the cochlea are also found in the retina, providing evidence for the potential functional significance of these variants in both neurosensory cell types (Lagziel et al., 2009; Zallocchi et al., 2012a,b). The trafficking of these variants has not been explored in the photoreceptor. A summary of Usher protein localization in the photoreceptor is shown in Fig. 3.

Based on the protein interactions and localization at the periciliary membrane complex, an area near the connecting cilium implicated in membrane trafficking to the outer segments, it has been proposed that the Usher protein complexes might play a role in the trafficking of vesicular cargo between the inner and outer segments of photoreceptors (Reiners et al., 2006; Maerker et al., 2008). Consistent with this notion, it has been reported that opsin accumulates in the connecting cilium of shaker 1 mice (Liu et al., 1999). While this observation was interpreted as suggesting a direct role for the actin binding molecular motor, myosin VIIA, in opsin transport through the cilium, it may reflect a general role for Usher proteins in the transport of cargo between the inner and outer segments of photoreceptors. A recent study demonstrated that shaker 1 mice have an elevated light threshold, following dark adaptation, for activating the transport of α-transducin from the outer segments to the inner segments of rod photoreceptors (Peng et al., 2011). The light dependent translocation of α-transducin is thought to play an important role in buffering the photoresponse from high light sensitivity to low light sensitivity (Sokolov et al., 2002). Shaker-1 mice are susceptible to light induced rod photoreceptor degeneration under conditions that do not affect strain matched wild type mice (Peng et al., 2011), suggesting that the defective transport and photoreceptor degeneration might be functionally linked in the shaker-1 mice. Myosin VIIA functions in the retinal pigment epithelium in the localization and motility of melanosomes (Gibbs et al., 2004). More recently it was shown that myosin VIIA functions light-dependent localization of the isomerase RPE65 to the central region of the retinal pigment epithelium. Mice lacking functional myosin VIIA were shown to have reduced RPE65 activity (Lopes et al., 2011). Thus, it is clear that myosin VIIA is likely functionally important for both the RPE cells and the photoreceptor cells. Additional work will be required to determine whether photoreceptor degeneration in USH1B is caused by the RPE defects, the photoreceptor cell defects, or both.

It has been suggested that the mouse is not a good model for retinal degeneration associated with USH1D because the longest isoform of cadherin 23, which is an essential component of the tip links in cochlear hair cells, is not expressed in the mouse photoreceptors, while it is expressed in primate photoreceptors (Lagziel et al., 2009). The functional importance of this

longest isoform of cadherin 23 in retinal photoreceptors is unknown, so this conclusion is based on untested assumptions. Among the Usher mouse models that do show a retinal degeneration phenotype, the knock-in mutation for the c.216G > A cryptic splice site mutation in exon 3 of the USH1C gene is an exception. This mouse shows abnormal electroretinograms by 1 month of age, and significant loss of photoreceptors between 6 and 12 months of age (Lentz et al., 2010). The *dfcr and ush1c*−/− mice, also harboring mutations in the USH1C gene do not have a strong retinal phenotype (Williams, 2008; Williams et al., 2009; Tian et al., 2010), but are deaf. These disparate observations suggest that the functionally important isoform(s) for harmonin in the retina and in the cochlea may be distinct.

A recent report suggests that calycal processes in photoreceptor cone cells are synonymous structures to hair cell stereocilia (Sahly et al., 2012). This same paper demonstrates, using immunofluorescence and scanning electron microscopy imaging, that the calycal processes present in frog and monkey cone photoreceptors are enriched in Usher type 1 proteins, while mouse cone photoreceptors, which do not have calycal processes, show a different distribution pattern of the Usher type I proteins, and complete absence of harmonin and cadherin 23. Based on these findings, the authors suggest that this is why Usher mouse models do not have a retinal degeneration phenotype. The published literature suggests problems with this assumption. First, several studies have demonstrated that all Usher proteins, and in most cases several protein variants, are indeed expressed in mouse photoreceptors. Most of these variants are present at the connecting cilia, periciliary region and photoreceptor synapses and in contrast to what was suggested by Sahly et al., 2012, Usher protein presence at the calycal processes is not restricted to type I only (Goodyear and Richardson, 1999; Reiners et al., 2003, 2005a,b, 2006; Lillo et al., 2006; Maerker et al., 2008; Lagziel et al., 2009; Williams et al., 2009; Zallocchi et al., 2010, 2012a,b). Importantly, recent studies demonstrate a robust light-dependent retinal phenotype for the shaker 1 mice (Peng et al., 2011). Finally, a harmonin knock-in mouse mutant showed a robust retinal degeneration phenotype (Lentz et al., 2010). Collectively, these studies clearly show that Usher proteins are expressed and functionally important in mouse photoreceptors.

