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In molecular diagnosis of Usher syndrome, one of the major challenges results in the identification of a large number of sequence variants of unknown clinical significance (Unclassified Variants or UVs). Although various in silico tools are available to study the effect of these UVs, analysis at RNA level remains an essential step to establish their clinical relevance.

Because, Cohn et al. (2006) has demonstrated the presence of eight Usher proteins in nasal ciliated epithelium, we hypothesised that these cells could be used to analyse Usher transcripts.

First, in order to test if Usher transcripts could be detected in nasal ciliated cells, we have realised specific nested RT-PCR for each gene implicated in this disorder. With this method, we have shown that these cells provide a good source of transcripts from 8 of the 9 known genes which cause Usher syndrome, namely MYO7A, USH1C, CDH23, PCDH15, USH1G for Usher type 1 and USH2A, GPR98, WHRN for Usher type 2.

Secondly, we have applied this approach to study the splicing effect of intronic or exonic variants carried by USH1 and USH2 patients. As expected, missplicing was confirmed for variations located at canonical sites. Furthermore, new splice variants (lying in exons or introns) were highlighted. In addition, absence of splicing alteration could be confirmed for variants susceptible to alter the protein structure.

The results obtained in this work show that nasal epithelial cells are reliable, accessible cells to discriminate Usher variants with and without effect on the RNA splicing process in a relative non-invasive way. In combination with in silico and minigenes analyses, this method will help in the interpretation of the UVs detected in Usher syndrome. Finally, because Usher syndrome is considered as a retinal ciliopathy, the approach presented here is susceptible to bring some highlights on splicing defects for alterations identified in other genes involved in this group of disorders.
Nº 2
Nasal ciliary beat frequency in retinal ciliopathies

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Purpose: The cilium in photoreceptors appears ultrastructurally very similar to the nasal ciliated epithelium. This similarity led to the hypothesis that an abnormality in ciliary function may be linked to both the nasal cilia abnormalities as well as to the retinal degeneration. The purpose of this study was to evaluate the nasal ciliary beat frequency in patients with RP and Usher syndrome type II and compare it to that of healthy control subjects.

Methods: A prospective, comparative control study. Fresh samples of nasal mucosa were obtained from 13 patients with typical forms of RP, and from 4 patients with Usher syndrome type II. The nasal ciliary beat frequency (CBF) and beat pattern were studied by high resolution digital high speed video. The control group included 32 fresh nasal mucosa samples from 32 patients without any other confounding diseases.

Results: The nasal ciliary beat frequency was lower in patients with Usher syndrome than in control subjects (Mann-Whitney U test, P<0.01). The nasal ciliary beat frequency was 9.28 ± 0.42 (mean ± SD) Hz in patients with Usher syndrome and 10.88 ± 1.31 Hz in patients of the control group. No significant difference was observed in the nasal ciliary beat frequency between the RP (10.59 ± 1.54 Hz) and control patients (Mann-Whitney U test, P =0.64).

Conclusions: The nasal ciliary beat frequency is diminish in patients with Usher syndrome type II, while remains normal in simplex RP patients. This results add evidence to the fact that Usher syndrome could be a primary ciliary disorder.
**Nº 3**

**Omega-3 fatty acid dietary intake and red blood cell (RBC) docosahexaenoic acid (DHA) levels in usher syndrome subtypes**

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**Background:** Reduced blood levels of the long-chain omega-3 polyunsaturated fatty acid, docosahexaenoic acid (DHA) have been inconsistently reported in patients with Usher syndrome. DHA is found at its highest concentration in the human body in rod and cone photoreceptors suggesting a potential functional role in phototransduction. Numerous studies have found defects in retinal and visual function of both animal models and humans deficient in omega-3 fatty acids including DHA.

**Purpose:** To confirm that blood levels of DHA (RBC-DHA) in patients with Usher syndrome were significantly different from normal, and further determine if differences in RBC-DHA were evident between clinical and genotypic subgroups. Concurrently, we assessed the relationship between dietary omega-3 intake and blood fatty acid status in the normal and Usher study cohorts.

