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Scaffold protein harmonin (USH1C) provides molecular links between Usher syndrome type 1 and type 2

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Usher syndrome (USH) is the most frequent cause of combined deaf-blindness in man. USH is clinically and genetically heterogeneous with at least 11 chromosomal loci assigned to the three USH types (*USH1A-G*, *USH2A-C*, *USH3A*). Although the different USH types exhibit almost the same phenotype in human, the identified *USH* genes encode for proteins which belong to very different protein classes and families. We and others recently reported that the scaffold protein harmonin (*USH1C*-gene product) integrates all identified USH1 molecules in a USH1-protein network. Here, we investigated the relationship between the USH2 molecules and this USH1-protein network. We show a molecular interaction between the scaffold protein harmonin (USH1C) and the USH2A protein, VLGR1 (USH2C) and the candidate for USH2B, NBC3. We pinpoint these interactions to interactions between the PDZ1 domain of harmonin and the PDZ-binding motifs at the C-termini of the USH2 proteins and NBC3. We demonstrate that USH2A, VLGR1 and NBC3 are co-expressed with the USH1-protein harmonin in the synaptic terminals of both retinal photoreceptors and inner ear hair cells. In hair cells, these USH proteins are also localized in the signal uptaking stereocilia. Our data indicate that the USH2 proteins and NBC3 are further partners in the supramolecular USH-protein network in the retina and inner ear which shed new light on the function of USH2 proteins and the entire USH-protein network. These findings provide first evidence for a molecular linkage between the pathophysiology in USH1 and USH2. The organization of USH molecules in a mutual 'interactome' related to the disease can explain the common phenotype in USH.

INTRODUCTION

Usher syndrome (USH) is an autosomal recessive disorder characterized by combined hearing loss and retinal degeneration. Three USH types (USH1–USH3) are distinguished mainly on the basis of the severity and progression of the clinical symptoms (1–3). In patients with USH1, profound congenital deafness and vestibular dysfunction are combined with retinitis pigmentosa (RP). In USH2, the most frequent type, the congenital hearing loss is milder, the onset of RP is slightly later and vestibular function is normal. USH3 is relatively rare and characterized by progressive hearing loss with variable vestibular dysfunction (1–3).

The gene products of the identified USH genes belong to different protein classes and families. Known USH1 molecules are the molecular motor myosin VIIa (USH1B), the two

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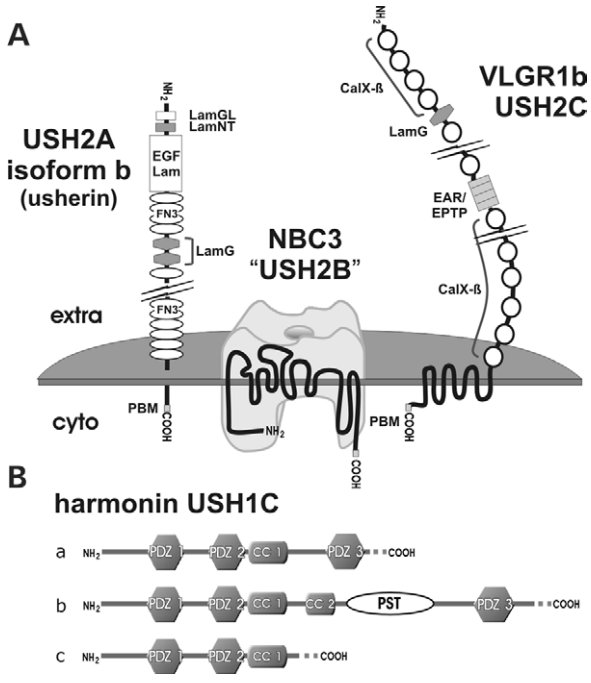

Figure 1. (**A**) Schematic representation of USH2A isoform b, VLGR1b and NBC3. All three proteins are transmembrane proteins containing N-terminal PBMs. Isoform b of the USH2A protein (previously also termed usherin) is composed of a short cytoplasmic tail, a transmembrane region and an extended extracellular part which contains the following characteristic extracellular domains: 32 fibronectin type III (FN3) repeats, two laminin G domains (LamG), 10 laminin EGF-like, one laminin N-terminal (LamNT) and one laminin G-like domain (LamGL). USH2A interacts via the extracellular laminin domains with the basement membrane protein collagen IV (39). The sodium-bicarbonate transporter NBC3, the candidate for USH2B, bears in addition to its 12 transmembrane regions and its C-terminal PBM no recognizable domains (16). The USH2C protein VLGR1b is a serpentine or seven-transmembrane protein, suitable to activate signaling cascades mediated by heterotrimeric G-proteins (15). VLGR1b has a very long extracellular N-terminal extension which contains potential protein-interaction domains, a laminin G domains (LamG) and a repeated set of EAR/EPTP motifs. The protein–protein interaction of these two domains is suggested to be mediated by 35 Ca^{2+}-binding CalX-β modules (15). (**B**) Schematic representation of harmonin (USH1C) isoforms. At least 11 isoforms can be divided into classes a–c (1,4). Harmonin a and b isoforms contain three PDZ (post-synaptic density 95, discs large, zonula occludens-1) domains. The longest class b isoforms contain also a second coiled-coil domain (CC2), and a proline, serine, threonine (PST)-rich region which binds to actin filaments (5). Class c, the shortest isoforms, lacks both the CC2 and the third PDZ domain.

cell–cell adhesion proteins, cadherin 23 (USH1D) and protocadherin 15 (Pcdh15, USH1F) and the scaffold proteins, harmonin (USH1C) and SANS (scaffold protein containing ankyrin repeats and SAM domain, USH1G). Molecular analysis of USH1 protein function revealed that all five USH1 proteins are integrated in a protein network by binding to protein-protein interaction domains, in particular to the PDZ domains of the USH1C-protein harmonin (Fig. 1B) (4–9). In the inner ear, these interactions are the basis for a USH1 network which is essential for the differentiation of hair cells and may participate in their signal transduction (5–12). In retinal photoreceptor cells, synaptic co-localization of all USH1 proteins suggests that these USH1-protein complexes exist at the synaptic terminals (4,9). USH2 comprises three subtypes USH2A–C. USH2A is caused by defects in USH2A, previously described as an extracellular matrix protein (13). More recently, we identified a novel USH2A isoform (isoform b) containing a transmembrane region and a short cytoplasmic part (Fig. 1A) (14). The gene defective in USH2C is *VLGR1* (very large G-protein coupled receptor 1), a member of the serpentine G-protein coupled receptor superfamily (Fig. 1A) (15). On the basis of the combined deaf-blindness phenotype of NBC3-deficient *Slc4a7*$^{-/-}$ mice, the sodium-bicarbonate co-transporter gene *NBC3* was suggested to be a candidate gene for USH2B (Fig. 1A) (16). Although USH1 and USH2 show clinical similarity, to date no relations between the molecular defects were reported.

