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Chapter 4

PROTEIN NETWORKS RELATED TO THE USHER SYNDROME GAIN INSIGHTS IN THE MOLECULAR BASIS OF THE DISEASE

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4.1. INTRODUCTION

The Usher syndrome (USH) is the most common form of inherited deaf-blindness with a prevalence of ~ 1/10,000, which represents 50 % of all deaf-blindness cases (Vernon 1969). USH was first reported by the German Ophthalmologists von Graefe and Leibreich nearly 150 years ago (von Graefe 1858, Liebreich 1861) and was later named after Charles Usher who reported it in 1914 (Usher 1914). USH is an autosomal recessive disorder. It is genetically heterogeneous involving at least 12 chromosomal loci (Table 4.1). Moreover, many USH patients do not show linkage to mutations in identified USH genes, indicating further genetic heterogeneity (Cohen *et al.* 2007). Three clinical subtypes USH1, USH2 and USH3 can be defined according to the severity of the hearing impairment, the presence or absence of vestibular dysfunction and the age of onset of retinitis pigmentosa (RP) (Petit 2001, Reiners *et al.* 2006, Kremer *et al.* 2006, Cohen *et al.* 2007, Saihan *et al.* 2009). Patients suffering from USH1, the most severe form of the disease, exhibit pre-lingual hearing loss, vestibular areflexia, and pre-pubertal onset of RP. In USH2, the most frequent type, congenital hearing loss is milder; the onset of RP is during or after puberty and vestibular function remains normal. USH3 is only relatively frequent in specific populations and is characterized by progressive hearing loss with large variability in vestibular dysfunction and in the onset of RP. Mutations in the genes related to USH1B, C, D, F and USH2D leading to missense, inframe alterations result in non-syndromic hearing loss, and mutations in the

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USH2A gene are the most frequent cause of autosomal recessive RP without hearing loss (Rivolta *et al.* 2000, Seyedahmadi *et al.* 2004, Saihan *et al.* 2009).

Table 4.1. Usher syndrome (USH) types, loci, genes, encoded proteins and their functions (for description see the text)

Type	**Locus**	**Gene**	**Protein**	**Function**	**Ref.**
1B	11q13.5	*MYO7A*	Myosin VIIa	Molecular motor	1
1C	11p14-15	*USH1C*	Harmonin	Scaffold protein	2-4
1D	10q21-q22	*CDH23*	Cadherin 23	Adhesion protein	5-7
1E	21q21	- -	- -	- -	8
1F	10q11.2-q21	*PCDH15*	Protocadherin 15	Adhesion protein	9, 10
1G	17q24-25	*SANS*	SANS	Scaffold protein	11,12
1H	15q22-23	*USH1H*	--	--	13
2A	1q41	*USH2A*	USH2A (usherin)	ECM, adhesion protein	14, 15
2C	5q13.	*VLGR1b*	GPR98 (VLGR1b)	GPCR, adhesion protein	16, 17
2D	9q32-q34	*WHRN/DFNB31*	Whirlin	Scaffold protein	18, 19
3A	3q25	*CLAR1*	Clarin-1	Adhesion protein	20, 21
3B	20q				

Ref. = References: USH1B: **(**1) Weil *et al.* (1995); USH1C: (2) Smith *et al.* (1992), (3) Bitner-Glindzicz *et al.* (2000); (4) Verpy *et al.* (2000); USH1D: (5) Bolz *et al.* (2001), (6) Bork *et al.* (2001), (7) Di Palma *et al.* (2001); USH1E: (8) Chaib *et al.* (1997); USH1F: (9) Ahmed *et al.* (2001), (10) Alagramam *et al.* (2001); USH1G: (11) Mustapha *et al.* (2002), (12) Weil *et al.* (2003); USH1H: (13) Ahmed *et al.* (2009); USH2A: (14) van Wijk *et al.* (2004), (15) Eudy *et al.* (1998); USH2C: (16) Weston *et al.* (2004), (17) Johnson *et al.* (2005); USH2D: (18) Mustapha *et al.* (2002), (19) Ebermann *et al.* (2007); USH3A: (20) Joensuu *et al.* (2001), (21) Adato *et al.* (2002)
ECM = extra cellular matrix protein; GPCR = G protein coupled receptor.

4.2. USH PROTEINS, DOMAIN STRUCTURES AND FUNCTIONS

The gene products of identified USH genes belong to various protein classes and families (Table 4.1, Figure 4.1, and Figure 4.2). The first identified USH gene, *MYO7* (USH1B) encodes the molecular motor myosin VIIa which uses energy from ATP hydrolysis so as to transport cargo along actin filaments (Udovichenko *et al.* 2002, Inoue and Ikebe 2003). The long tail of myosin VIIa contains several characteristic domains namely a coiled-coil dimerization domain, followed by two tandem repeats, each composed of a MyTH4 (myosin tail homology 4) domain and a FERM-(4.1-protein, ezrin, radixin, meosin)-like domain, which act as binding motifs for a number of proteins including other USH proteins (Wolfrum 2003, Senften *et al.* 2006) (Figure 4.1).

