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Current understanding of usher syndrome type II

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1. ABSTRACT

Usher syndrome is the most common deafness-blindness caused by genetic mutations. To date, three genes have been identified underlying the most prevalent form of Usher syndrome, the type II form (USH2). The proteins encoded by these genes are demonstrated to form a complex *in vivo*. This complex is localized mainly at the periciliary membrane complex in photoreceptors and the ankle-link of the stereocilia in hair cells. Many proteins have been found to interact with USH2 proteins *in vitro*, suggesting that they are potential additional components of this USH2 complex and that the genes encoding these proteins may be the candidate USH2 genes. However, further investigations are critical to establish their existence in the USH2 complex *in vivo*. Based on the predicted functional domains in USH2 proteins, their cellular localizations in photoreceptors and hair cells, the observed phenotypes in USH2 mutant mice, and the known knowledge about diseases similar to USH2, putative biological functions of the USH2 complex have been proposed. Finally, therapeutic approaches for this group of diseases are now being actively explored.

2. INTRODUCTION

Usher syndrome is the most common condition of combined blindness and deafness, occurring in about 1 in 23,000 people worldwide (1-3). This disease was first discovered by Albrecht von Grafe, a German ophthalmologist, in 1858 (4) and is named after Charles Usher, a British ophthalmologist, who reported the inheritance of this disease on the basis of 69 cases in 1914 (5). According to the clinical presentation in hearing, Usher syndrome is categorized into three types (6, 7). Type I (USH1) is manifested as congenital profound deafness as well as vestibular dysfunction; USH2 exhibits congenital moderate hearing loss and normal vestibular function; and USH3 is characterized by progressive hearing impairment and occasional vestibular dysfunction. The vision problem of the all three types is manifested as retinitis pigmentosa (2, 8-11), showing early night and peripheral vision loss and late central vision loss. To date, there is no cure for this disease. Patients with this disease mainly rely on early diagnosis and early education to adapt themselves to their dual sensory loss. Usher syndrome is a heterogeneous autosomal recessive genetic disorder. Twelve causative

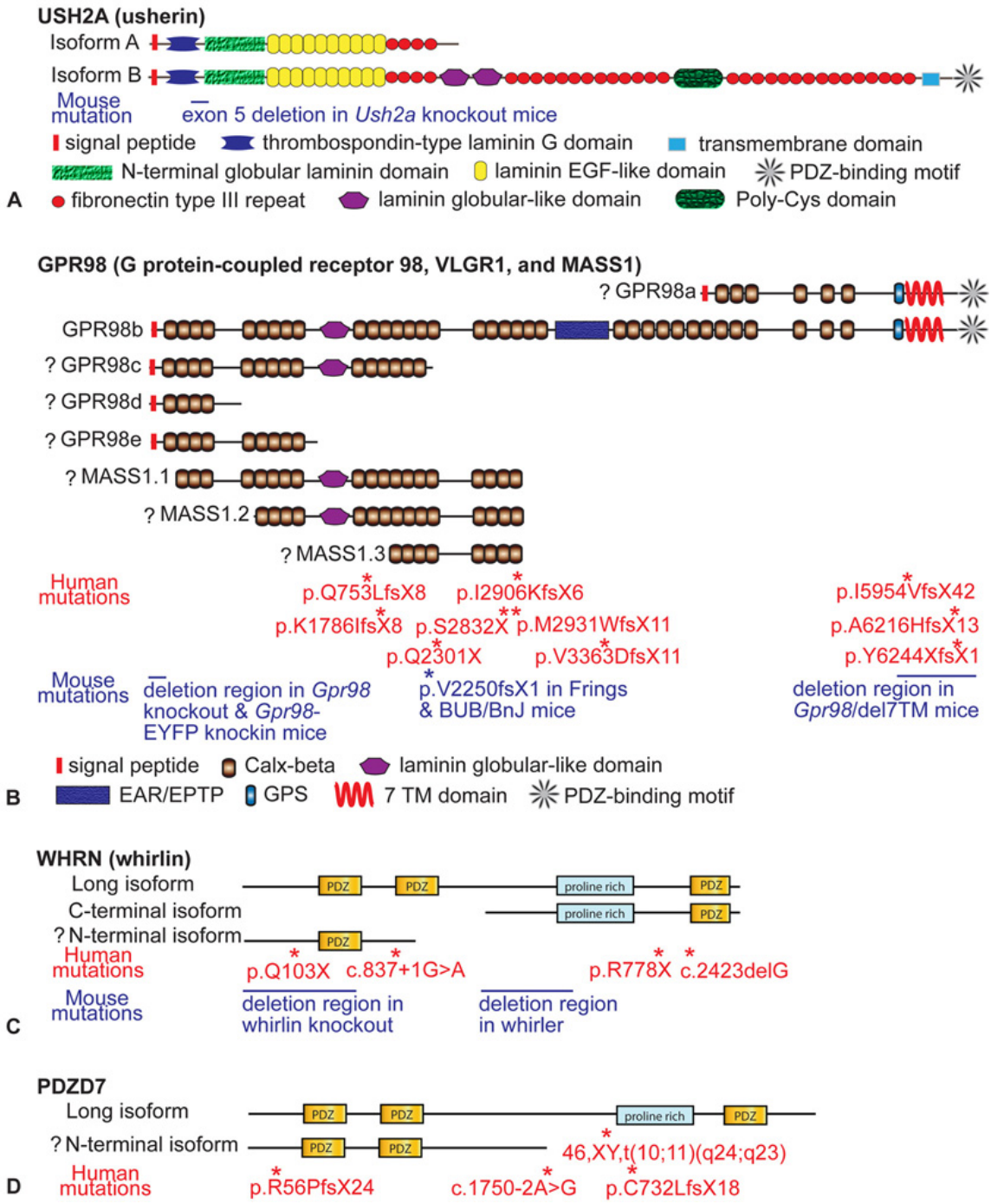

Figure 1. Schematic diagrams of USH2A, GPR98, WHRN and PDZD7. (A) USH2A has two protein isoforms. Isoform A is an N-terminal fragment of isoform B. Numerous *USH2A* mutations have been identified. They are evenly distributed along the entire gene in humans. Therefore, they are not marked here. Exon 5 replaced in *Ush2a* knockout mice is marked in blue. Domain names of various symbols are listed below. (B) GPR98 isoform b is a transmembrane protein with extremely long ectodomain. Although other GPR98 isoforms were found as RNA transcripts in humans and mice, their existence as functional proteins needs to be verified (question marks). *GPR98* mutations found in humans are marked in red, and its mutations in various *Gpr98* mutant mouse models are marked in blue. Domain names of each symbol are listed below. (C) Whirlin has two known protein isoforms, the long and C-terminal isoforms. The N-terminal isoform has not yet been found at the protein level in the retina or the inner ear (question mark). Whirlin mutations found in humans are marked in red, and its mutations in whirlin mutant mouse models are marked in blue. (D) PDZD7 has a long isoform. Its N-terminal short isoform has not been found at the protein level (question mark). PDZD7 mutations in humans are indicated in red.

loci, at least 9 genes, have been identified (12-14). However, the functions of these genes in either the inner ear or the retina are largely unknown. In this review, we focus on the current understanding of USH2, which accounts for about 70% of all Usher syndrome cases (2).