6. Therapeutic strategies in Usher syndrome

The retina is an attractive target for therapeutic intervention due to its immune-privileged nature and its accessibility for mildly invasive sub-retinal delivery of therapeutic agents. There have been several attempts to validate this approach for the treatment of retinal disease associated with Usher syndrome. Lentiviral-based gene delivery of human myosin VIIA has been shown to rescue the melanosome migration and opsin-mislocalization phenotypes in a knockout mouse model for USH1B (Hashimoto et al., 2007). Sub-retinal delivery of a functional whirlin construct transcribed from a photoreceptor cell-specific rhodopsin kinase promoter demonstrated the restoration of a VLGR1/whirlin/UsherinisoformB complex at the connecting cilium of the mouse (Zou et al., 2011), suggesting that this approach might rescue the retinal degeneration phenotype in humans. Although gene therapy is potentially suitable for some types of Usher syndrome, it is not suitable for the very large Usher genes, Usherin and VLGR1 (Liu et al., 2007; McGee et al., 2006). The use of aminoglycosides (specifically NB30 and NB54) and a chemically unrelated molecule, PTC124, has been shown *in vitro* to induce translational read through of stop codons caused by missense mutations in both the USH1C (Goldmann et al., 2010, 2012) and USH1D (Rebibo-Sabbah et al., 2007) genes. These translational read-through-inducing drugs have the advantages of not being gene-specific, allowing the treatment of a diverse range of genetic diseases; the size of the mutated gene is not important as well as the number and expression of the mutated isoforms and the expression of the gene is under endogenous control.

More recently, a targeted zinc finger nuclease was successfully used to actually correct a point mutation in the USH1C gene *in vitro*, providing proof of concept for the emergent technology of gene correction (Overlack et al., 2012). Just months ago, the first example of a therapy that successfully rescues both hearing and balance defects for an Usher gene was demonstrated. Here antisense oligonucleotide formulations directed at a splice mutation in the USH1C gene were injected intraperitoneally and shown to induce a sustained (several months) rescue of both phenotypes (Lentz et al., 2013). Collectively, these works predict that therapeutic intervention to slow or arrest the retinal (and possibly the inner ear) defects associated with Usher syndrome are forthcoming.

7. Concluding remarks

The major focus of Usher protein research on stereocilia development and mechanotransduction has yielded a wealth of evidence regarding the molecular origin of developmentally dynamic hair bundle links as well as an exquisite emergent molecular design for the hair cell mechanotransduction apparatus. It is clear from these works that Usher protein function is essential for both the development and function of the stereocilia. The role of these specific Usher protein variants in synaptic development and function is only beginning to be explored, although recent reports portend these distinct Usher protein complexes do play roles in the development and function of ribbon synapses. Although the delayed onset and progressive nature of the retinal phenotype in Usher patients make this organ a suitable target for therapeutic intervention, very little is known about Usher protein function in these cells beyond immunolocalization studies and hypothetical models based largely on *in vitro* protein interactions. Progress has been hampered by the weak to absent retinal phenotype in Usher mouse models, however recent data demonstrating protein transport defects and sensitivity to moderate light induced retinal degeneration in the shaker 1 mouse model could change this if indeed these phenotypes are generally applicable to other or all Usher mouse models. Finally, the highly dysmorphic stereocilia in Usher type 1 mice suggest that molecular functions of Usher proteins upstream of the initiation of stereocilia development (earlier than E16.5 in the mouse) might be at play, for example regulated trafficking of proteins to the apical microdomain of cochlear hair cells. Such a mechanism might apply to the protein trafficking defects that have been documented in photoreceptors of the shaker 1 mouse (and now *whirler*, manuscript under revision), which could explain the retinal degeneration phenotype, and thus provide a shared mechanistic paradigm for Usher protein function and pathology in both hair cells and photoreceptors.