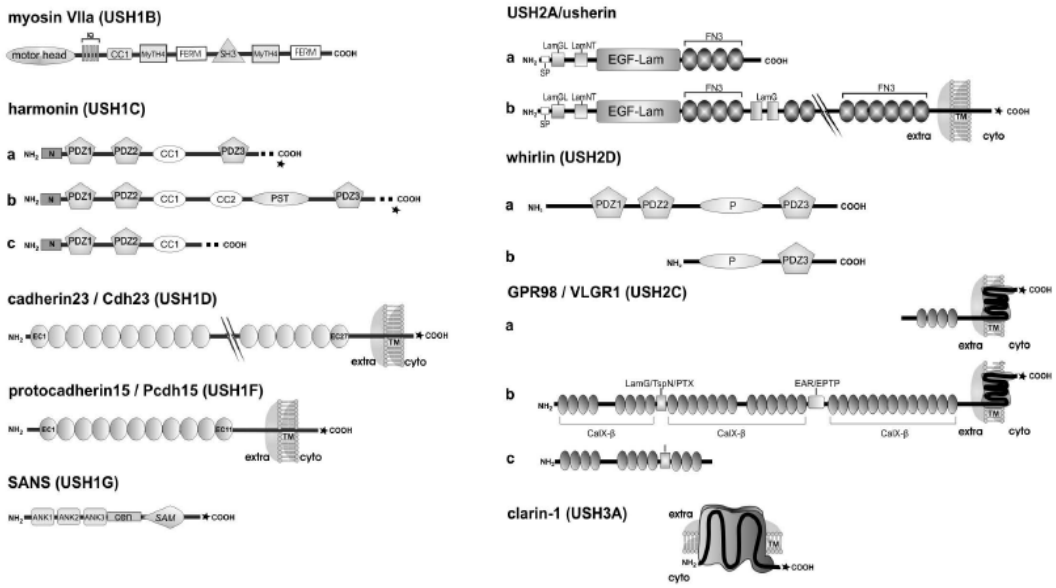

Figure 4.1. Domain structures of USH proteins. **Myosin VIIa** (USH1B): IQ: isoleucine-glutamine (IQ) motifs are binding sites for regulatory light chains, CC: coiled-coil domain, followed by two large repeats SH3 (src homology-3) domain. These repeats include a MyTH4 (myosin tail homology 4) and a FERM (4.1, ezrin, radixin, moesin) domain. **Harmonin** (USH1C): PDZ (PSD95, discs large, ZO-1) domains (PDZ1 – PDZ2) and one coiled-coil (CC1) domain. Harmonin class a isoforms consist of an additional PDZ (PDZ3) domain. The longest class b isoforms contain also a third PDZ domain (PDZ3), a second coiled-coil domain (CC2), and a proline, serine, threonine (PST)-rich region. Harmonin a1 and b4 possess a C-terminal class I PDZ-binding motif (PBM). **Cdh23** (Cadherin 23, USH1D) is composed of 27 extracellular cadherin repeats (EC1 - EC27), a transmembrane domain (TM) and a short cytoplasmic domain with a class I PBM (*asterisk*). **Pcdh15** (Protocadherin 15, USH1F) comprises eleven ectodomains (EC1–EC11), one transmembrane domain (TM) and a C-terminal class I PBM (*asterisk*). **SANS** (scaffold protein containing ankyrin repeats and SAM domain; USH1G) contains three N-terminal ankyrin repeats (ANK1 - ANK3), a central region (cen), a sterile alpha motif (SAM) and a C-terminal class I PBM (*asterisk*). **USH2A isoform b/Usherin** (USH2A): The extracellular domain contains a laminin G-like domain (LamGL), a N-terminal laminin domain (LamNT), 10 laminin-type EGF-like modules (EGF-Lam), 32 fibronectin type III (FN3) repeats (4 + 28), spaced by two laminin G domains (LamG) followed by a transmembrane region (TM) and the intracellular C-terminal domain containing a PBM. **Whirlin** (USH2D) is characterized by PDZ domains and a proline (P)-rich region. Short isoforms contain only a third PDZ domain (PDZ3). Full length **GPR98/VLGR1b** (very large G-protein coupled receptor 1b, USH2C) consists of extracellular N-terminal extension with a LamG/TspN/PTX-homologous domain, seven EAR/EPTP repeats, 35 Ca^{2+}-binding calcium exchanger β (CalX-β□) modules, one 7-transmembrane domain (TM) as well as a short intracellular domain containing a PBM (*asterisk*). **Clarin-1** (USH3A) is build up by four transmembrane-domains and contains a glycosylation consensus site. A C-terminal "TNV" signature may serve as a PBM (*asterisk*). "cyto" indicates the cytoplasmic and extra the extracellular side of the plasma membrane.

Two of the USH type 1 proteins, harmonin (USH1C) and SANS (scaffold protein containing ankyrin repeats and SAM domain, USH1G) are scaffold proteins. More recently a defect in a third scaffold protein, whirlin encoded by *DFNB31/WHRN* gene was identified as a cause for USH2 (Ebermann *et al.* 2007). All three USH scaffold proteins are composed of modules of protein-protein interaction domains (Adato *et al.* 2005, Maerker *et al.* 2008). SANS consists of a N-terminal domain of ankyrin repeats and a C-terminal sterile alpha motif

(SAM). Although, the central domain of SANS does not contain specialized protein binding motifs, it has been shown to be responsible for dimerization. Harmonin and whirlin are mainly characterized by PDZ domains, well known for their protein binding capacity in the proteins in which they were first identified and subsequently named as PSD95 (post-synaptic density protein 95), Disc large adhesion protein of *Drosophila* and the zonula oculans complex protein Zo-1. While a limited number of whirlin splice variants have been identified, *USH1C* is alternatively spliced to at least 12 different harmonin isoforms, grouped into the three groups a, b, and c (Figure 4.1).

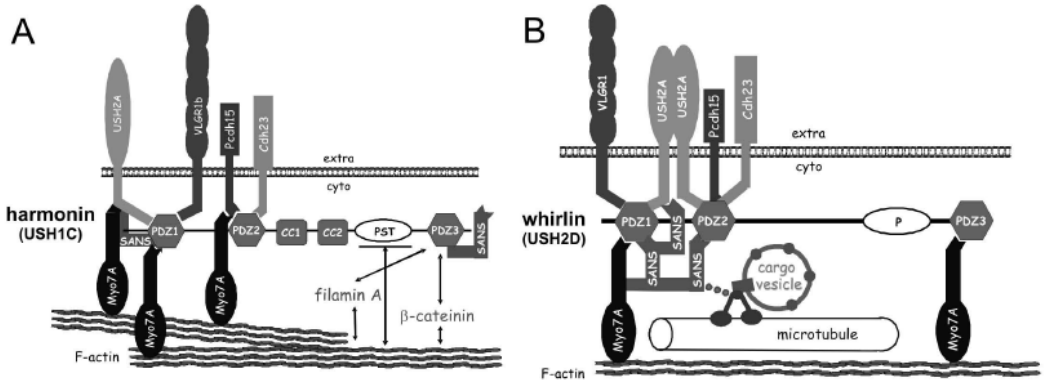

Figure 4.2. Schemata of protein networks organized by USH scaffold proteins. A. Harmonin (USH1C) protein network. B. Whirlin (US2D) – SANS (USH1G) protein network. "cyto" indicates the cytoplasmic and extra the extracellular side of the plasma membrane.

All other USH proteins are transmembrane proteins. Cadherin 23/Cdh23 (USH1D) and protocadherin 15/Pcdh15 (USH1F) are non-classical members of the huge cadherin super-family. They undergo frequent alternative splicing (Cdh23: isoforms a, b; Pcdh15: CD1 to CD4). Most variants are composed of a short cytoplasmic tail, a transmembrane domain and a much longer extracellular part characterized by Ca^{2+} binding cadherin domains suitable for dimerization and the formation of membrane-membrane adhesion (recently reviewed by Muller 2008, Overlack *et al.* 2010). *USH2A* encodes three known isoforms, the extracellular matrix protein usherin (Eudy *et al.* 1998) and two transmembrane protein USH2a isoform B and A (van Wijk *et al.* 2004). The gene for USH2C encodes an orphan adhesion G-protein coupled receptor GPR98/VLGR1b (very large G-protein coupled receptor 1b), the largest seven transmembrane receptor in the human body. Both of the transmembrane USH2 proteins USH2a and GPR98/VLGR1b possess extraordinary long extracellular domains which are suitable for protein binding and/or glycosylation. The membrane protein clarin-1 (USH3A), having four transmembrane domains belongs to the vertebrate specific clarin family of tetraspanine proteins and is so far the only member of USH3 which has been identified (Adato *et al.* 2002). This striking diversity of USH proteins raises the question why defects in such a heterogeneous group of proteins lead to similar clinical phenotypes, particularly amongst the group of USH1 and USH2 proteins.