3. USH2 GENES

Four USH2 loci were originally defined, *USH2A-D*. The genes responsible for *USH2A*, *USH2C*, and *USH2D* are *USH2A* (usherin) (15), *GPR98* (G Protein-coupled Receptor 98) (16), and *WHRN* (whirlin) (17), respectively. The gene for *USH2B* was once considered to be *NBC3* (sodium bicarbonate cotransporter) (18). However, further study of the consanguineous Tunisian family carrying the *USH2B* locus demonstrates that mixed mutations in the *GPR98* and *PDE6B* genes are responsible for the disease manifestation and, thus, the *USH2B* locus was withdrawn (19). Recently, *PDZD7* was shown to be a modifier gene for the retinal symptom in USH2A patients and also, together with *USH2A* or *GPR98*, to contribute to a digenic form of Usher syndrome (20). In addition to these above genes, a novel USH2 locus has been localized on the chromosome 15q, though the underlying gene has not yet been identified (21).

USH2A is the most predominant causative gene for Usher syndrome among different human ethnic populations (2, 15, 22-32). Its mutations lead to a wide spectrum of vision and hearing defects in humans. Some *USH2A* mutations, such as p.C759F and p.G4674R, are known to cause only nonsyndromic retinitis pigmentosa (30, 33, 34). *USH2A* has 72 exons and is expressed as isoforms A and B (Figure 1A). Isoform B, the major isoform in the retina (35), is an extremely large transmembrane protein with 5202 amino acids (aa) in humans (31). Its long extracellular region has multiple repeated functional domains common in cell adhesion proteins and extracellular matrix proteins. At its cytoplasmic C-terminus, there is a PDZ (postsynaptic density 95; discs large; zonula occludens-1)-binding motif (PBM). Isoform A is an N-terminal 1546-aa fragment of isoform B. USH2A is proposed to be involved in cell adhesion.

The *GPR98* gene is also known as *VLGR1* (Very Large G protein-coupled Receptor 1) and *MASS1* (Monogenic Audiogenic Seizure Susceptibility 1). It exists only in the vertebrate (36) and is one of the largest genes, with 90 exons (37). Its mRNA is present mostly in the brain and spinal cord during development (16, 37) and can also be found in many other tissues (16, 37-39). *GPR98* expresses multiple transcripts, including isoforms a, b and c in humans and isoforms b, d, e and Mass1 in rodents (Figure 1B) (37-40). Mutations in the longest isoform, isoform b, have been identified in patients with USH2C (16, 41, 42). Additionally, different mutations along the murine *Gpr98* gene share common phenotypes in vision and hearing (39, 40, 43-47). These findings suggest that isoform b is the major isoform in both the retina and the inner ear and is essential for vision and hearing. We have observed protein expression of this isoform in the retina (48). This isoform is 6306 aa long in humans. It has signature domains of family B of G protein-coupled receptors (GPCRs), i.e., a GPCR proteolytic site (GPS) and a 7-transmembrane domain (7TM). Therefore, GPR98 may function in signal transduction. GPR98 also has a PBM at its C-terminus. Along its long extracellular region, it has a laminin globular-like domain and multiple tandem-arranged Calx-beta domains. The laminin globular-like domain is a cell adhesion domain, and the Calx-beta domain is able to bind to Ca^{2+} with low affinity *in vitro* (38).

Different mutations of whirlin cause different diseases. Compound heterozygosity of p.Q103X and c.837+1G>A was discovered in patients with USH2D (17), and homozygous mutations of p.R778X and c.2423delG were found in patients with nonsyndromic deafness, DFNB31 (Figure 1C) (49, 50). Whirlin has multiple mRNA transcripts in the inner ear and the retina (49, 51-53), which can be conceptually translated into three groups of proteins, the long, N-terminal, and C-terminal isoforms (Figure 1C). The long isoform contains three PDZ domains and a proline-rich region. The N-terminal isoform has the first PDZ domain of the long isoform, and the C-terminal isoform has the proline-rich region and the third PDZ domain. At the protein level, whirlin mainly expresses the long isoform in the retina and the long and C-terminal isoforms in the inner ear (53). Because both the PDZ domain and proline-rich region are protein interaction modules, whirlin is believed to be engaged in the assembly of multi-protein complexes at specific subcellular locations.

Interestingly, the newly identified USH2 modifier and contributor gene, *PDZD7*, is a homolog of whirlin. In humans, it has 16 exons and several isoforms (Figure 1D) (20, 54). The long isoform has three PDZ domains and one proline-rich region. The two short isoforms are the N-terminal fragments of the long isoform with the first two PDZ domains. However, the short isoforms have not been confirmed at the protein level. Similar to whirlin, mutations in *PDZD7* are involved in either Usher syndrome or nonsyndromic deafness. A homozygous reciprocal translocation, 46,XY,t(10;11)(q24;q23), was found to disrupt the *PDZD7* gene at intron 10, which causes nonsyndromic congenital hearing impairment in a consanguineous family (54). A heterozygous p.R56PfsX mutation of *PDZD7* was found to exacerbate retinal degeneration in an USH2A patient, compared to her sibling carrying the same *USH2A* mutation. Additionally, heterozygous mutations of *PDZD7*, c.1750-2A>G and p.C732LfsX, are present in Usher patients with a heterozygous *USH2A* mutation, p.R1505SfsX, and with a heterozygous *GPR98* mutation, p.C732LfsX, respectively (20).

4. ANIMAL MODELS

Little access to patient retinas necessitates the establishment of various animal models in order to understand the biological functions of USH2 genes and the

Table 1. USH2 mutant mouse models

Model name	Mutation	Phenotype	References
Ush2a			
Ush2a knockout	replacement of exon 5 with a neomycin^r cassette	hearing impairment tested at 4 months of age; no circling; retinal degeneration evident at 20 months of age	(35)
Gpr98			
Gpr98 knockout	replacement of exons 2-4 with a neomycin^r cassette	audiogenic seizure susceptibility at P21; hearing impairment at P7; no circling; no report on retinal function	(40, 46)
Gpr98-EYFP knockin	replacement of exons 2-4 with a EYFP-neomycin^r cassette	defects in hair cell stereocilia at P4; no circling; no report on audiogenic seizure susceptibility or retina function	(47)
Frings & BUB/BnJ	a G deletion at 6864 bp (NM_054053) causing a p.V2250X truncation	audiogenic seizure susceptibility at P21; hearing impairment at P21; no circling; no report on retinal function	(39, 43)
Gpr98/del7TM	replacement of exon 82 with a HA-neomycin^r cassette	audiogenic seizure susceptibility at P21; hearing impairment at P21; no circling; mildly abnormal retinal function at 15 months of age	(44, 45)
Whrn			
Whrn knockout	partial replacement of exon 1 with a neomycin^r cassette	hearing impairment tested at 2 months of age; no circling; retinal degeneration starting at 28 months of age	(53)
whirler	a 592-bp deletion causing a p.H433fsX58 truncation	hearing impairment at P20; circling; no retinal degeneration	(49, 53, 56, 156)

disease mechanisms underlying USH2. Due to the well development of the transgenic techniques, the availability of extensive public genome resources, the similarity of retinal anatomy to humans', and the relative low maintenance cost, mouse models have been widely adopted. Currently, mouse models with either naturally occurring or transgenic mutations in *USH2A*, *GPR98* and *WHRN* are available (Table 1). In addition, studies on these USH2 genes in zebrafish using the morpholino knockdown technique are being actively pursued (20). According to the highly conserved USH2 protein sequences between these organisms, the findings in these animal models are expected to be supportive and complementary to each other.