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Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CD	cytoplasmic domain
CDH23	cadherin 23
CIB2	calcium integrin binging protein 2
dfcr	deaf circler
EAR	epilepsy associated repeat
FERM	4.1 protein, ezrin, radixin, moesin

GST	glutathione S-transferase
LTLD	lower tip link density
MyTH4	myosin tail homology 4
PCDH15	protocadherin 15
PDZ	post synaptic density protein (PSD95)
(Dlg1)	Drosophila disk large tumor suppressor
(zo-1)	Zonula occludens-1 protein
PDZD7	PDZ domain containing 7
RP	Retinitis pigmentosa
RPE	retinal pigment epithelium
RPE65	retinal pigment epithelium-specific 65 kDa protein
SNAP25	synaptosomal-associated protein 25
SNARE	soluble NSF attachment protein receptor
TMHS	tetraspan membrane protein of hair cell stereocilia
USH	Usher syndrome
UTLD	upper tip link density
VLGR1	very large G-coupled protein receptor type 1.

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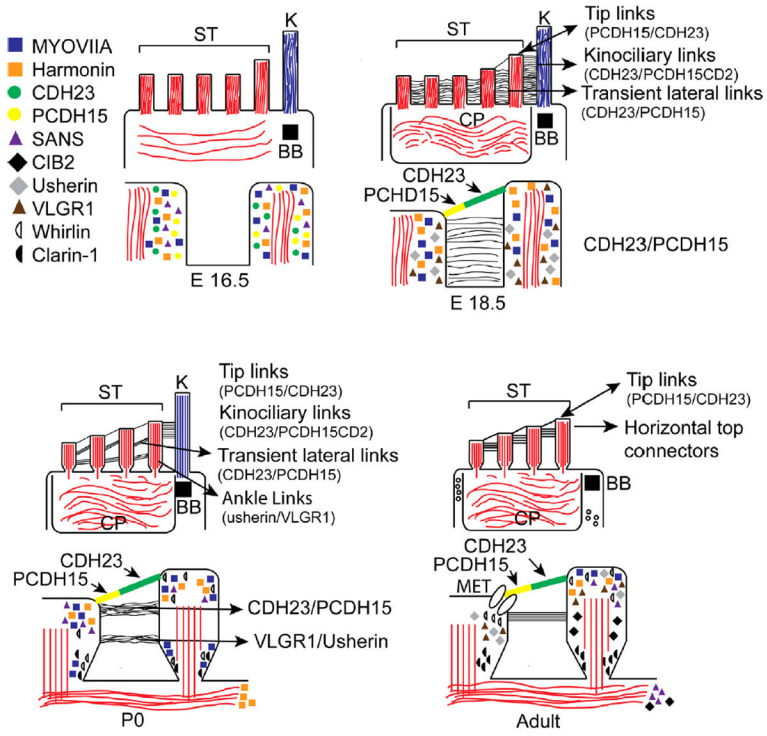

Fig. 1.

Early and late stages of cochlear hair bundle maturation showing expression and distribution of Usher proteins. The stereocilia (ST) that will form the mature hair bundle are held together by different links that vary during development. The first links can be detected at E18.5. Only the tip links and top connectors will persist in the adult hair cell bundle. By P0 the core of actin filaments insert rootlets into the cuticular plate (CP). In the adult cochlear hair cell bundle the kinocilia (K) regress and only its basal body (BB) remains. PCDH15, the lower component of the tip links, gates the mechanoelectrical transduction channels (MET). Usher protein localizations based on the following references: (Lagziel et al., 2005; Lefèvre et al., 2008; Boëda et al., 2002; Caberlotto et al., 2011; Grillet et al., 2009; Adato et al., 2005; Zallocchi et al., 2009, 2012a; McGee et al., 2006; Michalski et al., 2007; Reiners et al., 2005; van Wijk et al., 2006; Delprat et al., 2005; Kazmierczak et al., 2007).