**Methods:** The current study used a retrospective, cross-sectional design. Study participants were comprised of patients with Usher syndrome (*n* = 114; 14-80 yrs) recruited from multi-state and nationwide patient registries and a convenience sample of age-similar normal controls (*n* = 60; 13-67 yrs). Individuals taking oral supplements containing omega-3 fatty acids were excluded from recruitment. Blood samples were collected for RBC fatty acid analysis; specifically, DHA and the omega-3 precursor eicosapentaenoic acid (EPA) were determined by capillary column gas chromatography as percent of total fatty acids. The self-report food frequency dietary questionnaires were completed by each participant to document intake of omega 3-rich food sources (i.e., fish, shellfish, liver, poultry, and egg yolks) as well as intake of DHA and/or EPA due to dietary supplements (e.g., fish oil, DHA capsules).

**Results:** The Usher syndrome cohort had lower mean RBC-DHA levels compared to normal (3.70±1.0% vs. 4.09±1.0%; ±SD; *p* = .017). Overall, nearly 76% (i.e., 75/114) of the Usher cohort had genotyping data available for categorization (i.e., IB, IC, ID, IF, IIA, IIC & IIIA). DHA levels according to clinical type were reduced 8%, 11%, and 9% for types I (*n* = 26), II (*n* = 80), and III (*n* = 5), respectively. However, significant differences were not found in DHA levels between the clinical or genotypic subgroups. Calculated daily dietary DHA intake was not significantly different between the normal (112.1±111.9 mg/d) and Usher (103.2±71.8 mg/d) cohorts. Significant relationships were observed between DHA intake and RBC-DHA levels among both the normal and Usher cohorts (*r* = .470 and *r* = .433, respectively; *p* < .001). Blood EPA levels (0.57±0.28% vs. 0.53±0.25%) and calculated dietary EPA intake (60.7±47.2 mg/d vs. 64.7±60.1 mg/d) were also not different between the heterogeneous Usher cohort and the normal controls.

**Conclusions:** This study represents the first comprehensive comparison of blood DHA levels between each of the Usher clinical and genotypically-defined subtypes. Demonstrating equivalence of DHA intake was a vital element of establishing that dietary differences are likely not responsible for any observed differences in blood DHA levels. These results contribute to our understanding of the omega-3 fatty acid status among patients with Usher syndrome and the association of dietary intake of DHA from omega-3 rich food sources.
**Posters**

**Nº 4**

**Magi2 is a novel interaction partner of the USH1G protein sans in murine retina**

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**Purpose:** The human Usher syndrome (USH) is the most common form of inherited combined deaf-blindness. It is genetically heterogeneous and can be divided into three clinical subtypes (USH1-3). Mutations in USH proteins lead to profound inner ear defects and degeneration of retina. The so far identified USH genes encode proteins of diverse protein families. Previous studies revealed that all known USH1 and USH2 molecules are integrated in protein networks. In our studies we investigated the role of the USH1G protein SANS (scaffold protein containing ankyrin repeats and SAM domain) and its interaction partners in these USH networks in vertebrate photoreceptor cells. Photoreceptor cells are specialized, bipolar neurons with well defined structural and functional compartments. For their maintenance and function directed transport mechanisms are essential. Here, we identified and characterized Magi2 as a protein newly related to USH as a feasible component for development of cell polarity and transport processes.

**Results:** Y2H screens with the C-terminal SAMPBM domain (sterile alpha motif, PDZ-binding motif) of SANS identified three PDZ proteins, including the MAGUK protein Magi2 (membrane associated guanylate kinase inverted-2) as putative interactors of SANS. Co-IP and GST-pull down assays confirm direct interaction between PDZ5 domain of Magi2 and the C-terminus of SANS. Co-transfection assays in eukaryotic cells result in recruitment of interaction partners. Indirect immunofluorescence shows partial colocalization of both proteins in murine retina. Immunoelectron microscopy analyses show same subcellular localization of Magi2 and SANS and demonstrate association of Magi2 with transport vesicles in photoreceptor cells.

**Conclusion:** Direct binding of SANS to vesicle-associated Magi2 and the subcellular distribution of SANS-associated complexes strengthen our hypothesis that SANS participates in transport processes in photoreceptor cell inner segments. Furthermore, the interaction of SANS with the MAGUK protein Magi2 represents a molecular link of USH protein networks to establishment of planar cell polarity. This is closely associated with MAGUK protein function shown in other cellular context. Defects of complex partners may lead to dysfunction of the entire network causing photoreceptor degeneration as seen in USH patients.