To date, an *Ush2a* knockout mouse model has been generated by replacing exon 5 of *Ush2a* with a neomycin resistant cassette (Figure 1A) (35). Elimination of the USH2A protein expression in this mouse has been demonstrated by both western analysis and immunostaining. This mouse shows nonprogressive moderate hearing impairment at 4 month of age, the earliest time point tested, progressive late onset vision loss, which is evident at 20 months of age, and no circling behavior, indicative of no balance problem. These hearing, vestibular, and vision phenotypes are similar to the clinical symptoms in USH2A patients.

Four *Gpr98* mutant mouse lines have been reported (Figure 1B). The BUB/BnJ and Frings mice carry a naturally-occurring *Gpr98* mutation, c.6864delG (NM_054053), leading to a premature truncation of the protein, p.V2250X (39, 43). The second and third *Gpr98* mutant mouse lines are *Gpr98* knockout and *Gpr98*-EYFP knockin mice, generated by the same research group (40, 47). *Gpr98* exons 2-4 are replaced by a neomycin^r cassette in the knockout line and by an EYFP gene in a neomycin^r cassette in the EYFP knockin line. The fourth model was genetically manipulated to delete the transmembrane and cytoplasmic domains of GPR98 (del7TM) (45). Phenotypic analyses of these *Gpr98* mutant mice demonstrate that *Gpr98* mutations cause audiogenic seizure susceptibility at a very young age (around postnatal day (P) 21 to 27) (39, 40, 45). Although this phenotype in mice cannot be linked to the clinical symptoms in USH2C patients (16), it may be associated to patients with febrile and afebrile seizures (FFB4), which are caused by the *GPR98* nonsense mutation, p.S2832X (55). No circling behavior was observed in these *Gpr98* mutant mice. Further examinations show congenital hearing impairment in all four *Gpr98* mutant mice (43, 44, 46, 47). A mild vision defect, reduction in both a- and b-wave amplitudes of electroretinogram, was reported in the *Gpr98*/del7TM mouse at 15 months of age (44).

For whirlin, two mutant mouse lines are presently available, the whirler and whirlin knockout mice (Figure 1C). The whirler mouse occurred naturally with a large deletion between whirlin exons 6 and 9 (49). This mutant mouse has deafness and circling behavior (56) but no vision problem (53), resembling the nonsyndromic deafness DFNB31 patients. In the whirlin knockout mouse, a partial deletion of exon 1 was introduced. This knockout mouse has retinal degeneration starting from 28 months of age and early onset hearing loss at 2 months of age, the earliest time point tested. The mouse does not show vestibular defects (53). The phenotypes in the whirlin knockout mouse mimic the USH2 in humans. Interestingly, the mutation in the whirler mouse is located after the first two PDZ domains of whirlin, which is similar to the mutations causing DFNB31 in patients. Additionally, mutations in the whirlin knockout mouse and USH2D patients are both localized at the whirlin N-terminal region, which disrupt the first two PDZ domains. These findings

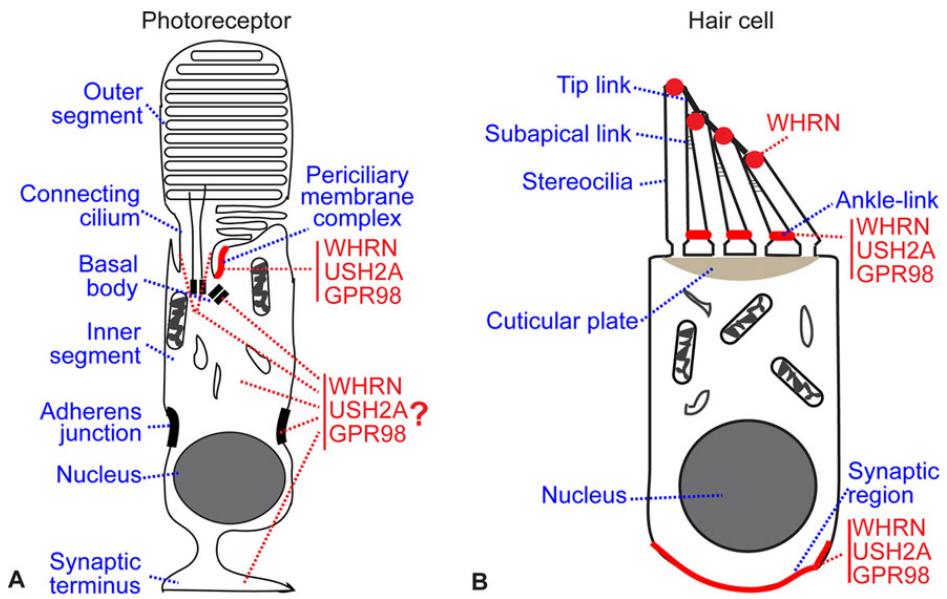

Figure 2. Cellular localization of USH2 proteins. (A) In photoreceptors, USH2 proteins were previously localized at the connecting cilium, basal bodies, inner segment, adherens junction, and synaptic terminus (question mark). They were recently demonstrated to be enriched at the periciliary membrane complex (red) between the outer and inner segment (Figure 3) (35, 53). A rod photoreceptor is presented here. The localization of USH2 proteins in cone photoreceptors is presumably similar to that in rod photoreceptors. (B) In hair cells, USH2A, GPR98, and whirlin (red) are localized at the ankle-link of the stereocilia during development. They are also present at the synaptic region of hair cells, except that whirlin is absent at the synaptic region in the inner hair cells. During development and in adulthood, whirlin is present at the tip of the stereocilia as well.

suggest that whirlin long isoform is important for vision, while both whirlin long isoform and C-terminal isoform are indispensable for hearing. Thus, the two whirlin mutant mouse lines are appropriate animal models for human diseases, DFNB31 and USH2D, respectively.

Unlike the USH1 mouse models that show roughly normal vision functions, mouse models with at least one mutation in each of the USH2 genes exhibit vision problems (12, 13, 57). However, these vision problems are generally very mild and late-onset, compared with those in USH2 patients. The reasons for this discrepancy are largely unclear. Many factors could be involved, such as differences between humans and mice in isoform combinations, mutation locations in USH2 genes, genetic backgrounds, redundant protein compensations, lifespans, photoreceptor structures and physiology, influence of non-genetic factors, sensitivities of diagnostic measures, etc. (58, 59). Additionally, although retinitis pigmentosa in USH2 is characterized to have an onset during puberty (6, 7), more and more atypical USH2 patients have been found with mutations in these three USH2 genes. It is common that these patients have severe vision loss much later than puberty (9-11, 60-62).

5. CELLULAR LOCALIZATION OF USH2 PROTEINS

USH2 proteins have been intensively investigated regarding their cellular location in the inner ear and the retina because of their involvement in Usher syndrome. Their cellular localization in other organs has not yet been systematically studied, except USH2A isoform A. This USH2A isoform was shown to be present in the basement membrane of a battery of tissues, including ovary, oviduct, testes, and intestine (63, 64).