In this study, we demonstrate the integration of USH2A, NBC3 and VLGR1b in a USH-protein network. We show that these three proteins bear a C-terminal PDZ-binding motif (PBM), which specifically binds to the PDZ1 domain of the USH1C-protein harmonin. Immunohistochemistry reveals the co-expression of the identified interaction partners of the USH-protein network mainly in the stereocilia of the inner ear hair cells as well as at the ribbon synapses of cochlear hair cells and retinal photoreceptor cells.

RESULTS

USH2 proteins and NBC3 are integrated into a USH-protein network via specific binding to the PDZ1 domain of harmonin

Recent studies indicated that USH1 proteins interact via class I PBMs with harmonin PDZ1 or PDZ2 domains (5–9). Sequence analysis revealed that a class I PBM is also present at the C-terminal ends of all three proteins, USH2A isoform b, VLGR1b and the candidate for USH2B, NBC3 (Figs 1A and 2A). We carried out *in vitro* and *in vivo* binding assays to test possible interactions between the USH2 proteins and NBC3 and harmonin. In glutatione S-transferase (GST)-pull down assays fusion proteins of the cytoplasmic regions of the USH2 proteins and NBC3 were found to bind to the most common harmonin isoform a1 (Fig. 2B). His-tagged harmonin was pulled down from bacterial lysates by all three GST-fused cytoplasmic tail fragments of USH2A, VLGR1b and NBC3 but not by GST alone. To confirm these interactions *in vivo*, COS-1 cells were co-transfected with plasmids encoding full-length HA-tagged harmonin a1 and the Flag-tagged cytoplasmic tail fragments of both USH2 proteins and NBC3 (Fig. 2C). For immunoprecipitation (IP) assays, extracts of these cells were incubated with anti-HA antibodies immobilized at sepharose beads. The tail fragments of USH2A isoform b, VLGR1b and NBC3 consistently co-immunoprecipitated with harmonin a1 (Fig. 2C). The absence of actin in the immunoprecipitates indicates the specificity of the assay (Fig. 2C, bottom panel). These results were further verified in yeast two-hybrid interaction assays (Fig. 2E).

In the next set of experiments, we pinpointed the interacting protein domains by using the separate PDZ domains of harmonin and fragments of the intracellular regions of the USH2 proteins and NBC3. GST-pull down and yeast two-hybrid assays revealed specific interactions between murine and human harmonin-PDZ1 and the cytoplasmic tails of USH2A

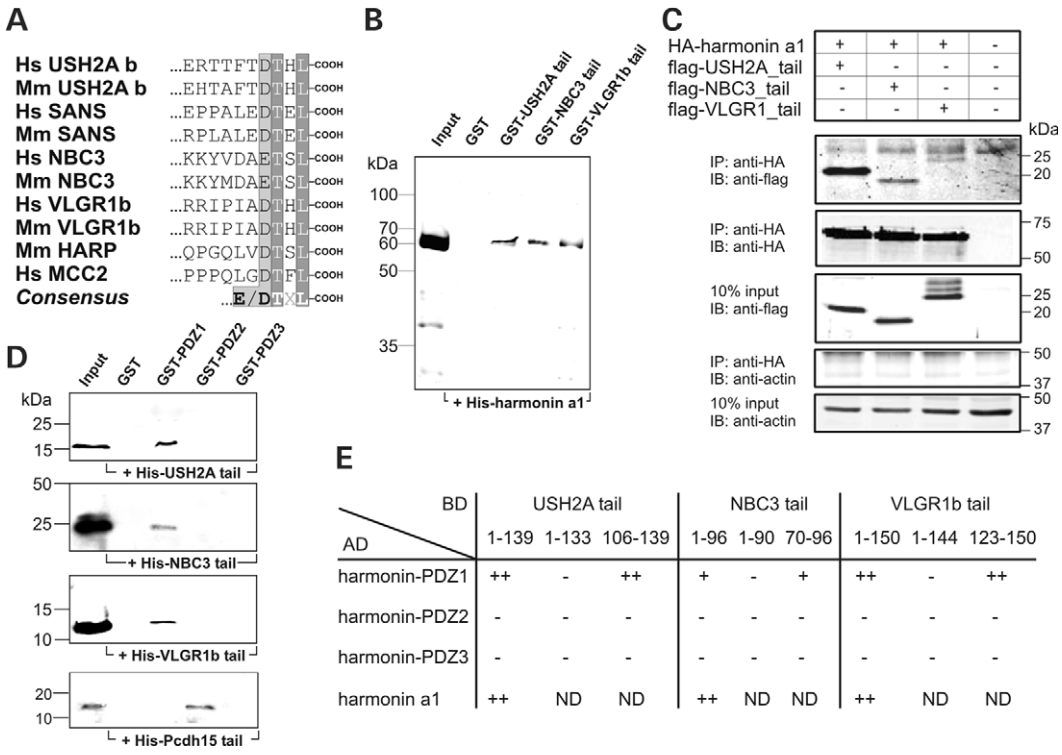

Figure 2. Harmonin interactions with USH2 proteins and NBC3. (**A**) Sequence alignment of the C-termini of human (Hs) and mouse (Mm) proteins, known to interact with PDZ1 of harmonin, shows the PBM consensus sequence -E/D-T-X-L-COOH. (**B**) His-tagged harmonin was pulled down by the GST-fused cytoplasmic tail fragments of USH2A, NBC3 and VLGR1b but not by unfused GST. (**C**) The top panel of the immunoblot (IB) shows that the cytoplasmic tails of all three USH2A, NBC3 and VLGR1b are co-immunoprecipitated with harmonin a1 (HA-harmonin a1, ~60 kDa, IP shown in the second panel, lanes 1–3) using a polyclonal antibody directed against the HA-epitope. Input material is shown in the third panel (3 × Flag-USH2a, ~20 kDa; 3 × Flag-NBC3 ~16 kDa; 3 × Flag-VLGR1, ~23 kDa). In the analysis of mock transfection (lane 4), no bands are visible. Actin detection was used to determine the specificity of the co-IP. Actin is not precipitated with anti-HA antibodies (fourth panel). (**D**) His-tagged tail fragments of USH2A, Nbc3, VLGR1b were pulled down by the GST-fused PDZ1 domain of murine harmonin but not by its PDZ2 or PDZ3 domain or unfused GST. In contrast, the cytoplasmic tail of Pcdh15 is pulled down with the GST-fused PDZ2 domain. (**E**) Yeast two-hybrid interaction assays were performed with full-length harmonin a1 and its separate PDZ domains fused to the GAL4 AD and different cytoplasmic fragments of USH2A, VLGR1b and NBC3, with or without C-terminal PBM, fused to the GAL4 DNA-binding domain (BD). Activation of the reporter genes, and thus interaction, was dependent on the presence of the PDZ1 domain of harmonin and the C-terminal PBM of USH2A, NBC3 or VLGR1b. Bait–prey swap experiments confirmed these results (data not shown). +, growth, ++, fast growth under selection conditions and α- and β-galactosidase production; −, no growth under selection conditions; ND, not determined.

isoform b, VLGR1b and NBC3, whereas the PDZ2 and PDZ3 domains of harmonin or GST alone did not interact (Fig. 2D and E). Bait–prey swap in the yeast two-hybrid assay did not prohibit the interactions (data not shown). The specificity of these interactions was further validated in control pull down assays with the cytoplasmic tail of Pcdh15 (USH1F) which also contains a C-terminal class I PBM, but binds to the PDZ2 domain of harmonin (Fig. 2D) (8,9).