Supports: DFG GRK 1044/2; FAUN; Pro Retina Deutschland; Forschung contra Blindheit-Initiative Usher Syndrom; Heinsius Houbolt Foundation, Oogfonds Nederland; Gelderse Blinden Vereniging
Nº 5

A microtubule-associated protein links to the Usher protein network

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Usher syndrome is the most common form of combined hereditary deaf-blindness. It can be caused by mutations in genes, encoding proteins with a variety of functions, but that all closely associate in a protein network. Nevertheless, the molecular disease mechanisms of Usher syndrome still remain largely elusive. To gain insight into the pathogenic pathways underlying Usher syndrome type 2A (USH2A), we have set out to determine the interacting repertoire of the USH2A isoform B protein in the retina.

The Y2H screen of a human retinal cDNA library, using the intracellular domain of USH2A as a bait, revealed several interesting interactors, including a microtubule-associated protein of 134 kDa (indicated as MAP134). The intracellular domain of USH2A specifically interacts with the two coiled coil domains of this protein. The interaction was confirmed by glutathione S-transferase pull-down assays. The transcripts of the gene were highly expressed in the brain and in the eye during mouse development. The MAP134 and USH2A proteins co-localize at several sub-cellular sites in photoreceptor cells, including the basal body. Immunoelectron microscopy showed presence of both USH2A and MAP134 in the apical inner segment, connecting cilium, basal body and accessory centriole of photoreceptor cells. Our data indicate that USH2A specifically interacts with MAP134 and that the two proteins co-localize in the photoreceptor cells, thus we introduce a novel member of the Usher protein network. As MAP134 is known to play a role in microtubule stabilization and cross-linking of microtubules to other components, it may serve as a scaffold for cross-linking regulatory and structural components. This may be important for the maintenance of the connecting cilium of photoreceptor cells, and/or for the microtubule-based transport towards and across the connecting cilium. Its direct association with USH2A indicates that the gene encoding MAP134 is a functional candidate for Usher syndrome and related retinal ciliopathies.
Influence of MYO7A, the Usher1B gene product, on the visual cycle protein RPE65

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Myosin VIIa (MYO7A) is an unconventional myosin. Its absence in humans results in Usher syndrome type 1B, which is characterized by deafness and progressive blindness. In this study, we tested the susceptibility of MYO7A-null mice to retinal light damage, and the effect of MYO7A on the visual cycle protein, RPE65.

MYO7A-null mice were exposed to acute light levels, and retinal degeneration was analysed by light microscopy of semi-thin sections, and ERG measurements. Protein levels were determined by western blot with specific antibodies against visual retinoid cycle proteins. ERG measurements and retinoid profiles were also obtained.

Acute light levels resulted in retinal degeneration in control animals, as seen in the reduction of nuclei number and ERG responses, while MYO7A-null animals were protected. Analysis of protein levels revealed that lack of MYO7A reduced RPE65 levels, but not other visual cycle proteins. Following a >50% photobleach, MYO7A-null retinas exhibit defects in retinal ester levels and ERG responses during the initial stages of dark recovery, consistent with a deficiency in RPE65 activity. Together, the results support a model where there is an involvement of the Usher 1B protein in the spatiotemporal organization of the retinoid cycle in vision.
The human Usher syndrome (USH) is the most frequent cause of inherited combined deaf-blindness. It is assigned to three clinical types and 12 genetically heterogeneous subtypes. USH is characterized by profound inner ear defects and retinitis pigmentosa. In contrast to USH patients, USH rodent models develop, if at all, a very mild retinal phenotype. So far, there is no explanation for this difference in the retinal phenotype. One possible explanation is that primates and rodent photoreceptor cells differ in structure and in the subcellular distribution of individual USH proteins. Here, we tested the latter hypothesis by analyzing the expression and subcellular localization of USH1/2 proteins by Western blots and by immunohistochemistry of macaque and human retinas. All USH1/2 proteins are expressed in photoreceptor cells of primate retinas. A combination of high resolution immunofluorescence and immunoelectron microscopy revealed localization of USH1/2 proteins at synapses and in the ciliary region or in the outer segment of primate photoreceptors. Interestingly, staining intensities of USH proteins significantly differed between rods and cones previously not described in rodents. Furthermore, a subset of USH proteins were identified in calycal processes which are prominent features of primate photoreceptor cells.