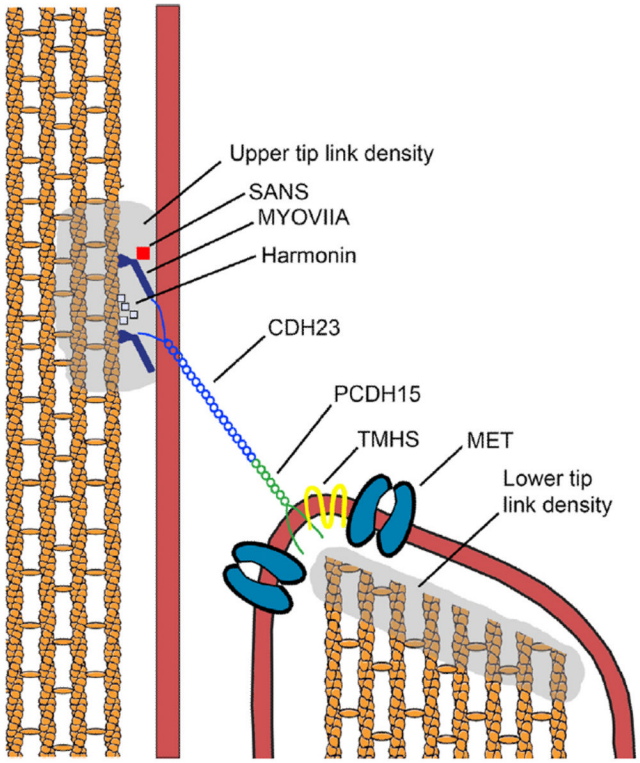

Fig. 2.

Usher proteins are building blocks of the hair cell mechanotransduction apparatus. Cadherin 23 homodimers and protocadherin 15 homodimers interact at their amino-terminal ends to form a rigid fiber that is anchored at the taller stereocilia via interaction of the carboxy-terminal ends of cadherin 23 with harmonin, which anchors the filament to the actin core. SANS and myosin VIIA are also implicated in the formation of this anchor at the upper tip link density. Protocadherin 15 carboxy termini are linked to the adjacent shorter stereocilia at the lower tip link density, where the as of yet unidentified mechanotransduction channels reside. Thus far, all that is known about this complex is that there is a functionally obligatory interaction with the tetraspanin TMHS (Xiong et al., 2012). The proteins that regulate the channel, as well as the channel itself, are an area of intense interest.

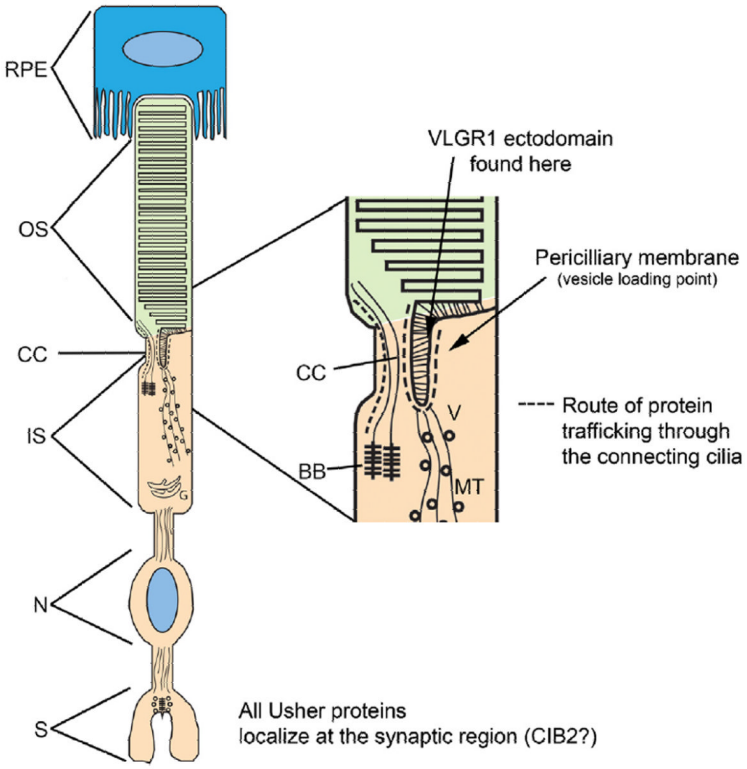

Fig. 3.