In the retina, USH2 proteins are localized specifically in photoreceptors (12, 35, 52, 53, 65, 66). The photoreceptor is a highly polarized sensory neuron converting light signals to electrical impulses. It consists of the outer segment, connecting cilium, inner segment, cell body, and synaptic terminus (Figure 2A). It contacts with Muller cells at the adherens junction. Initially, USH2 proteins were localized to the inner segment, adherens junction, connecting cilium, basal bodies, and synaptic terminus in photoreceptors (Figure 2A) (12, 52, 65, 66). However, among these subcellular structures, we have demonstrated that USH2 signals disappear largely only at the connecting cilium layer in the *Ush2a* knockout, whirlin knockout and *Gpr98*/del7TM mice, suggesting that these three USH2 proteins are highly enriched around the connecting cilium (35, 48, 53). Additional studies using immunoelectron microscopy show that all three USH2 proteins are at the mouse periciliary membrane complex (PMC) (35, 53), the plasma membrane of the apical inner segment directly facing the connecting cilium (Figure 2A) (67). The frog periciliary ridge complex (PRC), discovered more than twenty years ago (68), is an analogous structure to PMC. It is a morphologically-specialized structure with a symmetrical array of 9 ridges and 9 grooves. Whirlin is enriched at the PRC in frog photoreceptors (53, 65). Therefore, the subcellular locations of whirlin in mouse and frog photoreceptors are highly conserved.

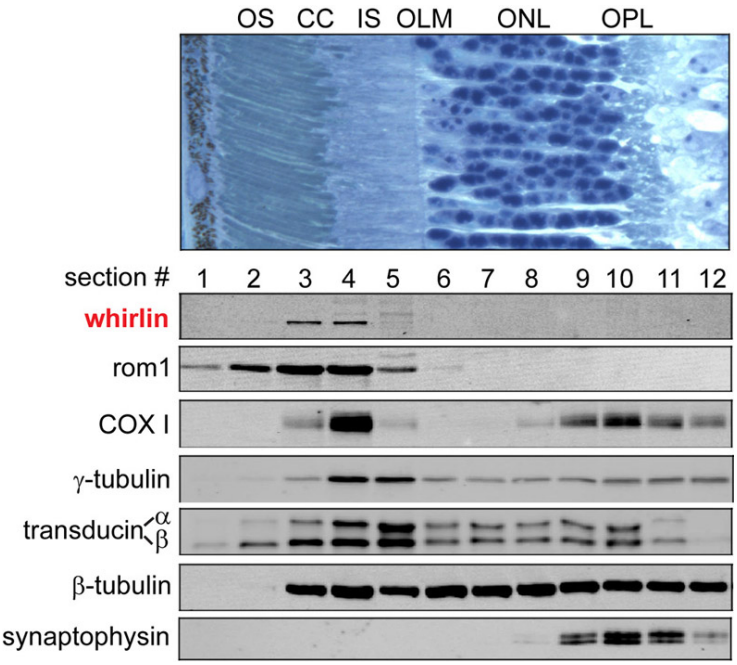

Figure 3. Subcellular localization of whirlin in photoreceptors as determined by western blot analysis of retinal serial sections. A mouse retina was flat-mounted, frozen, and tangentially sectioned at 10 µm, as previously described (69). The protein content of the serial sections 1-12 was analyzed by western blotting using antibodies against whirlin (Rabbit PDZIE) (53) and protein markers for various rod cellular compartments. The markers include the rod outer segment marker-rom1 (a gift from Dr. Andrew Goldberg, Oakland University), mitochondrial marker-complex IV subunit 1 (COX I, MS404, MitoSciences), rod photoreceptor markers-transducin alpha and beta subunits (sc-389, Santa Cruz Biotechnology and PA1-725, Applied BioReagents, respectively), synaptic marker-syntaptophysin (sc-12737, Santa Cruz Biotechnology), general cellular marker-beta-tubulin (T0198, Sigma), and marker for the basal bodies and centrioles-gamma-tubulin (T6557, Sigma). Specific whirlin band appeared in sections 3 and 4, corresponding to the border between the outer and inner segment, and was undetectable in other cellular compartments of photoreceptors. OS, outer segment; CC, connecting cilium; IS, inner segment; OLM, outer limiting membrane (adherens junction); ONL, outer nuclear layer; OPL, outer plexiform layer (photoreceptor synapse).

To further verify these findings, we recently employed the serial tangential sectioning technique coupled with western analysis (69). In the retina, different photoreceptor subcellular compartments are neatly organized into tangential layers. Western analysis of retinal sequential tangential sections clearly demonstrated that whirlin is highly concentrated at the connecting cilium region between the outer and inner segment, but not detectable in the inner segment, adherens junction (outer limiting membrane), or synaptic terminus (outer plexiform layer) in photoreceptors (Figure 3). The localization of USH2 proteins mainly at the PMC but not at the synaptic terminus of photoreceptors is further supported by the phenotypic analyses in USH2 mutant mice and the symptom manifestation in USH2 patients. For instance, electron microscopy shows ultrastructural abnormalities around the PMC region but not at the synaptic terminus of photoreceptors in whirlin knockout mice (53). Electroretinogram detects no defective waveforms, typically resulting from abnormal photoreceptor synaptic transmission, in *Ush2a*, *Gpr98* and whirlin mutant mice (35, 44, 53) or in USH2 patients.

The calycal processes in photoreceptors are thought as an analogous structure to the stereocilia in hair cells (70). It is interesting to know whether USH2 proteins are localized in these structures in photoreceptors. The calycal processes are well developed in humans, frogs and other species. In mice, only cone photoreceptors have obvious calycal processes (71-73). Previous studies using both mice and frogs did not report USH2 proteins in the calycal processes (12, 52, 65, 66). Our studies recently show that whirlin is not evident at the frog calycal processes, but GPR98 is localized at this structure in mouse cone photoreceptors (53).

The inner ear is composed of the cochlea and vestibular system for hearing and balance, respectively. In the vestibular system, hair cells exist in the maculae of the saccule and utricle and the cristae ampullares of the semicircular canals. In the cochlea, one row of inner hair cells and three rows of outer hair cells exist in the organ of Corti. The inner hair cells are responsible for mechanoelectric transduction, whereas the electromotile outer hair cells also perform an electromechanical transduction, thereby amplifying the sound-evoked vibrations of the entire sensory epithelium (74). All types of hair cells have stereocilia on their apical surfaces, which are modified microvilli filled with actin filament bundles. The stereocilia are well-organized into rows of different