There are significant differences between rodents and primates in the expression of USH1/2 proteins in retinal photoreceptors which can explain the lack of retinal USH phenotype in mice. USH proteins are differentially expressed in macaque photoreceptor synapses. Furthermore, in primates defects in USH molecules may lead to disorganization of the USH protein network in calycal processes and thereby to the destabilization of photoreceptor outer segments. Therefore, it is reasonable that the defects of the abundant USH1/2 molecules in cones may affect cone-dominated primate retinas more than rod-dominated rodent retinas.

Supports: FAUN, Fofö University of Mainz, Foundation Fighting Blindness
Nº 8

Genetic factors influence mechanosensory traits in humans

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Transduction of mechanical stimuli is achieved by different sensory systems. In this study we investigated the genetic influence on different mechanosensory systems, touch sensitivity, hearing acuity and the vascular baroreflex in a classical twin study. We also asked if common genetic mechanisms might equally influence different mechanosensory traits by looking at the correlation between the traits within healthy individuals. We looked more specifically for a link between touch sensitivity and hearing by studying touch related thresholds in patients that suffer from the Usher’s Syndrome. The two touch traits investigated in this study were tactile acuity and vibration sensitivity. Tactile acuity was measured as the ability to determine the orientation of grids with a different spacing (0.75 - 6 mm) with the fingertips using the “Tactile Acuity Cube”. Vibration sensitivity was measured as a vibration detection threshold of a 125 Hz vibration applied on the nailbed of the little finger (150 nm -500µm amplitude) using the “CASE IV” system. The auditory system was assessed by determining the hearing acuity as a threshold in decibel and by recording the evoked otoacoustic emissions (EOAEs) of the outer hair cells. We evaluated the reproducibility of the stimulus signal as well as the strength of the EOAEs in decibel. To assess the sensitivity of the vascular baroreflex we determined the spontaneous baroreflex during a 5 min period during which heart rate and blood pressure was recorded. Baroreflex slopes were calculated by performing cross-spectral analysis and also by the sequence technique. Here the actual slope and the number of detected sequences during the recording period were evaluated.

As thermosensory traits do not require mechanotransduction we tested temperature sensitivity on the volar forearm. Cold and warmth detection thresholds as well as heat pain and cold pain thresholds were determined using the TSA-II Neuro Sensory Analyzer (Medoc).

For most of the investigated traits we could show a significant heritable component with especially high estimates for hearing and touch traits. We could also show phenotypic correlations between mechanosensory traits of different modalities. For example, we found that the otoacoustic emission parameters are correlated to tactile acuity as well as to baroreflex sensitivity. In our study on the Usher’s Syndrome we found elevated thresholds for the detection of vibrations in a cohort of individuals carrying pathogenic mutations in the myo7a gene, whereas a cohort with Usher syndrome type 2 showed no significant touch impairment.

We could show a genetic influence that contributes significantly to the normal variation of different mechanosensory traits in humans. Furthermore, we found evidence for a common genetic influence on different mechanosensory traits in healthy people. We could also show for the first time that pathogenic mutation in one gene, myo7a, leads to a significant impairment of touch sensitivity.

Supported by the DFG
Nº 9
USH1C transcripts and harmonin protein expression in murine and human retina

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Purpose: The human Usher syndrome (USH) is the most frequent cause of inherited combined deaf-blindness. 9 identified USH genes encode proteins of diverse protein families. Former studies revealed that all known USH1 and USH2 proteins are organized in protein networks within the USH protein interactome. The USH1C scaffold protein harmonin is one of the organizers in those networks. Various alternative spliced transcripts are generated from the USH1C gene. Gene products are subgrouped into a, b and c isoforms depending on their modular composition of PDZ, PST and coiled-coil domains. So far, the specific functions and expression profiles of the various isoforms are unknown. Here we investigate whether harmonin b isoforms are expressed in murine retina and which isoforms are expressed in human retina in general.

Methods: The expression of harmonin isoforms in murine and human retina was analyzed by RT-PCR and Western blot analyses. Obtained PCR products were sequenced. The localization of harmonin in the human retina was studied by a combination of high resolution immunofluorescence and immunoelectron microscopy. Protein-protein interactions were investigated by GST-pull down assays.