4.3. USH PROTEIN NETWORKS AND THE INTERACTOME RELATED TO THE USHER SYNDROME

Protein-protein interaction assays revealed that all USH1 and USH2 proteins bind to the USH scaffold proteins, harmonin, whirlin, and SANS (reviewed in Reiners *et al.* 2006, Kremer *et al.* 2006, Maerker *et al.* 2008) (Figure 4.2). These interactions certainly provide the molecular links between the diverse USH proteins, and they also provide a base for the integration of other proteins into a protein interactome related to USH (Reiners *et al.* 2006) (Figure 4.3).

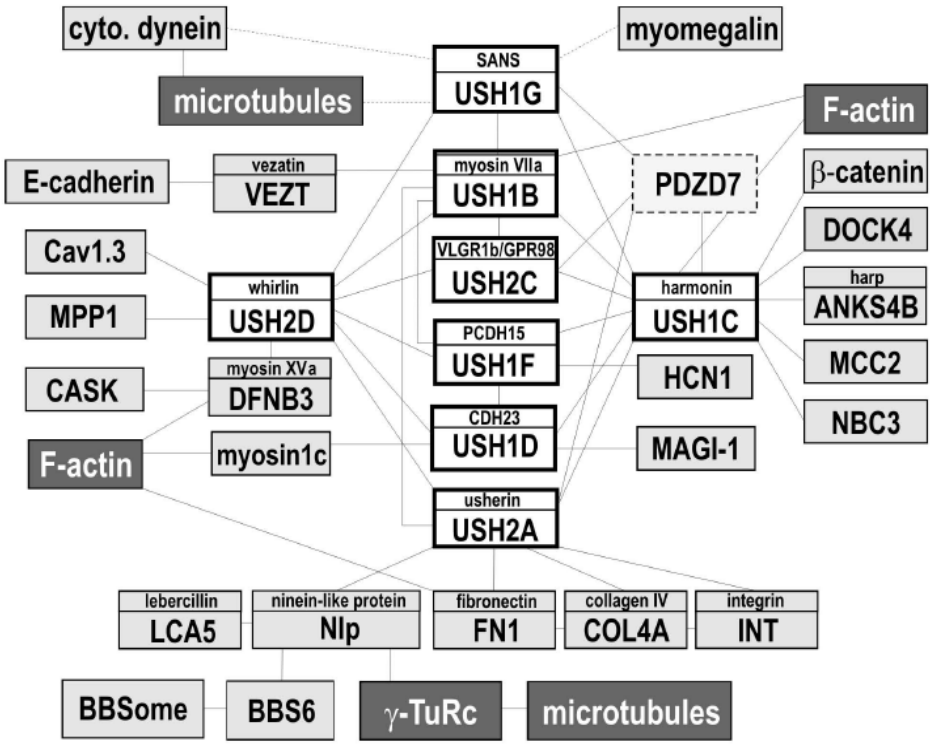

Figure 4.3. The protein interactome related to human USH. USH proteins are indicated by thick black boxes; PDZD7 is indicated as an identified USH modifier by dashed lines; cytoskeleton components are shown in dark grey boxes; other interaction partners in light grey. Confirmed interaction partners (by two or more independent methods) are indicated by solid lines; putative associations by dotted lines. Details of the interactome are found in the text and in references indicated there.

4.3.1. USH Scaffold Proteins Harmonin, Whirlin, and SANS Integrate USH1/2 Proteins in Protein Networks

In the following paragraphs I briefly describe the protein networks based on the three USH scaffold proteins harmonin, whirlin, and SANS illustrated in Figure 4.2. Homomeric interaction of the harmonin isoforms mediated by the binding of C-terminal PDZ binding motifs (PBM) and/or coiled-coil domains with two or three PDZ domains organizes a

harmonin net (Reiners *et al.* 2005a, 2005b, 2006, and unpublished results). This harmonin net provides the oligomeric scaffold for the binding of the USH1 and USH2 proteins predominantly to the PDZ domains of harmonin (Figure 4.2A): the cytoplamic tails of USH2a isoform b and GPR98/VLGR1b bind to harmonin´s PDZ1 domain via their C-terminal PBM (Reiners *et al.* 2005b). The two USH cadherins, Cdh23 and Pcdh15 also possess C-terminal PBMs, which directly interact with the PDZ2 domain of harmonin (Boëda *et al.* 2002, Siemens *et al.* 2002, Adato *et al.* 2005, Reiners *et al.* 2005a). The exclusion of exon 68 in the splice variant Cdh23-68 introduces an internal PBM, which binds to PDZ1 (Siemens *et al.* 2002) and to the N-terminal domain of harmonin (Pan *et al.* 2009). Myosin VIIa interacts through its FERM2 domain to both the PDZ1 and PDZ2 domain of harmonin (Boëda *et al.* 2002). This interaction provides an indirect molecular connection to the actin cytoskeleton which is further strengthened by PDZ3 binding partners (Reiners *et al.* 2006). In addition, the PST domain in the long harmonin b isoforms binds to actin filaments inducing actin filament bundling (Boëda *et al.* 2002). Furthermore, the PDZ1 of harmonin synergistically binds both the SAM and the carboxyl PBM of SANS, locking the two scaffold proteins into a highly stable complex (Yan *et al.* 2010). *In vitro* studies also show that harmonin´s PDZ3 can directly interact with SANS (Adato *et al.* 2005)

The scaffold protein SANS is also integrated into a different USH protein network facilitated by the PDZ domain containing USH2d protein whirlin (Figure 4.2B). In this network, the USH2 proteins USH2a isoform b and GPR98/VLGR1b directly bind with their C-terminal PBMs to the PDZ1 of whirlin (van Wijk *et al.* 2006). The cytoplasmic tail of USH2a isoform b can additionally interact with the PDZ2 domain of whirlin, which also binds both USH cadherins, Cdh23 and Pcdh15 (Kremer *et al.* 2006). The unconventional myosin VIIa can interact with both PDZ1 and PDZ3 terminal domains of whirlin. By binding to whirlin's PDZ1/2 the USH1g protein SANS establishes a link of this protein network to the microtubule cytoskeleton and to the microtubule based vesicular transport systems (Adato *et al.* 2005, Maerker *et al.* 2008, Overlack *et al.* 2008, Sorusch *et al.* in prep.).

In conclusion, there is no doubt that the molecular interactions between the USH proteins are the molecular basis for the similarity of clinical phenotypes caused by defects in the diverse USH proteins. Defects of a single component of a USH protein network may lead to dysfunction of the entire network.