The interactions with the PDZ1 domain are fully dependent on the presence of the C-terminal PBMs of USH2A, VLGR1b and NBC3 as determined in yeast two-hybrid assays in which all three PDZ domains of harmonin were expressed as fusion proteins with the GAL4 activation domain (AD) and used as bait. As prey, for both USH2 proteins and NBC3, the complete cytoplasmic tail and N- and C-terminally truncated fragments were expressed as fusion proteins with the GAL4 DNA-binding domain. Growth of the transformed yeast cells under selection conditions and α- and β-galactosidase production were observed with the full-length tail domains and the N-terminally truncated domains (Fig. 2E). The deletion of the C-terminal PBM in the cytoplasmic tail domain of USH2A, VLGR1b and NBC3 prohibited growth. Identical results were obtained in bait-prey swap experiments (data not shown). To confirm PBM-dependence of the interactions, we fused the proteins to different, spectrally separated, green fluorescent protein variants and visualized the localization of the proteins in COS-1 cells by fluorescence microscopy. We co-transfected COS-1 cells with a construct that encodes red fluorescent protein (mRFP)-tagged harmonin (HdCC1) and a construct that encodes the enhanced cyan fluorescent protein (ECFP)-tagged cytoplasmic C-terminal tail region of USH2A isoform b, VLGR1 or NBC3, with or without the C-terminal PBM (Fig. 3). The full C-terminal tails of USH2A and NBC3 localize to the nucleus when they are individually expressed (single transfected cells in Fig. 3A and C, respectively). The full C-terminal tail of VLGR1 localizes mainly to the cytoplasm and harmonin is dispersed in the cell, localizing to both the cytoplasm and the nucleus (single transfected cells in Fig. 3E and B, respectively). Co-expression of the full C-terminal tails of USH2A and NBC3 with harmonin recruited harmonin completely to the nucleus (Fig. 3A and C), indicating that these tail regions interact

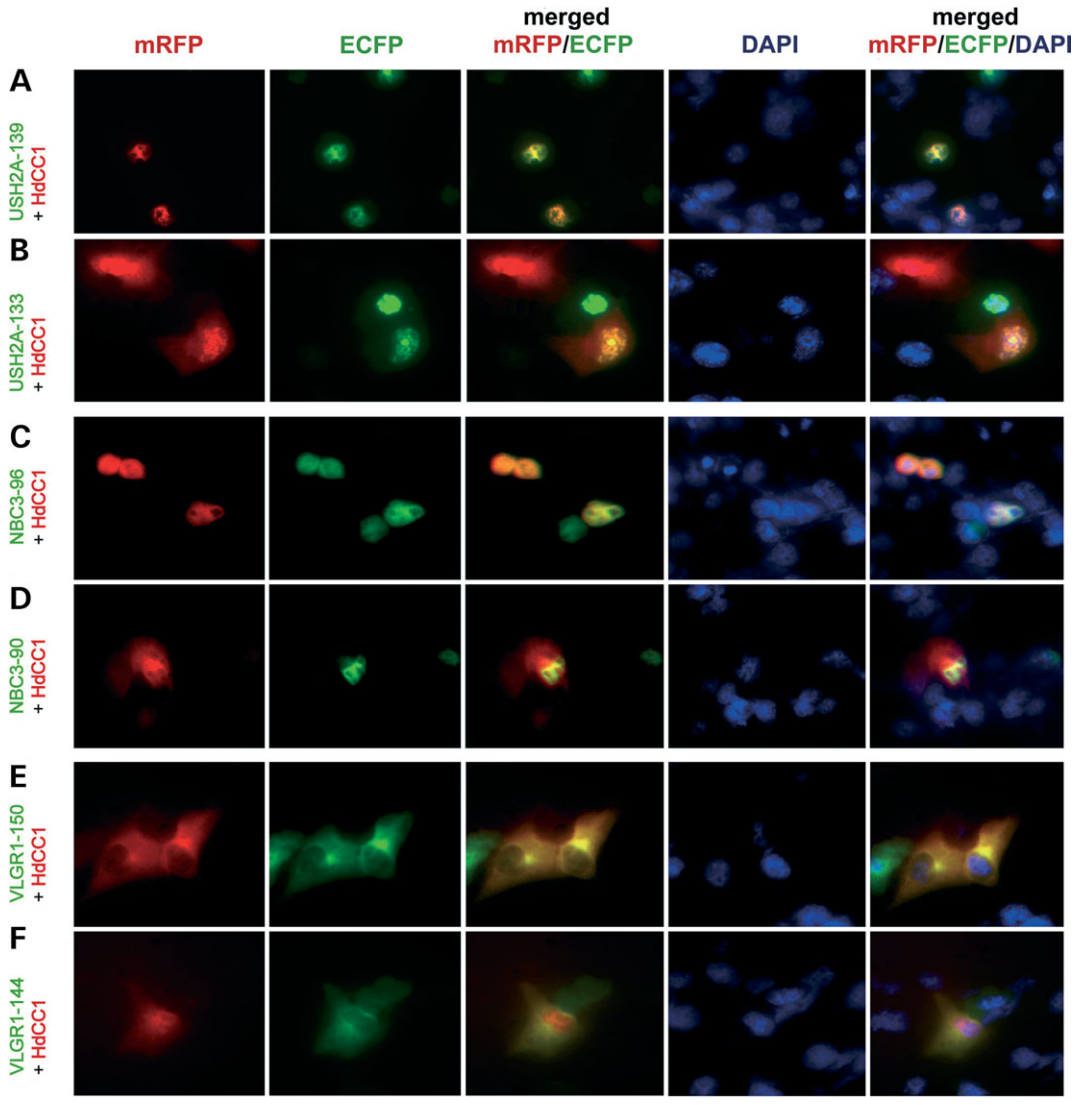

Figure 3. Co-transfection of COS-1 cells with mRFP-harmonin (HdCC1) and the ECFP-tagged tail domains of USH2A, VLGR1 and NBC3. Co-transfections of constructs encoding mRFP-HdCC1 and (**A**) ECFP–USH2A complete tail (USH2A-139), (**B**) ECFP–USH2A tail w/o C-terminal PBM (USH2A-133), (**C**) ECFP–NBC3 complete tail (NBC3-96) and (**D**) NBC3 w/o C-terminal PBM (NBC3-90) as well as (**E**) ECFP–VLGR1b complete tail (VLGR1-150), (**F**) VLGR1b w/o C-terminal PBM (VLGR1-144). In single transfected cells, USH2A and NBC3 tails were located in the nucleus (B and C), whereas the VLGR1b tail was mainly located in the cytoplasm (E). In single mRFP-harmonin transfected cells, harmonin was present in both the cytoplasm and the nucleus (B). In cells with both the complete tail of USH2A or NBC3 and mRFP-harmonin, the latter was recruited completely to the nucleus (A and C), whereas co-expression of the USH2A or NBC3 cytoplasmic tails lacking the PBM and mRFP-harmonin did not change the cellular distribution of harmonin (B and D). Co-expression of the complete VLGR1b tail and mRFP-harmonin lead to the localization of harmonin in the cytoplasm (E). In the absence of VLGR1b's PBM, harmonin was present in the cytoplasm and the nucleus (F). In conclusion, the co-transfection experiments indicate that the PBM of USH2A, NBC3 and VLGR1b is responsible for their interaction with harmonin. Scale bar: 15 μm.