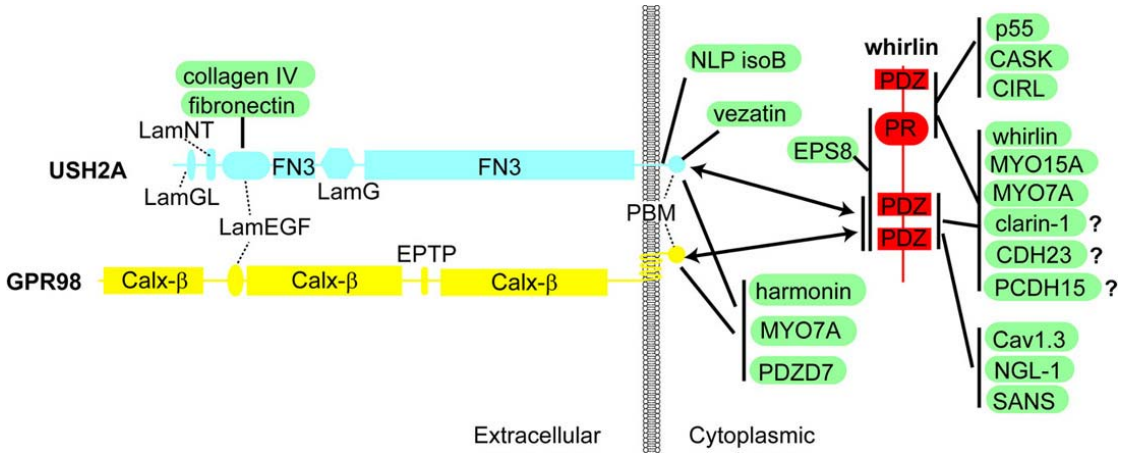

Figure 4. The USH2 protein complex and its putative components. USH2A (light blue) and GPR98 (yellow) interact with whirlin (red) through the PDZ-domain-mediated binding (arrows). Proteins (green) interacting with at least one of the three USH2 proteins are thought as putative components of the USH2 complex. The domains involved in the interactions are indicated by lines. LamGL, thrombospondin-type laminin G domain; LamNT, N-terminal globular laminin domain; LamEGF, laminin EGF-like domain; LamG, laminin globular-like domain; PR, proline-rich region.

lengths and form a staircase-like hair bundle. The mechanoelectric transduction channels are located at the tip of the stereocilia. Recent studies show that various links exist at the tip and along the entire length of the stereocilia during development and in adulthood (70, 75, 76).

All USH2 proteins are localized at the ankle-link of the stereocilia in hair cells (44, 46, 52, 53). This ankle-link is a transient structure at the base of the stereocilia, present from P2 to P9 and completely lost by P12 (Figure 2B) (76). Whirlin is also present at the tip of the stereocilia in hair cells throughout development and adulthood (51, 77, 78). In addition, the existence of USH2 proteins has been found in other regions of the inner ear, though it has not been verified using USH2 mutant mice as negative controls. The USH2 proteins are shown at the synaptic region of hair cells with an exception of whirlin absent at the synaptic region of the inner hair cells. The three USH2 proteins are also localized in the cell body of the spinal ganglia. Whirlin has been detected in various nervous fibers (52).

6. THE USH2 PROTEIN COMPLEX AND ITS PUTATIVE COMPONENTS

A PDZ domain, about 90 amino-acid long, has 5-6 beta-strands and 2 alpha-helices with a hydrophobic cleft. This cleft binds to a ligand, called the PDZ-binding motif (PBM). PBM exists usually at the C-terminus, sometimes in an internal region, or even at a PDZ domain of proteins (79). Whirlin has multiple PDZ domains, and both USH2A and GPR98 have a PBM at their C-termini. We and others, using a series of biochemical assays, have demonstrated that whirlin binds to USH2A and GPR98 *in vitro* through the PDZ domain-mediated interactions (26, 52, 53). The three USH2 proteins are colocalized to the ankle link of the stereocilia in hair cells and the PMC in photoreceptors (46, 53). In whirlin knockout, *Ush2a* knockout and *Gpr98*/del7TM mutant mice, we have discovered that loss of any one of these three USH2 proteins causes mislocalization and reduced expression of the other two proteins in photoreceptors and hair cells (53). These data strongly suggest that the three USH2 proteins, probably together with other interacting proteins, form a multi-protein complex *in vivo* (Figure 4) and that each one of the USH2 proteins is required for the assembly of this complex. However, the interdependence of the three USH2 proteins for their normal cellular localization and expression level is not exactly the same in hair cells and in photoreceptors, suggesting that the USH2 protein complex may not be assembled precisely in the same way or contain the exactly same pool of components between these two sensory neurons. Additionally, we found that the transgenic whirlin delivered by adeno-associated virus (AAV) can restore the normal localization and expression level of both USH2A and GPR98 in the whirlin knockout photoreceptor (48). This finding further confirms the existence of the USH2 protein complex *in vivo* and indicates that whirlin is involved in the organization of this USH2 complex. Supportively, USH2A and GPR98 were recently found to associate with each other in tracheal epithelial cells (80). However, the mechanistic details on the assembly of the USH2 protein complex are scarce.

Besides the three known USH2 proteins, USH2-interacting proteins could be potential components of this USH2 complex (Figure 4 and Table 2). *In vitro* biochemical assays have identified a scaffold protein, SANS, (65) and an actin-based motor protein, myosin VIIa (MYO7A) (77), as whirlin-interacting proteins. These two proteins are localized at the periciliary region of photoreceptors and close to or at the ankle link in hair cells (12, 13). They are implicated in human USH1G and USH1B, respectively (81, 82). Additionally, USH2A and GPR98 are shown to bind to MYO7A, harmonin and PDZD7 *in vitro* (20, 46, 83). Harmonin is encoded by the *USH1C* gene and is a homolog of both whirlin and PDZD7 (84, 85). Harmonin is known to bind to cadherin 23

Table 2. USH2-interacting proteins

Protein name	Involved domain	Involved domain in USH2 proteins	References
USH2A-interacting proteins			
vezatin	not determined	cytoplasmic region	(46)
MYO7A	MyTH4-FERM	cytoplasmic region	(46)
PDZD7	PDZ1 & PDZ2	PBM	(20)
harmonin	PDZ1	PBM	(83)
whirlin	PDZ1 & PDZ2	PBM	(26, 52, 53)
NLP isoform B	IF domain	cytoplasmic region w/o PBM	(97)
collagen IV	7S domain	loop b of the laminin EGF-like domain	(99)
fibronectin	not determined	loop d of the laminin EGF-like domain	(98)
GPR98-interacting proteins			
MYO7A	MyTH4-FERM	cytoplasmic region	(46)
PDZD7	PDZ1 & PDZ2	PBM	(20)
harmonin	PDZ1	PBM	(83)
whirlin	PDZ1	PBM	(26, 52, 53)
whirlin-interacting proteins			
Cav1.3	PBM	PDZ1 & PDZ2	(101)
SANS	PBM	PDZ1 & PDZ2	(65)
NGL-1	cytoplasmic region	PDZ1 & PDZ2	(77)
p55	GUK domain	PDZ3	(100, 102)
EPS8	PTB and PR	PDZ1, PDZ2 & PR	(103)
CASK	GUK domain	PR & PDZ3	(100)
CIRL/latrophilin1	C-terminal region	PR & PDZ3	(100)
MYO15A	PBM	PDZ3	(51)
	MyTH4-FERM	PDZ1 & PDZ2	(77)
	SH3	PR & PDZ3	(77)
MYO7A	not determined	not determined	(77)
whirlin	PDZ1 & PDZ2	PDZ1 & PDZ2	(53)
	PR & PDZ3	PR & PDZ3	(77)

MyTH4-FERM: myosin tail homology 4-protein 4.1, ezrin, radixin, moesin domain, NLP: ninein-like protein, IF: intermediate filament domain, Cav1.3: L type voltage-dependent calcium channel alpha 1D subunit, NGL-1: netrin-G1 ligand, GUK: guanylate kinase-like domain, CASK: calcium/calmodulin-dependent serine protein kinase, SH3: src homology 3, PR: proline-rich domain, PTB: phosphotyrosine binding domain

(CDH23) and protocadherin 15 (PCDH15), which have one or two PBMs (86, 87) and are involved in USH1D (88, 89) and USH1F (90, 91), respectively. Although the interaction between CDH23/PCDH15 and whirlin has not been reported, these two proteins could be whirlin-interacting candidates. Furthermore, clarin-1, encoded by the *USH3A* gene (92), has a predicted PBM (93). Thus, clarin-1 could also be a potential whirlin-interacting protein. Therefore, the USH2 proteins are able to associate with multiple USH1 and, possibly, USH3 proteins. Together with the finding that USH1 proteins interact with one another *in vitro* (94, 95), it has been hypothesized that proteins involved in different types of Usher syndrome are organized in one interactome *in vivo* (12, 65, 66, 96).