4.3.2. USH Is Linked to Diverse Cellular Processes and to other Hereditary Diseases

The interwoven interactions between the USH proteins also form the basis for the integration of non-USH proteins into the protein interactome related to USH (Reiners *et al.* 2006) (Figure 4.3). The molecular and cellular characteristics of the identified components of the USH interactome indicate that USH proteins participate in multiple cellular functions, such as molecular cargo transport, endocytosis, pH regulation, synapse organization as well as the establishment of cell adhesions and planar cell polarity or centriole function and ciliogenesis (Reiners *et al.* 2005b, Reiners *et al.* 2006, Gosens *et al.* 2007, Kremer *et al.* 2006, Maerker 2007, Lefèvre *et al.* 2008, van Wijk *et al.* 2008, Maerker *et al.* 2008, Sorusch *et al.* in prep; Bauß *et al.*, in prep).

Recent data demonstrate that the USH protein interactome branches to proteins known to be associated with other hereditary diseases. In particular links of USH proteins to other human ciliopathies including non-syndromic inner ear defects and isolated retinal dystrophies but also to kidney disease and syndromes like Bardet-Biedl syndrome (BBS) and Alport syndrome have been identified (Maerker 2007, van Wijk *et al.* 2008, 2009, in prep., Sorusch *et al.* in prep). Furthermore, genes encoding for proteins of the USH protein interactome are excellent candidates for genetic modifiers of the USH disease. Recently, *PDZD7* was identified as a modifier of the retinal phenotype of USH2A and USH2D (Ebermann *et al.*, 2010). The PDZ domain containing protein is a homolog of harmonin and whirlin. PDZD7 protein directly interacts with USH proteins, namely SANS, harmonin, USH2a isoform b and GPR98/VLGR1b (Schneider *et al.* 2009, Ebermann *et al.* 2010). In USH patients, an additional mutation in *PDZD7* increases the severity of *RP* and leads to an earlier onset of disease (Ebermann *et al.* 2010). Completion of the characterization of the USH interactome will provide further insight in the complexity of the USH disease and is imperative for the understanding of the disease and thereby for generation of rational treatments.

4.4. USHER PROTEIN NETWORK FUNCTIONS IN SENSORY ORGANS AFFECTED BY THE SYNDROME

As outlined in the introduction, mutations in USH genes mainly lead to dysfunction of the inner ear and the eye. However, there is some controversy whether the USH disease has consequences for olfaction (Zrada *et al.* 1996, Seeliger *et al.* 1999). Nevertheless, preliminary data indicate that all USH1 and USH2 proteins are expressed in the olfactory cells of the olfactory epithelium of mammals (Wolfrum *et al.* 1998, Mikosz 2005, Mikosz *et al.* unpublished results). However, further analyses are required to elucidate the role of USH proteins in olfactory cells. In the ear and the eye, USH protein networks seem to be mainly implicated in the mechanosensitive sensory cells in the auditory and vestibular hair cells and photoreceptor cells, respectively (Figures 4.4, Figure 4.5).

4.4.1. Usher Protein Network Function in the Inner Ear

In the inner ear, USH protein networks are essential for the development of stereocilia during hair cell differentiation (Reiners *et al.* 2006, Kremer *et al.* 2006). In differentiated hair cells, USH proteins are thought to participate in synaptic function and serve in mechanoelectric signal transduction (Gillespie and Muller 2009).

In mechanosensitive hair cells, the conversion from incoming mechanical stimuli into the intrinsic electrical signals is based on the deflection of the hair bundles composed of stereocilia, which insert at the apical surface of each hair cell (Gillespie and Muller 2009). Stereocilia are actually highly specialized microvilli, stereovilli, characterized by a rigid actin filament core anchored into the actin filament meshwork of the cuticular plate (Figure 4.4). Rows of stereovilli are arranged in a staircase array with the tallest row of stereovilli adjacent to a single kinocilium. This microtubule based “real” cilium does not participate in the mechanotransduction process but is essential for the organization of the stereovilli during hair cell differentiation. In mammals, it disappears during maturation of cochlear hair cells. In hair

cells of USH1 deficient mice, the position of the kinocilium and thereby, the spatial orientation of the hair bundles is altered (Lefèvre *et al.* 2008). The plasma membranes of adjacent stereovilli and the membrane of the kinocilium are connected by a number of extracellular linker filaments (Figure 4.4) (Muller 2008, Overlack *et al.* 2010). Transient ankle links, lateral links and kinociliary links are thought to assist the shape of differentiating hair bundles while mature hair cells possess horizontal top connectors and tip links. The tip links are physically connected to the mechanotransduction cation channels. Changes in the tip link tension, introduced by mechanically induced deflection of the hair bundle in the direction of the longest stereovillus, increases the open probability of mechanotransduction channels localized towards the stereovilli tips (Gillespie and Muller 2009).

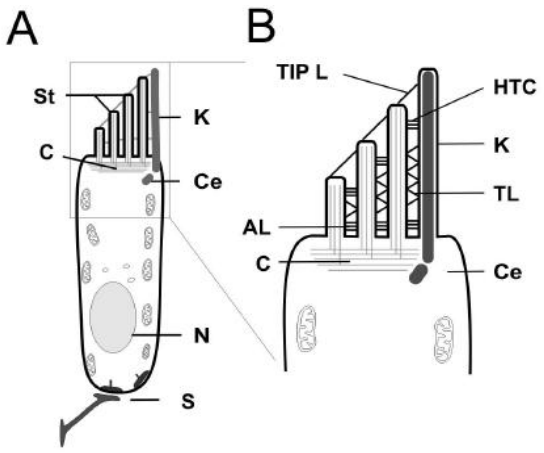

Figure 4.4. Schematic representation of an inner ear hair cell. A. The mechanotransduction in hair cells takes place at the stereovilli (= stereocilia, St), at the apical part of the hair cell. St are rigid microvilli-like structures that are organized in a staircase like manner of decreasing height. C: cuticular plate; Ce centriole; K: kinocilium; N: nucleus; S: ribbon synapse. B. The apical region of an inner ear hair cell where mechanotransduction takes place. Numerous links, interconnecting the growing stereovilli: transient links (TL), ankle links (AL) and tip links (TIP L). During maturation transient links and the kinocilium (K) disappear in mammalian cochlear hair cells; horizontal top connectors (HTC) and tip links persist.