with harmonin. In contrast, in the absence of the PBM in the C-terminal tails of USH2A and NBC3, harmonin was found also in the cytoplasm, similar to individually expressed harmonin (Fig. 3B and D). This indicates a disruption of the interactions. Similarly, co-expression of the full C-terminal tail of VLGR1b and harmonin completely retained harmonin in the cytoplasm (Fig. 3E), indicating an interaction of the proteins. In the absence of the PBM in the tail region of VLGR1b, harmonin was again detected throughout the cell (Fig. 3F). These results support the hypothesis that the interaction is essentially dependent on the PBM.

Localization of the network partners harmonin, the USH2 proteins and NBC in retinal photoreceptor cells and in inner ear hair cells

Previous studies demonstrate harmonin expression in the retina and the cochlea. In retinal photoreceptor cells, harmonin co-localizes with other USH1 proteins at the synaptic terminals (4,9) (Fig. 4B), whereas in the inner ear, this harmonin-organized USH1-protein network is essential for the stereocilia differentiation of hair cells and may participate in their signal transduction (5,6,8,10–12). Also USH2A, VLGR1

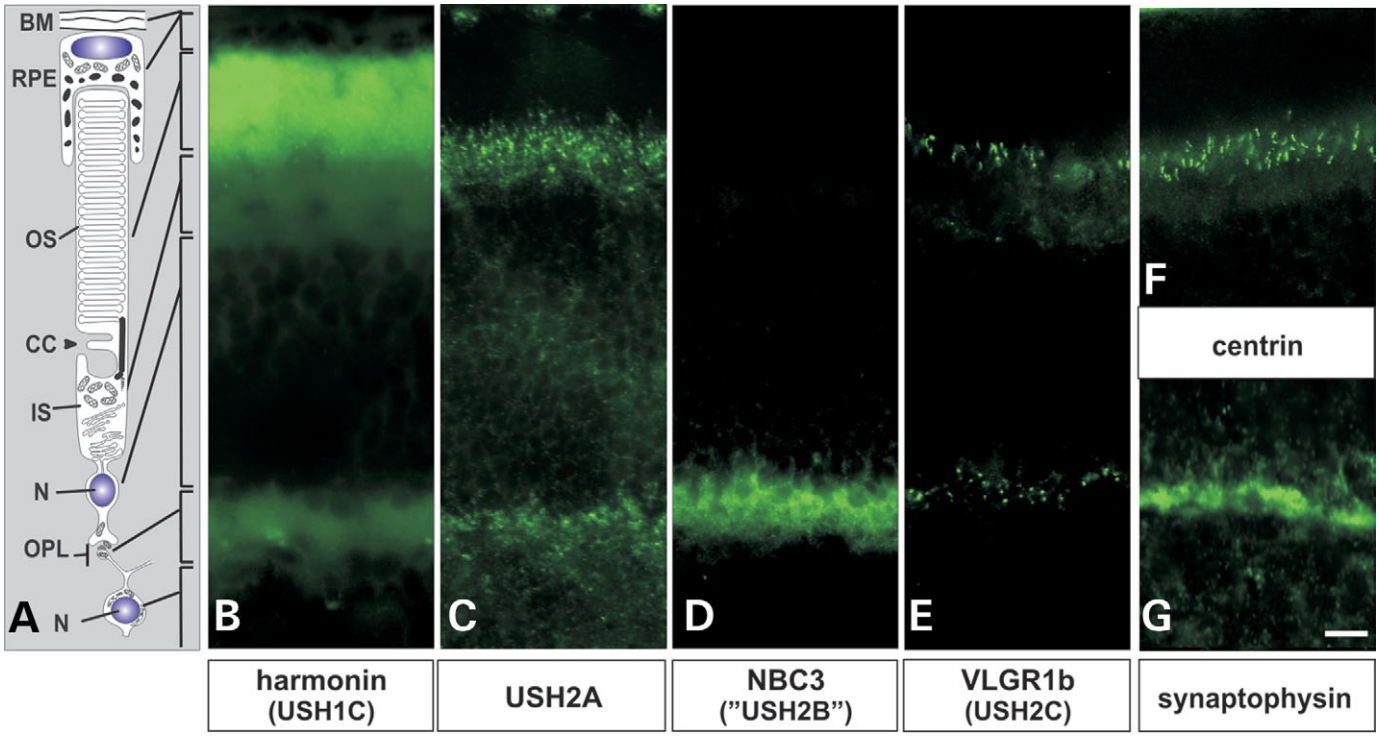

Figure 4. Localization of harmonin, USH2A, VLGR1 and NBC3 in photoreceptor cells of the mouse retina. (**A**) Schematic representation of a vertebrate rod photoreceptor cell. Vertebrate photoreceptors are composed of a light-sensitive outer segment (OS) linked via the connecting cilium (CC) to an inner segment (IS), which contains the biosynthetic and metabolic machinery. Synaptic junctions between photoreceptor cells and bipolar and horizontal cells are located in the outer plexiform layer (OPL) of the retina. OS tips are enveloped by apical extensions of the cells of the retinal pigment epithelium (RPE). N, nucleus. Indirect immunofluorescence of antibodies against harmonin (**B**), USH2A (**C**), NBC3 (**D**) and VLGR1b (**E**) in parallel longitudinal cryosections through the photoreceptor part of the mouse retina. (B) Harmonin is localized in OSs and ISs and at photoreceptor synapses of the OPL (4,9). (C) In addition to that in the extracellular Bruch's membrane (BM) (18), USH2A labeling was found in the OPL and the CC at the joint between the IS and OS of photoreceptor cells. (D) NBC3 localization in the OPL. (E) Anti-VLGR1b stained punctuated structures in the OPL and the CC localized. (**F**) CC localization was confirmed by double labeling with anti-centrin (35). USH2A, VLGR1b, NBC3 and harmonin are localized in the OPL where the ribbon synapses of photoreceptor cells are localized, stained by the anti-synaptophysin (**G**). Scale bar: 5 μm.

and NBC3 were previously demonstrated to be expressed in the retina and the cochlea (14–17). We elaborated on this by investigating the localization of USH2A, VLGR1b and NBC3 in the sensory epithelia that are affected in USH2. For this purpose, polyclonal rabbit and guinea pig antibodies against the recombinant expressed C-terminal regions of USH2A and VLGR1b were generated. Western blot analyses of mouse retina homogenates revealed that both affinity-purified antibodies detected a single band of the appropriate high molecular weight (Supplementary Material, Figs S1A and S2A). To validate the specificity of the affinity-purified antibodies, they were pre-adsorbed with 1 mg/ml of the bacterially expressed antigen before using them on western blots. The immunoreaction was completely abolished in the case of anti-VLGR1b antibodies (Supplementary Material, Fig. S1A) or drastically reduced for the anti-USH2A antibodies (Supplementary Material, Fig. S2A). For both antibodies, pre-treatment with recombinantly expressed unrelated antigens did not give any reduction of the immune reaction (data not shown).