The USH interactome is proposed to exist at the synapse in hair cells and photoreceptors, simply according to immunostaining data (12, 66). However, a variety of cellular and biochemical studies do not support that all USH2 proteins are present in the synapse of photoreceptors (see above and Figure 3) (48, 53). Recently, a novel USH interactome without harmonin has been proposed present at the periciliary region of photoreceptors (65, 96). This novel USH interactome has USH2 proteins together with MYO7A and SANS. MYO7A can interact with all three USH2 proteins *in vitro*. In hair cells, loss of MYO7A causes absence of these three USH2 proteins in the stereocilia in mice (*Myo7a*^{4626SB}). These findings suggest that MYO7A participates in transporting USH2 proteins to the ankle-link in hair cells (46). In photoreceptors, the localization of MYO7A partially overlaps with the PMC (13). But we found that whirlin expression and localization have no change in *Myo7a*^{sh1} (SH1/LeJ) (data not shown) or *Myo7a*$^{sh1\text{-}7J}$/J mice (Figure 5), which have a missense mutation at the motor domain or a 778-bp deletion immediately after the motor domain of MYO7A, respectively. Therefore, unlike in hair cells, MYO7A may not be the motor protein for the localization of USH2 proteins, or redundant motor proteins may exist in photoreceptors. Based on these data, we believe that the hypothesis of one USH interactome including various USH proteins needs to be tested carefully and thoroughly in both photoreceptors and hair cells.

A putative transmembrane protein, vezatin, is shown to bind to USH2A and localized to the ankle-link of hair cell stereocilia. In the *Gpr98* knockout mouse and the whirler mouse, vetazin is mislocalized from the ankle-link (46). Therefore, vezatin is probably a component of the USH2 complex at the ankle-link in hair cells. However, the presence of vezatin in the USH2 complex in photoreceptors has not been reported. USH2A also binds to the centrosomal ninein-like protein (NLP) isoform B (97). The latter protein links USH2A with lebercilin, a protein implicated in Leber's congenital amaurosis, an early onset retinal degenerative disease (97). Additionally, USH2A was found to associate with collagen IV and fibronectin (98, 99). For whirlin, it interacts *in vitro* with calmodulin-dependent serine kinase (CASK) (100), a voltage-dependant calcium channel α subunit (Cav1.3) (101), and a transmembrane protein (NGL-1) (77). Therefore, all these proteins are candidate components in the USH2 complex at the PMC in photoreceptors and the ankle-link in hair cells (Figure 4). However, further investigation and confirmation

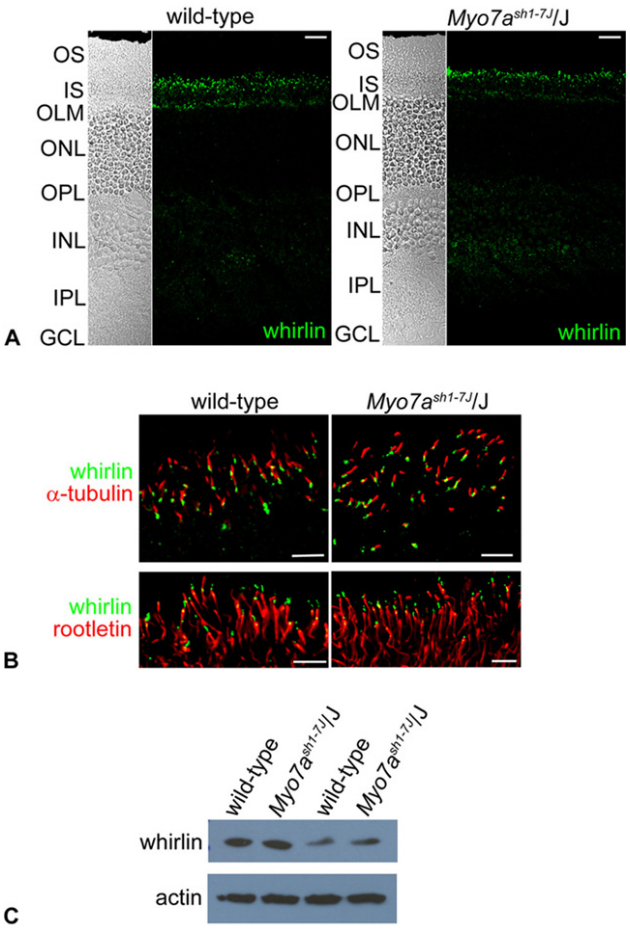

Figure 5. Whirlin expression in the *Myo7a*$^{sh1\text{-}7J}$/J retina. (A) Whirlin signals in the wild-type and *Myo7a*$^{sh1\text{-}7J}$/J mice are similar to each other across the various retinal layers. The signals at the OLM are nonspecific signals according to our previous studies (48, 53). OS, outer segment; IS, inner segment; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars, 20 μm. (B) Observation of whirlin signals at a high magnification shows no difference between the wild-type and *Myo7a*$^{sh1\text{-}7J}$/J photoreceptors. Whirlin (green) is localized beneath acetylated α-tubulin (red, top panels) and above rootletin (red, bottom panels) in photoreceptors. Scale bars, 5 μm. (C) Western analysis demonstrates that there is no difference in the whirlin expression level between the wild-type and *Myo7a*$^{sh1\text{-}7J}$/J retinas. Actin signals were used as a loading control. The *Myo7a*$^{sh1\text{-}7J}$/J mouse was purchased from the Jackson Laboratory and was discovered by genomic DNA sequencing in our lab to have a 778-bp deletion (between 4348 – 5127 bp in NM_008663) immediately after the motor domain.

of these proteins in the USH2 complex *in vivo* are essential and urgent. Besides studying the above putative components of the USH2 complex, we believe that an unbiased protein interaction screening is an alternative way to generate a more complete picture of the molecular composition of the USH2 complex.

Whirlin also binds to a scaffold protein, p55 (102), an actin-regulatory protein, EPS8 (103), and an actin motor protein, myosin XVa (MYO15A) (51, 77, 78). These three proteins are colocalized with whirlin at the tip of hair cell stereocilia (51, 77, 78, 102, and our unpublished data). In the shaker2 mutant mouse (*Myo15a*^{sh2}), which has a mutation in the *Myo15a* gene, all whirlin, EPS8 and p55 are mislocalized. In the whirler mouse, the expression of p55 and EPS8 at the tip of stereocilia is ablated or reduced, but MYO15A expression is not changed. These findings suggest that the interactions of whirlin with p55, EPS8 and MYO15A exist in hair cells and that MYO15A is essential for the delivery of whirlin to the tip of hair cell stereocilia. However, p55, EPS8 and MYO15A are probably not the components of the USH2 complex in hair cells, because the USH2 complex is localized at the ankle-link, the base of hair cell stereocilia. In photoreceptors, the localization of p55, EPS8 and MYO15A are not clear.