Results: RT-PCRs revealed expression of harmonin a and b isoforms in murine retina using different primer sets confirming previous data on protein expression in mice. Harmonin PDZ domains utilized in GST-pull down assays recovered isoforms a and b from mouse retina extracts supporting their expression as well. Moreover we could show that harmonin is able to form heteromeric harmonin protein networks. In another set of experiments, we investigated harmonin expression in human retina. DNA sequencing of obtained transcripts clearly demonstrated the expression of A and B isoforms in human retina. Furthermore, we got evidence for other harmonin isoforms not described so far in human. The investigation of the subcellular localization of harmonin in human retina revealed that harmonin is localized in inner and outer segments of photoreceptor cells. Co-staining of harmonin with cone-specific fluorescent peanutagglutinin as well as immunoelectron microscopy illustrated that harmonin was prominent in outer segments of primate rod photoreceptor cells. Additionally, harmonin was differentially expressed in primate rod and cone synapses.

Conclusions: Present data reveal that different harmonin isoforms of all types are expressed in murine and human retinal photoreceptor cells. We assume that harmonin functions as an organizer of protein networks in the outer segments of primate rod photoreceptor cells and in primate cone synapses.

Supports: DFG GRK 1044/2 (UW); FAUN (UW); Pro Retina Deutschland (UW); Forschung contra Blindheit (UW); Forschungsförderung University of Mainz (KNW)
In Humans, mutations in the \textit{USH1c} gene can cause the most severe type of Usher Syndrome, which is characterized by an early onset of retinitis pigmentosa, balance defects and congenital deafness. Previous studies have shown that \textit{Ush1c} gene is required for the correct development of the acoustic and vestibular stimulus receptor: the hair bundle. The hair bundle (HB), or stereocilia, is a specialized organelle localized at the apical side of each mechanosensory cell in the inner ear, and, it is composed of interlinked actin filaments. In vertebrates, at least three canonical \textit{Ush1c} isoforms or transcript variants (tv) have been identified (tvA, tvB and tvC). These three variants encode for proteins containing PDZ protein interaction domains and are thought to act as scaffold binding proteins required for the assembling of Usher macro molecular complexes.

As their vertebrate counterparts, the \textit{ush1c/-} mutant and \textit{ush1c} morphant zebrafish has vision and hearing impairment, vestibular defects, and, fragmented or bent stereocilia at 5 days-post-fertilisation (dpf). In addition to this, a striking phenotype, present in both mutant and morphant at this stage, is the decreased number of hair bundles in the sensory patches of the zebrafish ear. Cell death and cell proliferation analysis suggest that this decrease is neither due to an increase of apoptosis or necrosis nor to a decrease in cell proliferation, suggesting that \textit{ush1c} is required for initial stereocilia development. Moreover, in the \textit{ush1c/-} mutant and \textit{ush1c} morphant, some Usher proteins are mislocalized such as ush2a and cip98a, suggesting that ush1c is required for the correct sub-cellular localisation. Interestingly, to date, the only canonical transcript variant detected in zebrafish, from 24 hour post-fertilization to 5 dpf, is the variant A (\textit{ush1c TvA}). Therefore, we hypothesize that \textit{ush1c_TvA} plays a major role during the biogenesis of the mechano-receptor, and we are currently investigating its role during this process.
Posters

Nº 11
Expression of the Usher syndrome type 3 gene (CLRN1) in zebrafish sensory cells

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\textbf{Purpose:} Usher syndrome (USH) is the most common form of combined blindness and deafness. Usher syndrome type 3 is the rarest subtype caused by mutations in the \textit{Clarin 1} gene (CLRN1). In this study, we investigate the expression and localization of \textit{clrn1} in zebrafish to elucidate the function of clrn1 in hearing, balance, and sight.

\textbf{Methods:} Zebrafish \textit{clrn1} expression was studied in sectioned zebrafish eyes and ears with \textit{in situ} hybridization. Clrn1 protein localization was studied with an antibody specific to zebrafish Clrn1. Morpholino oligonucleotides (MO) specific to \textit{clrn1} were used to inhibit \textit{clrn1} expression, and the functional and morphological effects of Clrn1 absence were studied with behavioural tests and histological studies.

\textbf{Results:} The \textit{in situ} hybridization studies showed zebrafish \textit{clrn1} expression in hair cells of the larval ear and neuromasts. In larval retina \textit{clrn1} expression was concentrated in the inner nuclear layer (INL), and present at lower levels in photoreceptor cells. This expression pattern persists in the adult retina. The clrn1 antibody studies showed similar localization dynamics with Clrn1 present in the INL and in photoreceptors of larval and adult retinas. When Clrn1 production was inhibited with a \textit{clrn1} specific morpholino, the injected larvae showed reduced levels of protein in the retina, slower optokinetic response and significant balance problems.