To determine the subcellular localization of USH2 proteins in the retina, cryosections of mouse and rat eyes were analyzed by indirect immunofluorescence microscopy using affinity-purified antibodies against USH2A, VLGR1b and NBC3. We confirmed the localization of USH2A in Bruch's membrane as previously described (18) (Fig. 4C). In addition, specific anti-USH2A immunofluorescence was present at the synaptic terminals of photoreceptor cells in the outer plexiform layer of the retina (Fig. 4C), which was counterstained with the synaptic marker antibodies against synaptophysin (Fig. 4G). The present immunohistochemistry with antibodies against NBC3, VLGR1b and harmonin further demonstrated that these three proteins are also localize at the synaptic regions in the outer plexiform layer of the retina (Fig. 4B, D and E) (16). Furthermore, double-labeling experiments with the connecting cilium marker antibodies against centrin (Fig. 4F) revealed the localization of USH2A and VLGR1b in the connecting cilium of photoreceptor cells (Fig. 4D and E). Although precursors of retinal pigment epithelium (RPE) cells express VLGR1b (19), no protein was detected in the mature RPE (Supplementary Material, Fig. S1B–D). Pre-adsorption of the affinity-purified antibodies against USH2A and VLGR1b with the appropriate recombinant antigen completely abolished the indirect immunofluorescence on cryosections through the mouse retina (Supplementary Material, Figs S1B–F and S2B–F).

Immunohistochemical staining of USH2A, NBC3, VLGR1b and harmonin in the rat inner ear revealed their localization in the stereocilia of hair cells (Fig. 5). The localization of the

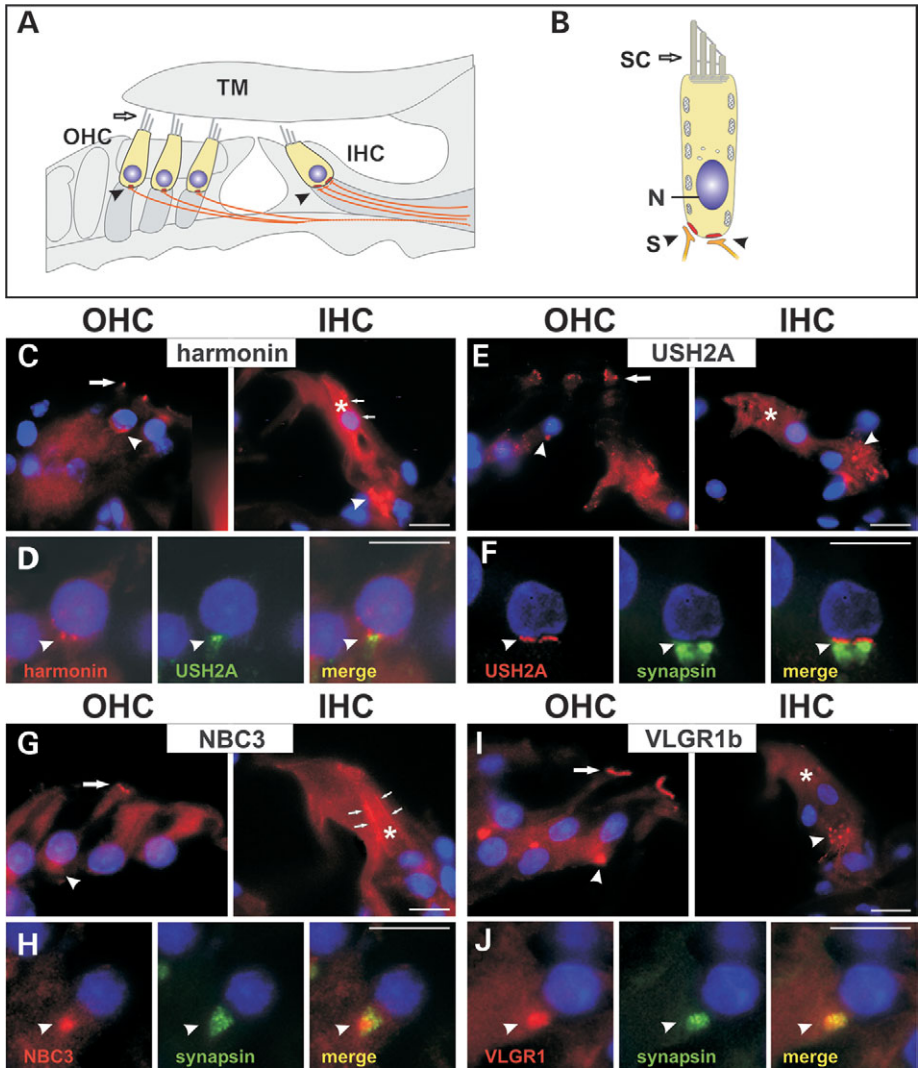

Figure 5. Localization of harmonin, the USH2 proteins and NBC3 in rat cochlear hair cells. Schematic representation of the organ of (**A**) Corti and (**B**) a mechanosensitive outer hair cell (OHC). In the organ of Corti, three rows of OHCs and a single row of inner hair cells (IHC) are enclosed by various types of supporting cells. The apical part of hair cells carries numerous rigid microvilli, improperly named stereocilia (SC, arrows), where the mechanotransduction takes place. The project towards the tectorial membrane composed of extracellular matrix material. N, nucleus; Arrowheads point to synaptic junctions (S) between OHC/IHC and efferent and afferent neurons. (**C–J**) Indirect immunofluorescence of antibodies against harmonin (USH1C) (C and D), USH2A (E and F), NBC3 (G and H) and VLGR1b (USH2C) (I and J) in longitudinal cryosections through OHC/IHC of the adult (P26) rat cochlea. NBC3, harmonin, NBC, USH2A and VLGR1b were found in the hair cell stereocilia (large arrows) as well as at the synapses (arrowheads) of OHCs. The localization in the synaptic region of the OHCs was confirmed by triple labeling with the DNA marker DAPI, anti-harmonin and anti-USH2A (D) as well as with DAPI, anti-USH2A (F), anti-NBC3 (H), anti-VLGR1b (J) and the synaptic marker of hair cells and efferent fibers anti-synapsin. In addition, anti-USH2A, anti-VLGR1b and anti-harmonin immunofluorescences were detected at the synapses of efferent and afferent fibers projecting to the IHC (arrowheads, right images in C, E and I). Synaptic labeling was confirmed by counter staining with anti-synaptophysin (synaptic marker of hair cells and efferent fibers) and anti-bodies against the neurofilament protein NF-200 (marker for afferent fibers) (data not shown). Furthermore, a localization of harmonin and NBC3 was present at the lateral IHC cell membrane indicated by small arrows (right panel in C and E). The cell bodies of IHCs are indicated by asterisks. Scale bars: 5 μm.