4.4.1.1. During Hair Cell Development USH Proteins are Essential for the Formation of Transient Interstereovilli Links

Intensive analyses of inner ear hair cells in USH animal models revealed an essential molecular function of USH proteins and their interacting partners in the formation, maintenance and function of all types of interstereovilli links described above (El-Amraoui and Petit 2005, Muller 2008, Gillespie and Muller 2009). Scanning electron microscopy revealed disorganization and disorientation of the hair bundle stereovilli at the surface of hair cells in all USH mouse models analyzed to date indicating deficits in the cohesion between adjacent stereovilli during inner ear development (El-Amraoui and Petit 2005, Williams 2008, Geller *et al.* 2009, Caberlotto, E., Foucher I, Michel, V. *et al.* 2009, Mechanoelectrical transduction current characteristics and morphogenetic phenotype in USH1 mouse mutants. *7th Molecular Biology of Hearing and Deafness* 119). These findings are supported by results obtained in zebrafish mutants of ortholog USH genes showing disorganized hair bundles on the hair cells in the lateral line system (reviewed in El-Amraoui and Petit 2005). Studies

analyzing the localization of USH proteins in stereovilli during hair cell development in mutant and wild type USH animal models have led to the following hypothesis (El-Amraoui and Petit 2005, McGee *et al.* 2006, Michalski *et al.* 2007, Muller 2008, Lefèvre *et al.* 2008): the transmembrane USH proteins Cdh23, Pcdh15, USH2a and GRP98/VLRG1 are components of the transient interstereovilli links forming the fiber core by their extracellular domains. The cytoplasmic tails of all four transmembrane molecules are anchored by the USH scaffold proteins harmonin and whirlin as well as myosin VIIa to the differentiating actin filament core of the stereovilli. Based on the mislocalization of interacting USH network partners in myosin VIIa deficient *shaker-1* mice, myosin VIIa was suggested as the molecular motor essential for the transport of components of the diverse linker complexes to their indented destination (Boëda *et al.* 2002, Michalski *et al.* 2007). Furthermore, in hair cells of USH1 deficient mice the position of the kinocilium and thereby the spatial orientation of the hair bundle is dependent on the links between stereovilli and kinocilium (Lefèvre *et al.* 2008). The absence or dysfunction of any of the USH proteins from the transient linker complexes leads to the deficit of the transient linker filaments and thereby to disorganized hair bundle stereovilli and hair cell dysfunction resulting in the auditory and vestibular USH phenotype.

4.4.1.2. USH Cadherins are Core Components of the Mechanotransductive Tip-Link

The USH cadherins Cdh23 and Pcdh15 are not only part of the transient lateral links and kinociliary links but also compose the tip-links which are directly involved in the mechanotransduction (Muller 2008, Gillespie and Muller 2009). Recent studies provide strong evidence that the tip-link filament is composed of a Cdh23 homodimer interacting in trans-orientation with a Pcdh15 homodimer (Kazmierczak *et al.* 2007 and citations in Muller 2008). At both ends of the asymmetric tip-link filament the cytoplasmic tails of both USH cadherin homodimers are fixed within anchoring complexes at the adjacent stereovilli. USH scaffold proteins are present in both the anchoring complex of the upper tip-link density and of the lower tip-link density of the stereovilli. In the upper tip-link density complex the USH1c protein harmonin anchors the Cdh23 dimer. Recent studies on harmonin deficient *deaf circler* mice indicated that the long harmonin b isoform participates in slow adaptation of the hair cells (Grillet *et al.* 2009, Michalski *et al.* 2009), a process functionally linked to the upper tip-link density complex (Gillespie and Muller 2009). The lower tip-link density comprises besides the mechanosensitive cation channel and different unconventional myosins (III and XV) the USH2d scaffold protein whirlin. It is worth while to speculate that whirlin may anchor the tip-links in the lower tip-link density by interacting with the C-terminals tails of Pcdh15 previously demonstrated in *in vitro* binding assays (Kremer *et al.* 2006). From these data it is evident that USH proteins participate in the mechanoelectrical signal transduction suggesting that defects in USH proteins also compromise the process of signal transduction.

4.4.1.3. The Role of the USH Protein Network at Hair Cell Ribbon Synapses is Unknown

Although USH proteins have been frequently found at the ribbon synapse of hair cells (Reiners *et al.* 2005b, Reiners *et al.* 2006, van Wijk *et al.* 2006, Kremer *et al.* 2006) little is known about their function in this cellular compartment. Interestingly, USH proteins are not restricted to the pre-synaptic differentiations but are also found in post-synaptic boutons of

the dendritic processes of auditory neurons (Reiners *et al.* 2005b). It has been suggested that the transmembrane proteins may form trans-synaptic filaments out of their large extracellular domains projecting from the pre-synapse membrane through the synaptic cleft to the post-synapse specialization (Reiners *et al.* 2006, Overlack *et al.* 2010). Nevertheless, future comprehensive analyses are necessary for important insights into the function of USH protein networks at the ribbon synapses.

Clearly mutations in USH genes cause inner ear defects that occur during prenatal development. USH protein complexes establish cohesion between the stereovilli and the kinocilium essential for the correct hair bundle morphogenesis during the development of hair cells. Defects in USH proteins cause hair bundle disorganization with prenatal onset, which is manifested in existing USH mouse models, and also reflects the time course of the USH disease in human patients.

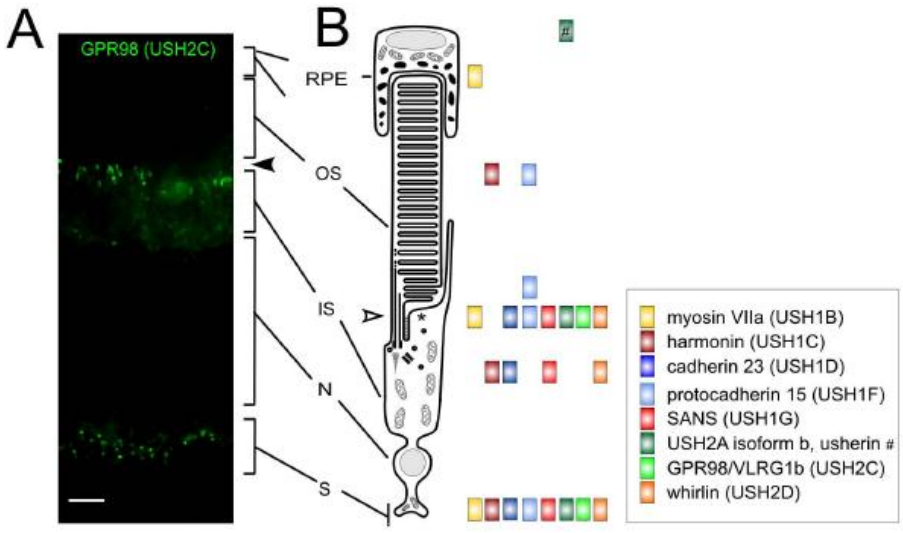

Figure 4.5. Subcellular localization of USH proteins in retinal photoreceptor cells. A. Indirect immunofluorescence of anti-GRP98/VLGR1b in the photoreceptor cell layer of a mouse retina. B. Schema representing a vertebrate rod photoreceptor cell. Vertebrate photoreceptor cells are composed of distinct morphological and functional compartments. Photosensitive outer segment (OS) is connected with the biosynthetic active inner segment (IS) via the connecting cilium (*arrowhead*). At the ciliary base the basal body and the adjacent centriole are present. The proximal OS and the connecting cilium are enclosed by the periciliary specialization of the apical IS (*asterisk*). Calycal processes extend from the apical IS and project parallel to the OS. Ribbon synapses (S) link photoreceptor cells with bipolar and horizontal cells. N: nucleus; RPE: retinal pigment epithelium. Subcellular localization of USH proteins in the diverse compartments of photoreceptor cells is indicated by color code.