Usher protein localization in rod photoreceptors. All Usher proteins identified thus far have been shown to localize at two regions in the photoreceptors by immunofluorescence microscopy; the region near the connecting cilium and the synaptic region. Two Usher proteins, myosin VIIA and usherinisoform a localize to the retinal pigment epithelium as well (Bhattacharya et al., 2001; Gibbs et al., 2003). Usherinisoform b, VLGR1, whirlin, and SANS have been shown to localize to the periciliary membrane region by immunogold labeling studies, which is a docking area for vesicular trafficking (Maerker et al., 2008). The EAR domain of VLGR1, which is in the middle of the molecule, has been shown to localize to the space between the periciliary basement membrane and the ciliary plasma membrane (McGee et al., 2006). Based on the presence of Usher proteins near the vesicle loading point at the periciliary membrane it has been proposed that they may play a role in vesicle transport between the inner segments and the outer segments of photoreceptors. BB, basal bodies; CC, connecting cilium; G, Golgi; IS, inner segment; MT, microtubules; N, nucleus; OS, outer segment; RPE, retinal pigment epithelium; S, synaptic region; V, vesicles.

Table 1

Usher genes and their associated disease.

Usher gene	Protein	Associated diseases	Mouse models	Zebrafish models	References
MY07A	Myosin VIIA	USH1 BDFNA11 DFNB2, non-syndromic RP	*shaker1; Myo7A null*	*mariner*	Sang et al. (2013); Ammar-Khodja et al. (2009); Weil et al. (1995); Ernest et al. (2000); Ben Rebeh et al. (2010); Lopes et al. (2013)
USH1C	Harmonin	USH1C DFNB18	*dfcr; ush1c−/−; harmonin-PDZ2; Ush1c216AA*	*Ush1c^{fh293}*	Johnson et al. (2003); Verpy et al. (2000); Lefevre et al. (2008); Grillet et al. (2009); Lentz et al. (2010); Tian et al. (2001); Seiler et al. (2004);
CDH23	Cadherin 23	USH1D DFNB12	*Waltzer*	*sputnik*	Bolz et al. (2001); Schultz et al. (2011); Sollner et al. (2004)
PCDH15	Protocadherin15	USH1F DFNB23	*Ames Waltzer; noddy; pcdh15-CD1/CD2/CD3*	*arbiter*	Ahmed et al. (2003); Ahmed et al. (2001); Seiler et al. (2004); Webb et al. (2011); Geng et al. (2013)
USH1G	SANS	USH1G DFNA20/26	*Jackson shaker/Ush1g$^{js/js}$*		Weil et al. (2003); Mustapha et al. (2006); Liu et al. (2007);
CIB2	Calcium integrin binding protein 2	USH1F DFNB48	*Cib2tm1a(EUC0MM)^{Wtsi}*		Riazuddin et al., 2012; MGI
USH2A	Usherin	USH2A non-syndromic RP (RP15)	*Ush2a−/−*	*Ush2a^{sa1881}*	Ebermann et al. (2009); Kremer et al. (2006); Liu et al. (2007); Xu et al. (2011); ZIRC
GPR98	Vlgr1/Gpr98	USH2C	*VLGR1/del7TM; VLGR1 ko*		Weston et al. (2004); McMillan and White (2004); Yagi et al. (2005)
DFNB31	Whirlin	USH2D DFNB31	*Whirler; whirlinko*		van Wijk et al. (2006); Mogensen et al. (2007); Mburu et al. (2006); Zou et al. (2011)
CLRN1	Clarin-1	USH3A	*Clrn1(−/−)*		Adato et al. (2002); Zallocchi et al. (2009)