four proteins was also seen in the synaptic region of outer hair cells (OHC) demonstrated by counterstaining with the synaptic marker anti-synapsin and the nuclear DNA marker DAPI (Fig. 5). In addition, we obtained anti-USH2A, anti-VLGR1b and anti-harmonin immunofluorescence at synapses of efferent and afferent fibers projecting to the inner hair cells (IHC) (Fig. 5). These findings indicate that the interaction of these network proteins also occurs in the cytomatrix of inner ear synapses. Furthermore, anti-NBC3 and anti-harmonin immunohistochemistry revealed localization of both proteins at the lateral membrane of cochlear IHC (Fig. 5C and G). Present analysis further confirmed the localization of the ion-cotransporter NBC3 in regions beneath the *stria vascularis* (data not shown) as described before (16).

DISCUSSION

Genetic and phenotypic heterogeneity are well-known common features of many inherited sensory disorders. Less

well known are the molecular links between different monogenic syndromic sensory disorders with distinctive phenotypic similarities. Previous studies of the USH, combining inner ear and retinal dysfunction, have demonstrated the expression of the USH1-protein harmonin and its co-localization and physical interaction with other USH1 proteins in the retina and the inner ear. In the retina, they co-localized in the photoreceptor cells at the synaptic terminals (4,9) (Fig. 4B). In the inner ear, they were identified in the mechanosensitive hair cells where the interactions within the USH1-protein network are essential for the hair cell differentiation and may participate in their signal transduction (5,6,8,10–12). Harmonin is therefore thought to act as a scaffold protein, integrating all known USH1 molecules in a USH1-protein network (8). Interestingly, besides the USH1 proteins, the proteins that are involved in the more common USH2 phenotype, USH2A, VLGR1 (USH2C) and the USH2B-candidate NBC3, were previously demonstrated to be expressed in the retina and cochlea (14–17), but a common denominator of these syndromes remained elusive. In the present study, we identified the two USH2 molecules, USH2A and VLGR1b, and the USH2B-candidate NBC3 as novel meshes of this USH-protein network. The present data obtained by *in vitro* and *in vivo* binding assays demonstrate that these three proteins specifically interact with harmonin. Further tests revealed that all three proteins bind to the PDZ1 domain of harmonin which supports the previously suggested key role of harmonin's PDZ1 domain in the formation of most of the identified harmonin interactions (8). The illustration in Figure 6 summarizes the various deciphered protein-protein interactions within the USH-protein network.

PDZ domain mediated protein-protein interactions mainly involve PDZ binding to the C-terminus of interacting partners but can also occur through PDZ-PDZ interactions, as well as through binding to other internal peptide sequences or even to lipids (20). Previous studies showed that harmonin's PDZ1 domain binds to a not yet identified internal sequence in the tail of myosin VIIa (USH1B) (5). A homo- or heteromeric network of harmonin isoforms can be formed via the interaction of a C-terminal PBM in some harmonin isoforms (e.g. harmonin a1, b4) with PDZ1 (6) or by binding of the PDZ1 or PDZ2 domain, respectively, to the second coiled-coil domain (CC2) in harmonin b isoforms (8) (Fig. 6). Here, we analyzed the deletion constructs of the cytoplasmic tail domains of the USH2 proteins and NBC3 in *in vitro* and *in vivo* binding assays and identified that the interactions of USH2 proteins and NBC3 with harmonin's PDZ1 domain are fully dependent on the PBM at the C-terminus of their cytoplasmic tail domain. Sequence comparisons between the C-termini of the different interaction partners of harmonin's PDZ1 domain reveal a PBM class I consensus sequence for the PDZ1 domain of harmonin: -D/E-T-X-L, containing a charged amino acid [aspartate (D) or glutamate (E)] at the fourth position (Fig. 2A). Suggesting that this consensus sequence is the specific binding motif for harmonin's PDZ1 domain, it is an interesting future issue to elucidate how the binding of the different partners (including four USH1, two USH2 proteins and NBC3) to this particular PDZ domain is regulated or modulated and how this contributes to the dynamics within the USH-protein network (5–9,21,22).

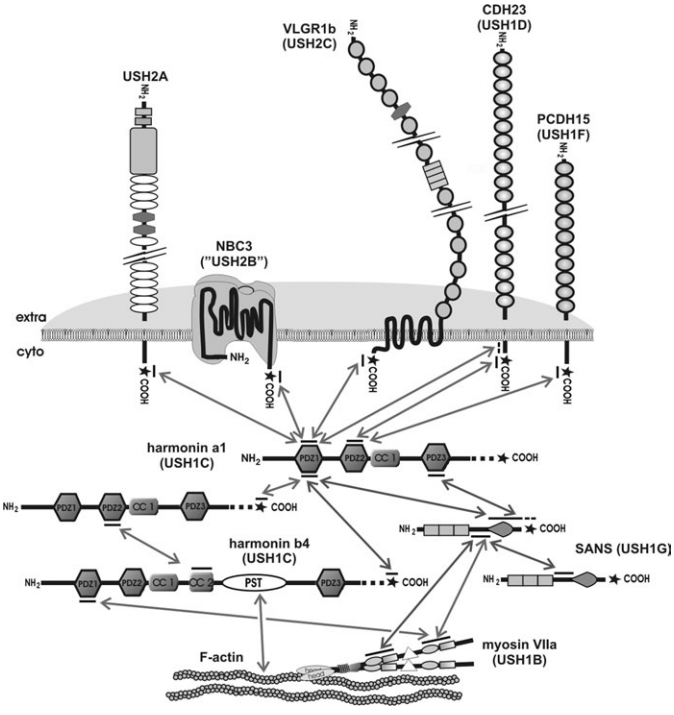

Figure 6. Schematic diagram illustrating the deciphered interactions within the USH-protein network-interactome. The three USH2-related transmembrane proteins USH2A, NBC3 ('USH2B' candidate) and VLGR1b (USH2C) bind via their C-terminal PBM to PDZ1 of harmonin. The cadherins Cdh23 (USH1D) and Pcdh15 (USH1F) interact via their C-terminal PBM with PDZ2 of harmonin (5,6,8,9) [note: a splice variant of Cdh23 binds also to PDZ1 (6)]. The scaffold protein SANS (USH1G) is suitable to form homomers via its central domain (8). It interacts with its C-terminal part with PDZ1 and PDZ3 of harmonin, whereas its central domain also binds to the MyTH-FERM domains in the tail of myosin VIIa (USH1B) (8). Homo- and hetromeric interactions between the harmonin isoforms can occur via binding of PDZ1 to the C-terminal PBM of some splice variants (e.g. harmonin a1 and b4) and/or through interactions of PDZ1 and PDZ2 with the second coiled-coil (CC2) domain restricted to harmonin b isoforms (4,5,8). The USH-protein network is directly connected to the actin cytoskeleton through the PST domain present in harmonin b isoforms and/or via the motor domain of myosin VIIa dimers (5).