4.4.2. Usher Protein Network Function in the Retina

The vertebrate retina is organized into well defined layers composed of specific retinal cells or their cellular compartments. With the exception of the USH3A protein clarin-1 all known USH proteins are expressed in the photoreceptor layer of the retina (Reiners *et al.* 2006, van Wijk *et al.* 2006, Geller *et al.* 2009). Studies examining the subcellular localization of USH1 and USH2 proteins in photoreceptor cells have shown that USH protein networks

are organized in distinct cellular compartments (Figure 4.5). The USH1b protein myosin VIIa is the only USH protein with a confirmed expression in the retinal pigment epithelium (RPE). In RPE cells myosin VIIa serves in melanosome translocation and participates in the phagocytosis of photoreceptor outer segment membranes (Williams and Gibbs 2004).

Vertebrate photoreceptor cells are highly polarized sensory neurons, which consist of morphological and functional distinct cellular compartments (see Figure 4.5A). The light sensitive outer segment is a highly specialized modified cilium. It is characterized by stacked membrane disks, which contain all components of the visual transduction cascade.

Throughout the lifetime of these cells the membranes of the outer segment disks are continually renewed (Young 1976). Aged disk stacks at the outer segment tips are phagocytozed by cells of the apposing RPE, whereas the disks synthesized *de novo* are added at the base of the outer segment. This continuous membrane turnover requires a high rate of biosynthesis, which is accomplished by the organelles localized in the inner segment. Newly synthesized outer segment components are transported from these organelles with biosynthetic activity through the inner segment and across the connecting cilium to the outer segment (Besharse and Horst 1990, Roepman and Wolfrum 2007). At the base of the connecting cilium, cargo destined for the outer segment are transferred from the inner segment transport machinery to the ciliary transport complexes localized at a specialized compartment of the apical inner segment, the so-called periciliary ridge complex (Papermaster 2002, Roepman and Wolfrum 2007). The periciliary ridge complex was first described in frogs (e.g. Peters *et al.* 1983). More recently a homologous region with a matching molecular complexity was identified in mammalian photoreceptor cells (Liu *et al.* 2007, Maerker *et al.* 2008, Nagel-Wolfrum, K., Goldmann, T., Sehn, E., Stern-Schneider, G., Overlack, N., Wolfrum, U. 2009, Subcellular localization of proteins related to the Usher syndrome in human and primate retinas. *Invest. Ophthalmol. Vis. Sci.* **50**, E-Abstract 2718; Wolfrum, U., Goldmann, T., Overlack, N., Müller, C., Vetter, J. M., Nagel-Wolfrum, K. 2010, Subcellular localization of Usher syndrome proteins in the human retina. *Invest. Ophthalmol. Vis. Sci.* **51**, E-Abstract 2494).

The apical inner segment membrane is also the source of slender membrane appendages, the so called calycal processes, which are arranged like the calyx of a flower around the outer segment and project alongside the outer segment to the RPE (Cohen 1963, Nagle *et al.* 1986, Dose *et al.* 2004). The calycal processes are derived from the stable cytoskeletal actin filament core and have been suggested to stabilize the outer segments by keeping the disk stacks in shape and protecting them against mechanical forces. Within the vertebrates there are notable interspecies differences in the number of the calycal processes, which seem to correlate with the diameter and length of the outer segment. This is in contrast to rodents which possess only a single calycal process, whereas amphibians have ~ 20 and primates have up to 8 calycal processes.

The basal inner segment extends into the perikaryon and the synaptic region where the electrical signal generated in the photoreceptors is transmitted to bipolar and horizontal cells of the inner retina.

4.4.2.1. The USH Phenotypes Differ between Human and Mouse

Unlike the inner ear, where defects of USH mouse models are analogous to those present in human USH patients, the retinal phenotype of *RP* as seen in human USH is absent in rodent models of USH (Williams 2008). The reason of this discrepancy between human and

rodents is unclear and possible explanations range from a functional molecular redundancy in the rodent retina or structural differences between human and rodent photoreceptor cells to the relatively slow progression of the disease which may not fully develop the retinal phenotype within the short lifespan of rodents (Reiners *et al.* 2006, Williams 2008). Although mild retinal phenotypes have been reported in some USH1 rodent models (Johnson *et al.* 2003, Williams 2008, Lentz *et al.* 2010), only recently a clear visual phenotype has been identified in USH2 mouse models, namely in USH2A and whirlin null mice (Liu *et al.* 2007, Yang *et al.* 2010). Consequently, the lack of mouse models has hampered comprehensive functional analyses of USH proteins and their related interactome in the retina of a mammalian model.

4.4.2.2. USH Proteins are Found in the Pre- and Post-Synapse of Retinal Ribbon Synapses

As in hair cells there is evidence for the presence of a USH protein network at the synaptic contacts between photoreceptor cells and secondary retinal neurons (Reiners *et al.* 2006, Kremer *et al.* 2006, Jacobson *et al.* 2008) (see also Figure 4.5). It is noteworthy that the ribbon synapse, specialized for sustained neurotransmitter release (Wagner 1997), is one of the major similarities between mechanosensitive hair cells and photoreceptor cells. Although other studies failed to confirm these data (Williams 2008), we have recently observed USH proteins in the outer plexiform layer of primates in further support of the findings obtained in rodents (Reiners *et al.* 2006, Nagel-Wolfrum, K., Goldmann, T., Sehn, E., Stern-Schneider, G., Overlack, N., Wolfrum, U. 2009, Subcellular localization of proteins related to the Usher syndrome in human and primate retinas. *Invest. Ophthalmol. Vis. Sci.* **50**, E-Abstract 2718; Wolfrum, U., Goldmann, T., Overlack, N., Müller, C., Vetter, J. M., Nagel-Wolfrum, K. 2010, Subcellular localization of Usher syndrome proteins in the human retina. *Invest. Ophthalmol. Vis. Sci.* **51**, E-Abstract 2494). In particular, network components integrated by harmonin are enriched in the synaptic pedicles of cone photoreceptors. Other USH proteins like GPR98/VLGR1b are clearly anchored with its cytoplasmic tail in the post-synaptic bouton of bipolar cells (Reiners *et al.* 2005b, Specht *et al.* 2009). Ongoing studies may provide further insights into the role of USH1 and USH2 proteins in synaptic function in the retina and inner ear.