7. POTENTIAL FUNCTIONS OF THE USH2 COMPLEX AND DISEASE MECHANISMS UNDERLYING USH2

Disruption of the USH2 complex is now believed to be the primary cause underlying USH2 pathogenesis in

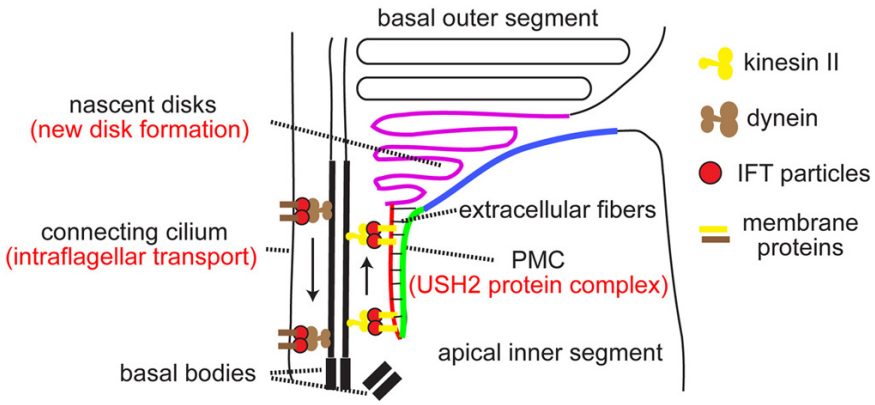

Figure 6. A schematic diagram of subcellular structures around the PMC in photoreceptors. The PMC (green) directly faces the plasma membrane of the connecting cilium (red). There are fiber-like connections between these two structures. The plasma membrane of the apical inner segment (blue) neighboring the PMC is in close proximity of the membrane of the nascent disks (purple) at the base of the outer segment. Intraflagellar transport occurs along the connecting cilium (arrows). Formation of new membrane disks is at the basal outer segment.

humans and the Usher syndrome-like phenotypes in mice. Therefore, this complex is critical for the cell biology and survival of both photoreceptors and hair cells. However, its exact biological function has not yet been revealed. Because of the normal electroretinogram in USH2 mutant mice and the normal whole cell recording in *Gpr98* mutant vestibular hair cells and cochlear inner hair cells (35, 44, 46, 53), this USH2 complex may not directly contribute to phototransduction in photoreceptors or mechanotransduction in hair cells. The predicted functional domains in USH2 proteins, i.e., the cell adhesion domains in USH2A and GPR98, the GPCR domains in GPR98, and the protein interaction modules in whirlin, suggest that the USH2 complex may link the extracellular matrix and the intracellular cytoskeletons and may transduce signals of mechanical stresses between the exterior and the interior of photoreceptors at the PMC and of hair cells at the ankle-link, like the dystrophin glycoprotein complex and the integrin supramolecular complex (104-110). In support of this hypothesis, USH2A was found to associate with collagen IV and fibronectin (98, 99), the two common proteins in the extracellular matrix; GPR98 was demonstrated as a component of the extracellular fibers at the ankle-link in hair cells and between the PMC and the connecting cilium in photoreceptors (44, 46, 65); whirlin was shown to bind to p55 and myosins, the actin-binding proteins; and adenylyl cyclase 6 was suggested to be involved in GPR98 signaling in hair cells (46).

The occasional finding of abnormal sperms in USH patients and the localization of USH proteins around the connecting cilium and basal body lead to the classification of Usher syndrome as one of retinal ciliopathies (57, 111, 112). In photoreceptors, the outer segment is a large specialized cilium filled with many membrane disks, where phototransduction occurs (Figure 2). This cellular compartment undergoes continuous and rapid renewal (113-116), which requires a large amount of proteins and membrane lipids synthesized in the inner segment to be quickly transported to the outer segment through intraflagellar transport along the connecting cilium (Figures 2 and 6). Recent studies indicate that defects in the connecting cilium and intraflagellar transport are the culprit for retinal degeneration in retinal ciliopathies, such as the ones caused by RPGR and RPGRIP mutations and in Bardet Biedl syndrome (111, 112, 117, 118).

The PRC in frog photoreceptors has been proposed, based on immunocytochemistry and freeze-fracture electron microscopy, as the membrane fusion site for post-Golgi vesicles carrying rhodopsin and docosahexaenoyl (DHA)-phospholipids before these cargos are transported from the inner to outer segment (68, 119-121). Additionally, Rab8, rac1, Sec8, moesin, syntaxin 3 and SNAP-25 have been localized around the PRC in frog photoreceptors (122, 123). These proteins are proposed, though not verified using mouse genetics, to participate in and/or regulate the docking and membrane fusion of post-Golgi vesicles to the plasma membrane at the PRC. Therefore, the USH2 complex at the PMC, the analogous structure of PRC in mouse, might play an either direct or indirect role in the docking/membrane fusion between the post-Golgi vesicles and plasma membrane at the base of the connecting cilium (65, 96). This proposed function can also be applied in hair cells. The ankle-link exists when stereocilia grow and differentiate from small microvilli. At this time, many vesicles are at the base of stereocilia (124), which could be the post-Golgi vesicles carrying proteins and membrane lipids from the cell body to the growing stereocilia. Supportively, the *Gpr98* knockout mouse shows delocalization of some CDH23 long isoforms at the tip of the stereocilia and possibly loses some apical links between the stereocilia (46). However, solid evidence supporting this putative function of the USH2 complex is still missing. For instance, obvious mislocalization of rhodopsin has not been observed in whirlin knockout and *Ush2a* knockout mice (35, 53), and vesicles fused with the plasma membrane have not been demonstrated at the ankle link.

On the other hand, the USH2 complex may play a role in structural maintenance as the USH1 proteins do, based on the similar symptoms in USH1 and USH2

patients. Studies on USH1 proteins, CDH23 and PCDH15, have demonstrated that they interact to form transient interciliary links and the tip link in the hair bundle and play a role in cohesion and stiffness of the hair bundle. Mutations in their genes cause hair bundle fragmentation and misorientation and, subsequently, affect mechanotransduction. Other USH1 proteins may also contribute to the cohesion and stiffness of hair bundle. For instance, SANS and MYO7A are required to transport harmonin. Harmonin is essential for normal localization of SANS and PCDH15 and probably anchors CDH23 and PCDH15 to actin filaments in the stereocilia (59, 95, 125). Among the USH2 proteins, USH2A and GPR98 probably interact with each other or with some unidentified cell adhesion proteins to form the ankle link. In the wild-type mouse, the stereocilia of outer hair cells are organized into a V-shaped, three-row, staircase-like hair bundle. However, in Ush2 mutant mice, the hair bundle exhibits various disorganized arrangement, mainly having a U-shape (35, 46, 53). This phenotype suggests that the USH2 complex probably keeps intact the membrane invaginations at the base of stereocilia during stereocilia growth and subsequently ensures the cohesion and orderly-arrangement of stereocilia in adulthood.