\textbf{Conclusions:} Our studies showed that both \textit{clrn1} RNA and protein are present in the zebrafish larval ear and eye, and that blocking \textit{clrn1} translation and thus presence of Clrn1 protein produces defects in vision, hearing, and balance.

\textbf{Support:} American Heart Association, European Molecular Biology Organization, De Blindas Vänner Foundation, NIH grants DC04186 and HD22486, Research Foundation of the University of Helsinki, The Eye and Tissue Bank Foundation (Finland) and Hope for Vision
Sensorineural deafness and progressive blindness associate to Igf-I deficit

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Insulin-like growth factor I (IGF-I) is a member of the family of insulin-related factors and plays a fundamental role in the development of sensory systems. Both IGF-I and its high affinity receptor IGFR1 are expressed in specific spatiotemporal patterns in the developing inner ear, postnatal cochlea and vestibular ganglia. Human IGF-I deficiency is associated with syndromic sensorineural deafness, infertility, mental retardation and decreased intrauterine-postnatal growth rates (ORPHA 73272; OMIM608747). Similarly, Igf1 null mice present severe hearing loss, aberrant inner ear morphology and key alterations in IGF-I intracellular targets, which includes lower activation of Akt and ERK1/2 and stronger activation of p38 kinase in the cochlea of E18.5 embryonic Igf1⁻/⁻ null mice. The comparison of Igf1⁻/⁻ and Igf1⁺/⁺ transcriptomes in E18.5 mouse cochleae using RNA microchips showed alterations in 231 genes, which were confirmed by using complementary techniques. Up-regulation and activation of the forkhead box M1 (FoxM1) transcription factor correlated with decreased nuclear expression of its target cyclin-dependent kinase inhibitor p27kip1. IGF-I deficit also caused a delayed maturation of neural cells, which was evidenced by the misexpression of the neural progenitor transcription factors Six6, Mash1 and of the myocyte enhancing factor 2 (MEF2) in the cochlea¹.

To explore the potential role of IGF-I in other sensory dysfunctions, we have studied the morphology and electroretinographic (ERG) responses of the Igf1⁻/⁻ null mouse retina during ageing. P360 Igf1⁻/⁻ null mice have an almost flat scotopic ERG response and a reduced photopic ERG response, while heterozygous Igf-1⁻/+ mouse still preserve both responses but decreased when compared with Igf1⁺/⁺. Besides this, the study of retinal function reveals that P360 Igf1⁻/⁻ null mice suffer important structural modifications in the first synapse of the retinal pathway that affect mainly the postsynaptic processes from horizontal and bipolar cells, while the Igf1⁻/+ mice just show small alterations.

In summary, IGF-I deficit associates to sensorineural deafness and early progressive blindness in the mouse, and support the use of the Igf-1⁻/⁻ mouse as a new model for the study of human syndromic sensory alterations.

PTC124-mediated read-through of a nonsense mutation causing Usher type 1C

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The Usher syndrome (USH) is the most frequent cause of inherited combined deaf-blindness. It is clinically and genetically heterogeneous, assigned to three clinical USH types of which the most severe type is USH1. The USH1C gene encodes the PDZ containing scaffold protein harmonin which is expressed in form of numerous alternatively spliced variants. Harmonin binds directly to all USH1 and USH2 proteins and is a key organizer in the USH protein network. So far no effective treatment for the ophthalmic component of USH exists. PTC124 is a promising compound for translational read-through of nonsense mutations leading to a premature termination stop. It currently gauged in clinical phase II for nonsense mutation in non-ocular diseases.

Here we investigated the potential of PTC124 as a treatment option for patients carrying a nonsense mutation in the USH1C gene (p.R31X) causing the USH1 disease. We demonstrated PTC124-mediated translational read-through of the p.R31X mutation in USH1C not only in cell culture but also in retinal explants. Our functional assays showed that the recovered harmonin expression restored harmonin’s scaffolding function and F-actin bundling activity. Furthermore, we compared the biocompatibility of PTC124 with the clinical approved read-through inducing aminoglycoside gentamicin. In this comparison PTC124, showed a much better biocompatibility in murine and human retinal explants.