4.4.2.3. Harmonin, a Scaffold for a Protein Network in the Rod Outer Segment?

At the other pole of the cell, the USH1c scaffold protein harmonin was also found to be localized in the photosensitive outer segment of rodents (Reiners *et al.* 2003). Recently, localization of harmonin in the outer segments of rods but not in cones has been confirmed in human and Macaque retinae (Nagel-Wolfrum, K., Goldmann, T., Sehn, E., Stern-Schneider, G., Overlack, N., Wolfrum, U. 2009, Subcellular localization of proteins related to the Usher syndrome in human and primate retinas. *Invest. Ophthalmol. Vis. Sci.* **50**, E-Abstract 2718; Wolfrum, U., Goldmann, T., Overlack, N., Müller, C., Vetter, J. M., Nagel-Wolfrum, K. 2010, Subcellular localization of Usher syndrome proteins in the human retina. *Invest. Ophthalmol. Vis. Sci.* **51**, E-Abstract 2494). These findings were affirmed by the analyses of subcellular fractionations of retinal tissue as well as horizontal sections through the retina (Reiners *et al.* 2003). In addition, these studies have indicated that the presence of the long b isoforms of harmonin is restricted to the outer segment compartment. It is noteworthy that

components of the signal transduction machinery are often organized by scaffold proteins. Examples include the mechanotransduction in the hair cells as discussed above and also the *Drosophila* INAD protein, which gathers signaling molecules into a transducisom in the rhabdomeric photoreceptor cells of invertebrates (Zuker and Ranganathan 1999). Although harmonin is so far the only PDZ containing scaffold protein identified in the signal transductive outer segment compartment of vertebrate photoreceptor cells, no interaction of harmonin with any molecule of the visual signal transduction has been demonstrated so far. Direct binding of Pcdh15 to PDZ2 domain of harmonin and the co-expression of both interaction partners in the outer segment makes it feasible that Pcdh15 anchors harmonin molecules at outer segment membranes (Reiners *et al.* 2005a).

4.4.2.4. Protocadherin Relations at the Outer Segment Region of Disk Neogenesis

However, recent data shows that Pcdh15 is more prominent at the apical membrane of the inner segment, exactly where the membrane attaches to the rim of the newly formed membrane disks of the outer segment (Overlack *et al.* 2010). It has been speculated that Pcdh15 interacts with the photoreceptor cell specific protocadherin Pcdh21, which has been localized to these rims (Rattner *et al.* 2001). The fragile newly formed disks may be stabilized by resulting heteromeric asymmetric adhesion fibers fixing the rim at the adjacent inner segment membrane. Whirlin may anchor Pcdh15 in the apical inner segment by direct interaction with the Pcdh15 C-terminal PBM (Kremer *et al.* 2006, Overlack *et al.* 2010).

4.4.2.5. The Ciliary-Periciliary USH Protein Network Channels the Cargo Transport to the Outer Segment

A large number of USH1 and USH2 proteins appear to be co-localized in the ciliary and periciliary region of photoreceptor cells (Figure 4.5). In the absence of harmonin, whirlin and SANS serve as the core scaffold proteins of this USH protein network (van Wijk *et al.* 2006, Maerker *et al.* 2008, Yang *et al.* 2010).

Myosin VIIa was the first USH protein found in the connecting cilium of rod and cone photoreceptor cells (Liu *et al.* 1997). The abnormal accumulation of opsin in the connecting cilium of myosin VIIa deficient *shaker-1* mice indicates that myosin VIIa participates in the transport of opsin across the connecting cilium (Liu *et al.* 1999, Wolfrum and Schmitt 2000). The large number of identified myosin VIIa binding partners include the USH proteins whirlin, SANS and USH2a, which are also found in the connecting cilium (van Wijk *et al.* 2006, Maerker *et al.* 2008). These proteins may support function of myosin VIIa in the ciliary transport.

In the periciliary region of mammalian photoreceptor cells the transmembrane USH proteins, USH2a isoform b and GPR98/VLGR1b, and the scaffold proteins whirlin and SANS are localized in the collar-like extension of the apical inner segment which wraps the connecting cilium (Liu et al 2007, Maerker et al 2008, and Yang *et al.* 2010). Ultra-structural analyses have demonstrated that fibrous links connect the membrane of the apical inner segment with the adjacent membrane of the connecting cilium (e.g. Maerker *et al.* 2008). Immunoelectron microscopic decoration of these fibers by antibodies against the extracellular domain of GPR98/VLGR1b in wild type and the absence of these fibers in VLGR1/del/TM mice have shown that the extraordinarily long extracellular domain of GPR98/VLGR1b forms a core component of these fibrous links. Studies on the subcellular localization of

USH2a also reveal its presence at the periciliary membrane (Liu *et al.* 2007, Maerker *et al.* 2008) suggesting that the extracellular domain of USH2a isoform b also is part of these fibrous links. Based on these results it is evident that the ciliary-periciliary membrane adhesion of photoreceptor cells are analogous to the ankle links connecting neighboring stereovilli in the developing hair bundle of inner ear hair cells described above.

As in the ankle link complex of hair cells the cytoplasmic tails of GPR98/VLGR1 and USH2a isoform b are anchored by whirlin in the cytoplasma of both the cilium and the periciliary collar-like inner segment extension of photoreceptor cells (Maerker *et al.* 2008). This hypothesis is strengthened in earlier reports (Yang, J., Liu, X., Zhao, Y., Adamian, M., Pawlyk, B., Li, T., 2006, Subcellular localization of whirlin and its interaction with Ush2a in the photoreceptors. *Invest Ophthalmol Vis Sci* **47**, E-Abstract 2848 and Yang, J., Liu, X., Adamian, M. *et al.* 2008, N-terminal mutation of whirlin disrupts the Usher syndrome II (USH2) protein complex and causes retinal and inner ear defects in mice. *Invest Ophthalmol Vis Sci* **49**, E-Abstract 4405) and recently published data (Yang *et al.* 2010) confirming that the periciliary localization of both transmembrane proteins, GPR98/VLGR1b and USH2a, is whirlin dependent. The presence of SANS which directly binds whirlin and USH2a completes the USH proteins in the ciliary/periciliary USH protein network. SANS further provides links to the microtubule cytoskeleton and microtubule associated transport.

Comparative molecular analysis of mammalian and *Xenopus* photoreceptor cells showed that the mammalian periciliary USH protein network is also part of the periciliary ridge complex in frogs (Maerker *et al.* 2008). The periciliary ridge complex is thought to define the target membrane for docking and fusion of transport vesicles containing outer segment cargo. This conclusion has driven the hypothesis that the periciliary USH protein network facilitates the transport of vesicles after their passage from the Golgi apparatus through the inner segment to its apical membrane. There is growing evidence that SANS connects the post-Golgi vesicles to microtubule transport routes and the cytoplasmic dynein motor complex which drives the inner segmental transport in photoreceptor cells (Tai *et al.* 1999). It is worth while to speculate that the binding of SANS to whirlin triggers the release of the cargo form the transport system of the apical inner segment, and thereby, facilitates the vesicle docking and fusion at the periciliary target zone of the apical inner segment. By this, the cargo is placed in a fine spatial initial position for subsequent transport across the connecting cilium to its destination in the outer segment.