Previous studies indicated that during the differentiation of the stereocilia in the developing hair cells, harmonin interacts with the USH1 proteins, myosin VIIa, cadherin 23 and Pcdh15 (5,8,9). Furthermore, underneath the cuticular plate, it also co-localizes with the USH1G protein SANS, previously shown to interact with harmonin (7,8). The USH2 proteins and the USH2B-candidate NBC3 are likely to be part of this USH-protein network in the stereocilia. The extended extracellular portions of USH2A and VLGR1b are determined to perform homo- or heteromeric interactions (14,15). These might contribute to the anchorage of stereocilia within the extracellular matrix or to interconnections between neighboring stereocilia as previously demonstrated for cadherin 23 and Pcdh15 (8,10–12). VLGR1b is the largest known cell surface protein and its extremely long N-terminus probably serves as the basis for extracellular filaments harboring numerous Ca^{2+}-binding motifs (15). This feature of VLGR1b is shared with the tip links, elastic structures between neighboring stereocilia of hair cells which are proposed to serve in

gating the mechanosensitive channel (23). Nevertheless, VLGR1b may also play a role in Ca^{2+}-homeostasis by functioning as a Ca^{2+}-sink at the surface of stereocilia because of its large number of CalX-β modules (15). These characteristics of VLGR1b might be the molecular basis for the failure of normal development of the stereocilia and an early onset of hearing impairment in VLGR1b-deficient mice carrying the *Mass1*^{frings} mutation (24).

Several USH1 molecules were previously identified at synapses in the CNS as well as in the sensory cells of the retina and the inner ear (2,4,9). On the basis of the subcellular co-localization of all known USH1 proteins, we recently proposed a USH1-protein network at photoreceptor synapses organized by harmonin (4,9). The present data indicate that the USH2 proteins and NBC3 are additional partners in this synaptic supramolecular USH network not only in the retinal photoreceptor cells but also in the inner ear hair cells. Whereas cytoplasmic USH1 proteins (e.g. myosin VIIa and SANS) may participate in synaptic molecule trafficking or/and targeting (4,8), the transmembrane USH1 cadherins (Pcdh15 and Cdh23) are suggested to be involved in synaptic adhesion (4,5,9,25). In synaptic terminals of neuronal cells, cell adhesion molecules in the pre- and post-synaptic membrane interact via their extracellular domains and keep the synaptic cleft in close register (26). As mentioned earlier, USH2A and VLGR1b are pre-destined to perform homo- or heteromeric interactions via their extracellular domains and may also contribute to the synaptic organization in retinal photoreceptor cells and inner ear hair cells. The VLGR1b may also participate in cell adhesion mediated G-protein signaling which is known to regulate the organization of the synaptic cytomatrix especially during synaptogenesis (27–29). An important role of the sodium-bicarbonate co-transporter NBC3 in the pH-regulation at synaptic terminals was previously discussed in Bok *et al*. (16). NBC3-mediated bicarbonate-flux may be essential for an efficient buffering of H^+-loads in synaptic terminals, necessary for the maintenance of a normal rate of the plasma membrane Ca^{2+}-ATPase (PMCA)-mediated Ca^{2+}-efflux and the function of L-type voltage-gated Ca^{2+}-channels (16). Especially in photoreceptor synapses, spatial integration of NBC3 and PMCA1 may enhance the efficiency of H^+-buffering, possibly via binding of the C-terminal PBM of the PMCA1 (30) to one of harmonin's PDZ domains. Analogous to this, a functional association between NBC3 and PMCAs can be postulated to occur in the stereocilia, the synaptic region and lateral membrane of hair cells where major Ca^{2+}-fluxes take place (31).

Our findings emphasize the essential central role of harmonin in the supramolecular network of all known USH1 and USH2 molecules (including the putative USH2B protein, NBC3). In its function as a potent scaffold protein, harmonin provides the first molecular link between the USH types, USH1 and USH2. As the USH3 protein clarin-1 also contains a PBM (32), it would be no surprise to find that also this protein binds to harmonin and thus that it is also part of the USH-protein network. The decipherment of the USH network further indicates a common pathophysiological pathway for the sensoneuronal degeneration in USH. Dysfunction or absence of any of the proteins in the mutual 'interactome' related to the Usher's disease may lead to the disruption of the network causing the degeneration of the sensory epithelium of the inner ear and the neuronal retina, the clinical symptoms characteristic for USH patients. Further unravelling of this interactome in the future will provide candidate genes for syndromic and non-syndromic forms of deafness and retinal degeneration.

MATERIALS AND METHODS

Animals

All experiments using C57BL/6J mice or Wistar rats conformed with the statement by the Association for Research in Vision and Ophthalmology (ARVO) as to care and use of animals in research.

Constructs for expression of cDNA

cDNAs for the expression of proteins were obtained by RT–PCR or from EST-clones and were sub-cloned into the appropriate expression vectors as previously described (9,33,34). The human harmonin a1 was derived from the clone ICRFc100D12145Q3 obtained from the RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH (Berlin, Germany).

The numbers of the given amino acids are according to the following GenBank entries. Harmonin a1 (mouse), AF228924; harmonin a1 (human), AAH16057; USH2A isoform b (human), NP_99681; NBC3 (mouse), BC038373.1; NBC3 (human), NP_003606; VLGR1b, NP_115495; Pcdh15 (mouse), AF_281899.

Antibodies and fluorescent dyes

The antibody against VLGR1b was generated against a part of the cytoplasmic tail, bacterially expressed in pDEST15 GST-tag expression vector (Gateway cloning system). Anti-USH2A antibodies were generated in rabbits and guinea pig against the FN domains of mouse USH2A and against a 139 amino acid fragment of the cytoplasmic tail of human USH2A. Expression of the fusion proteins and purification of the antibodies were performed as described (4). The antibodies against harmonin (H3), NBC3, synaptophysin, NF-200, and centrin (clone 20H5) were previously described (4,9,35–37). The HA- and Flag-tag antibodies as well as the anti-actin antibody were derived from Sigma (Germany). The monoclonal anti-synapsin antibody was purchased from Promega (Germany). The secondary antibodies were purchased from Molecular Probes (The Netherlands) or Rockland (USA).