In photoreceptors, subcellular structures around the PMC are very intricate (Figure 6). The apical inner segment stays in close proximity with the connecting cilium and the basal outer segment. Extracellular fiber-like connections with GPR98 as a component exist between the PMC and connecting cilium (65). Because of the continuous intraflagellar transport of membrane proteins and lipids along the connecting cilium (126-130) and the active new membrane disk formation at the base of the outer segment (131, 132), the plasma membrane of the connecting cilium and the basal outer segment could be very flexible. It is reasonable to speculate that the USH2 complex may function in maintaining the integrity of the dynamic plasma membranes of the connecting cilium, the apical inner segment, and the basal outer segment (Figure 6). This speculation is supported by the presence of subcellular structural abnormalities around the PMC in USH2 mutant mice, such as membrane fusions between the apical inner segment and its surrounding subcellular structures in whirlin knockout mice and enlarged gaps between the PMC and the connecting cilium in *Gpr98*/del7TM mice (53, 65). Finally, the USH2 complex might function as a diffusion barrier at the plasma membrane between the cell body and the connecting cilium/stereocilia. However, evidence is needed to support this possibility.

8. THERAPEUTIC STUDIES FOR USH2

Because of the wide clinical application of the well-developed cochlear implant for hearing loss (133, 134), more attention is currently focused on seeking effective treatments for retinitis pigmentosa in Usher syndrome. The easy access to the retina allows local delivery, small amount of administration, minimum immune response, and non-invasive monitoring of the treatment, thereby lending favorable advantages for a variety of therapeutic options. Gene therapy, drug application, cell transplantation, and nutritional supplements have been extensively attempted in cultured cells, animal models, or even in patients. However, only two reports have been published on testing potential treatments specifically for USH2 (48, 135).

In the first report, human neural progenitor cells from the post mortem fetal cortical brain tissue were tested (135). This type of progenitor cells has previously been shown to survive and integrate into neural tissues. They postpone cellular and/or functional loss in animal models of multiple neurodegenerative diseases, including the Royal College of Surgeons rat for retinitis pigmentosa (136). In the *Ush2*a knockout mouse, the transplanted progenitor cells exist between photoreceptors and retinal pigment epithelium (RPE) cells at 10 weeks after subretinal injection. This transplantation delays the cellular changes in photoreceptors and alleviates retinal functional deterioration before photoreceptor loss. This effect lasts for 10 weeks, the longest time point examined (135). However, the underlying mechanism for this treatment is not quite clear at this stage.

Compared to the cell-based therapy, replacement of the mutant gene in the retina is a straightforward therapeutic approach. In the last decade, numerous achievements have been made in this field. Three clinical trials are now ongoing in patients with Leber's congenital amaurosis, who carry mutations in the *RPE65* gene (137-141). The preliminary results from these clinical trials are very promising in efficacy. Therefore, gene therapy could hold promise in treating retinal degenerative diseases. Recently, the efficiency and efficacy of a lentivirus-mediated gene replacement of MYO7A have been studied in the *Myo7a*^{4626SB} mutant mouse, an animal model for USH1B (142). Although the delivery of MYO7A into photoreceptors and RPE cells is not quite efficient, the treated mutant retina does show correction of the histological phenotypes in these two cells. In addition, our laboratory has been studying gene therapy for USH2D. We utilized a combination of AAV and a photoreceptor-specific promoter to efficiently target whirlin into both rod and cone photoreceptors. The expression of the whirlin transgene in the whirlin knockout mouse is comparable to the endogenous whirlin in the wild-type mouse. Most importantly, the transgenic whirlin was found to restore the USH2A and GPR98 expression in the whirlin knockout retina (48). These encouraging progresses in the USH1B and USH2D mouse models lay a solid foundation for a further and detailed exploration of gene therapy for other subtypes of USH2. However, the main obstacle for designing viral constructs carrying the USH2 genes is the extremely large size of *USH2A* and *GPR98*, which far exceeds the packaging capacity of AAV and lentivirus.

Aminoglycosides and their derivatives have been actively investigated as drugs to treat genetic diseases caused by nonsense mutations, such as cystic fibrosis, Duchenne muscular dystrophy and Usher syndrome. Some of them have already been tested in clinical trials (143, 144). These drugs can induce a read-through of nonsense

mutations by inserting an amino acid at the stop codon, though the inserted amino acid is not necessary to be the correct one as in the wild-type sequence. The extent of suppression of nonsense mutations by these drugs is variable. Aminoglycosides and their derivatives have been tested in test tubes, cell cultures and retinal explants to suppress the nonsense mutations found in USH1F (PCDH15) and USH1C (harmonin) patients (145-148). However, the high cellular toxicity of these drugs and the low efficiency of their read-through activities set a hindrance for their further application to patients. Efforts are being made to discover new generations of aminoglycoside derivatives or their substitutes with low cellular toxicity and high efficacy. A recent research has shown a great improvement of these two aspects using PTC124, a drug unrelated to aminoglycosides (149).

The nutritional supplementation, daily intakes of vitamin A at a dose of 15,000 international units (IU) and vitamin E less than 400 IU, has been shown to slow down the progression of common forms of retinitis pigmentosa, as monitored using electroretinogram (150-153). A further study following patients on this nutritional supplement therapy demonstrates safety of this high dose of vitamin A up to 12 years (154). Therefore, it is thought that this high-dose daily supplementation of vitamin A could be an effective therapy for retinitis pigmentosa in Usher syndrome. However, controversies exist regarding the validity of the conclusion drawn from the original data and the toxicity of the high dose of vitamin A (155, 156). In addition, the underlying mechanism of this vitamin A supplement therapy is uncertain, though retinal, a derivative of vitamin A, is an essential chromophore for phototransduction in photoreceptors.

9. SUMMARY AND PERSPECTIVE

The proteins encoded by the three identified USH2 genes have been demonstrated to form a protein complex *in vivo*. This complex is localized mainly at the PMC in photoreceptors and the ankle-link of the hair cell stereocilia. Formation of this protein complex is crucial for the normal localization and stability of individual USH2 proteins. Whirlin, as a scaffold protein, is involved in the assembly of this complex. Although many proteins are presently thought as potential components in this USH2 complex, further careful studies using various animal models are essentially necessary to confirm their existence in this complex *in vivo*. To date, the biological function of the USH2 complex is largely unknown, though it is believed that disruption of this complex is the primary molecular mechanism underlying retinal degeneration and hearing impairment in USH2. USH2 is an incurable disease. Effective treatments using different approaches are still being sought and explored.

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Abbreviations: GPR98: G protein-coupled receptor 98, VLGR1: very large G protein-coupled receptor 1, MASS1: monogenic audiogenic seizure susceptibility 1, WHRN: whirlin, GPCR: G protein-coupled receptor, GPS: GPCR proteolytic site, 7TM: 7-transmembrane domain, PDZ: postsynaptic density 95/discs large/zonula occludens-1 domain, PBM: PDZ-binding motif, RPE: retinal pigment epithelium, PRC: periciliary ridge complex, PMC: periciliary membrane complex, AAV: adeno-associated virus, EYFP: enhanced yellow fluorescent protein

Key Words: USH2A, GPR98, VLGR1, whirlin, periciliary membrane complex, the ankle-link, retinitis pigmentosa, photoreceptors, hearing loss, and hair cells

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