The spatial organization and its putative function in ciliary transport places the periciliary USH protein network right next to the protein complexes and networks known to be essential for ciliary transport, namely the BBSome, mainly composed of molecules related to Bardet-Biedl syndrome (BBS) (Nachury *et al.* 2007) and the intra-flagellar transport (IFT) system recently analyzed in photoreceptor cells (Sedmak and Wolfrum 2010, and included citation). Prospective further characterization of the USH protein interactome will certainly provide insights into its relation to the processes related to the BBSome (van Wijk, E., Kersten, F. F. J., Zaghloul, N. *et al.* (2008) A centrosomal protein molecularly links Usher syndrome to Leber congenital amaurosis and Bardet-Biedl Syndrome in the Retina. *Invest. Ophthalmol. Vis. Sci.* **49**, E-Abstract 4033) and the sub-complexes of the IFT system in photoreceptor cells. Furthermore, it will be of importance to elucidate the interplay with circuit of small Rab GTPases known for the regulation of the intracellular transport in photoreceptor cells (Deretic 2006).

Consistent with the observation of abnormal cilia described in the photoreceptor cells of USH2 patients (Barrong *et al.* 1992) the prominent ciliary-periciliary USH protein network classifies USH as a ciliopathy. Although USH lacks a number of systemic features associated with ciliopathies, abnormalities related to ciliary defects in non-ocular tissues of USH patients (Arden and Fox 1979, Hunter *et al.* 1986, Bonneau *et al.* 1993, Baris *et al.* 1994, Zrada *et al.*1996) and the expression of USH proteins in a variety of ciliated cells (Wolfrum *et al.* 1998, Reiners 2004) is consistent with a general ciliary function of USH related molecules.

4.4.2.6. The USH Protein Network in Calycal Processes May Explain the Discrepancy in the Retinal USH Phenotype between Human and Mice

Calycal processes are another specialization of the apical inner segment. Differences between the number of the calycal processes in humans and rodents have previously been suggested to underlie the incoherent retinal USH phenotypes of human patients and USH mouse models (Petit 2001, Reiners *et al.* 2006). So far this hypothesis had lacked any molecular basis.

Only recently our laboratory has demonstrated that the USH1 protein SANS as well as the USH2 proteins USH2a, VLGR1b/GPR98 and whirlin, are indeed expressed in calycal processes (Maerker 2007, Nagel-Wolfrum, K., Goldmann, T., Sehn, E., Stern-Schneider, G., Overlack, N., Wolfrum, U. 2009, Subcellular localization of proteins related to the Usher syndrome in human and primate retinas. *Invest. Ophthalmol. Vis. Sci.* **50**, E-Abstract 2718; Wolfrum, U., Goldmann, T., Overlack, N., Müller, C., Vetter, J. M., Nagel-Wolfrum, K. 2010, Subcellular localization of Usher syndrome proteins in the human retina. *Invest. Ophthalmol. Vis. Sci.* **51**, E-Abstract 2494). The scaffold complex of SANS and whirlin may anchor USH2a and VLGR1b/GPR98 in the cytoplasm of the calycal processes. The extracellular domains of both transmembrane proteins may form fibrous links between the adjacent membranes of the calycal process and the outer segment. This is supported by electron microscopy data indicating that the plasma membranes of the outer segment and of the calycal processes are connected by extracellular fibers. However, in comparison to the interstereovilli links and the ciliary-periciliary adhesion complex, the distance between the plasma membranes of the outer segment and the calycal processes is relatively short. To date no protein counterparts, which interact with the extracellular domains of transmembrane proteins of calycal processes, was found in the outer segment membrane. However, it is possible that a putative membrane integrating domain present in the extracellular part of VLGR1/GPR98 directly binds to the plasma membrane of the outer segment. Interspecies comparative analysis revealed that the USH protein network in the calycal processes is not restricted to primates but also found in lower vertebrates indicating that it has been conserved within the vertebrates during evolution (Wolfrum *et al.* 2010). It seems likely that in species with higher numbers of calycal processes the dysfunction of the calycal processes could destabilize the structure of the photoreceptor outer segments.

4.5. SUMMARY AND CONCLUSION

Usher syndrome (USH), the most common form of combined hereditary deaf-blindness is a genetically and clinically heterogeneous disease. To date 12 identified genes are grouped

into three USH types based on slight differences in the phenotype and the progression of the symptoms. Mutations in these genes exhibit similar phenotypes in patients, namely hearing loss, balance impairments and retinal degeneration. Analyses of the corresponding gene products surprisingly revealed that USH proteins belong to diverse protein classes and families. Molecular analyses of USH proteins demonstrated that all proteins are organized within complex protein networks present in the sensory cells of the inner ear and the eye. The main organizers of these networks are the three scaffold proteins harmonin (USH1C), SANS (USH1G) and whirlin (USH2D).

Localization studies of USH proteins indicated that USH protein networks are mainly present in stereovilli bundles of hair cells and the ciliary region of photoreceptor cells as well as at the synapses of both sensory cell types. Studies in mouse models of USH showed that the interplay between USH proteins is essential for the formation of fibrous links between the neighboring stereocilia and to the kinocilium of hair cells and thereby, for the precise organization of the hair bundle. Furthermore, recent data showed an association between USH proteins and the molecular machinery for mechanoelectrical signal transduction.

At the synapses of the hair cells and photoreceptor cells, homomeric and heteromeric interactions between harmonin isoforms may provide a platform for binding of all known USH1 and USH2 proteins mediated by PDZ domains of harmonin. Harmonin also connects the USH protein network to the actin cytoskeleton either directly via a PST domain and/or indirectly via binding to actin associated proteins.

In the ciliary-periciliary region of photoreceptor cells, a USH protein network is organized in the absence of harmonin by the two other scaffold proteins, SANS and whirlin. As in the stereovilli bundles of hair cells, the molecules of this periciliary network arrange fibrous links between adjacent membranes and thereby define the target membrane domain for transport vesicles. Furthermore, there is evidence for a role of the periciliary USH protein network in the regulation of the microtubule based cargo transport through the inner segment, the transfer of cargo to the ciliary transport machinery and the subsequent cargo delivery to the photoreceptor outer segment.

Dissection of the protein interactome related to USH has shed more light on the molecular and cellular function of USH proteins. Furthermore, our studies revealed molecular links to other diverse hereditary neuronal diseases and ciliopathies suggesting common cellular networks and pathophysiological pathways for USH and related diseases. Last but not the least, the molecular and functional characterization of the USH protein interactome identifies candidate genes for so far unknown USH genes as well as for genetic modifiers of USH genes and conclusively elucidates potential targets for treatments and cures.

In summary, USH is a complex disease. Nevertheless, the characterization of the USH proteins and their molecular interplay in diverse protein networks has elucidated the pathology underlying USH in the ear and the eye. This knowledge is imperative for the evaluation of therapeutic targets and strategies. The identified USH interactome outlined in the present review shows strong links to non-syndromic diseases of the peripheral visual and auditory systems as well as other systemic syndromes. Genes encoding USH protein interacting molecules are interesting candidate genes for so far unidentified USH genes and as well as genetic modifiers of the USH phenotype. Furthermore, the analyses of the molecules related to USH and their interplay in the protein networks described in the review also provide important insights for understanding the molecular function of the sensory cells of the inner ear and the eye.

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