Specificity test of anti-USH2A and anti-VLGR1b antibodies

For denaturing gel electrophoresis, retina homogenates were mixed with SDS-PAGE sample buffer (62.5 mM Tris–HCl, pH 6.8; 10% glycerol; 2% SDS; 5% mercaptoethanol; 1 mM EDTA; 0.025% bromphenol blue). Per lane, 25 μg of protein was separated on 3–8% Tris–acetate-buffered (125 mM, pH 7.0) polyacrylamide gradient gels following the NuPAGE system protocol (Invitrogen, Germany) and blotted onto PVDF membranes (Amersham Biotech/GE Healthcare, Germany). Immunereactivities

were detected on western blots, with the appropriate primary antibodies and secondary antibodies coupled to IRDye680 (donkey-anti-rabbit) or IRDye800 (goat-anti-mouse IgG) employing the Odyssey Infrared Imaging System (LI-COR, USA). For the competition experiment, the affinity-purified anti-USH2A and anti-VLGR1b antibodies were pre-incubated for at least 3 h at room temperature in the presence of 1 mg/ml bacterial expressed antigens used to immunize the rabbits. As a molecular marker PeqLab Gold Marker IV (Peqlab, Germany) ranging from 10 to 170 kDa was used. Band sizes were calculated using the TotalLab software (Phoretics, UK).

GST-*pull down* assay

Constructs encoding harmonin domains were PDZ1 (82–168 amino acid), PDZ2 (207–292 amino acid) and PDZ3 (419–537 amino acid) cloned in the vector pGEX-4T-3 as well as the full-length sequence of murine harmonin a1 (1–548 amino acid) cloned in the vector pDEST17 (Gateway cloning system, Invitrogen, USA) (9). The cDNAs encoding C-terminal fragments of USH2 proteins were human USH2A isoform b (5063–5202 amino acid), murine NBC3 (702–1255 amino acid) and human VLGR1b (6198–6307 amino acid) were cloned in the vectors pDEST17 and pDEST15. The murine Pcdh15 fragment encoding part of the intracellular domain consisted (1845–1944 amino acid) was cloned in the vector pDEST17 (9). Equal amounts of GST or GST-fusion protein were mixed with lysates of His-tagged fusion proteins, and the GST-*pull down* assay was performed as described (9).

Co-IP assay

Human HA-tagged harmonin a1 (1–534 amino acid) was expressed from vector pcDNA3-HA/DEST (Gateway cloning system). Flag-tagged fragments from the human proteins USH2A isoform b (5064–5202 amino acid), NBC3 (1119–1214 amino acid) and VLGR1b (6158–6307 amino acid) were expressed by using p3 × Flag-CMV/DEST (Gateway cloning system).

COS-1 cells were transfected by using the Nucleofector kit V (Amaxa, USA) and program A-24 according to manufacturer's instructions. Twenty-four hours after transfection cells were washed with PBS and subsequently lysed on ice in lysis buffer [50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100 supplemented with a complete protease inhibitor cocktail (Roche, Germany)]. HA-tagged harmonin a1 was immunoprecipitated from cleared lysates overnight at 4°C by using anti-HA polyclonal antibody and Protein A/G PLUS-sepharose (Santa Cruz Biotechnology, USA). After four washes with lysis buffer, the protein complexes were analyzed on immunoblots using the Odyssey Infrared Imaging System (LI-COR, USA). Tagged molecules were detected by anti-HA or anti-Flag monoclonal antibodies.

Fluorescence microscopic analysis of co-transfected COS-1 cells

To determine the cellular localization in COS-1 cells of the cytoplasmic tails of human USH2A isoform b, human VLGR1b and human NBC3 as well as human harmonin a1 without coiled-coil 1 (CC1; 299–358 amino acid) domain, we cloned the tails of human USH2A, VLGR1b and NBC3 with and without their C-terminal PBMs in pDEST501 (Gateway cloning system), resulting in N-terminally fused ECFP-fusion proteins. Harmonin-CC1 was cloned in pDEST733 (Gateway cloning system), resulting in an N-terminally fused mRFP-harmonin-CC1 fusion protein. COS-1 cells were co-transfected with pDEST501-USH2A tails (USH2A-139: 5064–5202 amino acid; USH2A-133: 5064–5196 amino acid), pDEST501-VLGR1b tails (VLGR1-150: 6158–6307 amino acid; VLGR1-144: 6158–6301 amino acid) or pDEST501-NBC3 tails (NBC3-96: 1119–1214 amino acid; NBC3-90: 1119–1208 amino acid) together with pDEST733-harmonin-CC1 by using Lipofectamine (Invitrogen) according to the manufacturer's instructions. After 24 h, cells were washed in PBS, fixed with 4% paraformaldehyde, mounted with Vectashield containing DAPI (Vector Laboratories Inc., UK) and analyzed by epifluorescence microscopy.

Yeast two-hybrid assays

The GAL4-based yeast two-hybrid system (HybriZAP, Stratagene) was used to identify the interactions between harmonin and USH2A, VLGR1b and NBC3 as previously described using Gateway-adapted vectors (9,33,34). The yeast strain PJ69-4A was used as a host, which carried the *HIS3* (histidine), *ADE2* (adenine), *MEL1* (α-galactosidase) and *LacZ* (β-galactosidase) reporter genes. Interactions were analyzed by assessment of reporter gene activation, using growth on selective media (*HIS3* and *ADE2* reporter genes), an X-β-gal colorimetric plate assay (*MEL1* reporter gene) and an X-α-gal colorimetric filter lift assay (*LacZ* reporter gene). Complete human harmonin a1 (1–534 amino acid) was expressed and fragments thereof, PDZ1 (72–188 amino acid), PDZ2 (198–311 amino acid) and PDZ3 (422–534 amino acid). Human USH2A tail fragments were USH2A 1–139 (5064–5202 amino acid), USH2A 1–133 (5064–5196 amino acid) and USH2A 106–139 (5169–5202 amino acid). Human NBC3 tail fragments were NBC3 1–96 (1119–1214 amino acid), NBC3 1–90 (1119–1208 amino acid) and NBC3 70–96 (1188–1214 amino acid). VLGR1b tail fragments were VLGR1b 1–150 (6158–6307 amino acid), VLGR1b 1–144 (6158–6301 amino acid) and VLGR 123–150 (6185–6307 amino acid).

Fluorescence microscopy

Eyes of adult mice were cryofixed in melting isopentane, cryosectioned and treated as described (4,37). For cochlear immunocytochemistry, cochleae of postnatal rats (P14) were isolated, dissected and fixed as described previously (38). Samples were decalcified after fixation in Rapid Bone Decalcifier (Fisher-Scientific, Germany). Cryosections and COS-1 cells labeled by indirect immunofluorescence or fluorescent proteins and counterstained by DAPI were imaged with an Olympus AX70, a Zeiss Axioplan2 or a Leica DMRP microscope equipped with epifluorescence illumination. In the appropriate control sections, in no case, a reaction was observed. Images were obtained with a Hamamatsu ORCA

ER charge-coupled device camera (Hamamatsu, Germany) and processed with Adobe Photoshop (Adobe Systems, USA).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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