PTC124’s high retinal compatibility combined with its transcriptional read-through efficacy emphasize the high potential of PTC124 as a therapeutic agent for the p.R31X nonsense mutation in USH1 as well as in other retinal genetic conditions.

Supports: FAUN, DFG (GRK1044), Foundation Fighting Blindness
Evidence for a regulation of SANS’ scaffold function by phosphorylation

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Purpose: The human Usher syndrome (USH) is clinically divided into 3 types (USH1-3) which are genetically heterogeneous: 12 identified USH genes encode proteins of diverse protein categories. All USH1 and USH2 proteins are organized in protein networks within the USH protein interactome. Previous analyses revealed direct binding partners of USH1G protein SANS (scaffold protein containing ankyrin repeats and SAM domain) indicating its crucial role in integrating USH protein networks and the involvement in cargo/vesicle transport processes in photoreceptor cells. Nevertheless, little is known about the composition of protein complexes organized by SANS and the regulation of assembly of USH protein complexes. We apply tandem affinity purification (TAP) strategies to analyze the composition and integration of complex compounds via direct physical binding and indirectly by bridging molecules. The combination of the two small tags StrepII and FLAG (SF-TAP) allows a fast but gentle in situ isolation of protein complexes in their cellular context without digestion.

Methods: SF-SANS-TAP was performed and proteins of recovered complexes were analyzed by mass spectrometry. SANS- and p-Serine-antibodies were used in Western-Blot analyses. Phosphorylation and dephosphorylation of SF-N-SANS was tested in vitro with protein kinase 2 (CK2), serine/threonine phosphatase 2A (PP2A) and light/dark adapted bovine retina extracts. Co-localization studies in the murine retina were performed by immunohistochemistry. Binding capacity of untreated, de- and phosphorylated SF-N-SANS was analyzed by in vitro pull down analysis.

Results: LC-MS/MS analyses of SF-N-SANS and SF-C-SANS TAPs did not show significant differences. However, we identified numerous potential complex partners belonging to different protein categories, e.g. cytoskeletal, scaffold-, and signaling proteins, including previously described SANS-interacting proteins (harmonin and tubulins). We additionally discovered several novel proteins, e.g. serine/threonine phosphatase 2A (PP2A). Further assessment revealed that serine/threonine sites of SANS are light dependently phosphorylated by CK2 and dephosphorylated by PP2A. Immunocytochemistry revealed partial co-localization of SANS with CK2 and PP2A in photoreceptor cell compartments. Finally, binding capacity of SANS was changed depending on its phosphorylation status.

Conclusion: The SF-TAP procedure is a powerful tool for the in situ decipherment of protein complex compositions and enables identification of potential SANS complex partners under physiological conditions. Present data confirmed the association of SANS with the microtubule cytoskeleton and the interaction with USH1C protein harmonin. Furthermore, we gathered first evidence for the regulating mechanisms of USH protein complex assembly. Our findings further enlighten USH protein networks and thereby pathophysiological mechanisms causing sensorineuronal degenerations in human USH.

Supports: DFG-GRK 1044/2, FAUN Stiftung, Pro Retina Deutschland; Forschung contra Blindheit-Initiative Usher Syndrom
Nº 15
The effect of the administration of Buthionine sulfoximine and glutathione ethyl ester in a model of retinitis pigmentosa

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Purpose: We have administered Buthionine sulfoximine (BSO) an inhibitor of gamma-glutamylcysteine synthetase to wild type and rd1 mice, to study its influence over retina development and retinal pathology. We have also administered glutathione ethyl ester to wild and rd1 mice. Since by itself GSH is not effectively transported into cells, several alternatives are available to increase GSH within cells and GSH esters are hydrolyzed to GSH in cells.

Methods: Two groups of wild type and rd1 mice received an intraperitoneal (ip) injection of BSO (1.5 g/kg body weight) once daily for eight consecutive days starting from postpartum day 3. In addition, another two group of wild type and rd1 pups received ip injections of BSO and a combination of antioxidants orally or glutathione ethyl ester. Malondialdehyde (MDA) and glutathione (GSH) concentrations as well as glutathione peroxidase (GPx) and glutathione reductase (GSSG-R) activities were measured and TUNEL, as well, as GSH immunostaining were performed.

Results: BSO treatment decreased GSH in wild type and rd1 retinas as well as the ratio GSH/GSSG, the administration of antioxidants to BSO treated animals produced an small but non significant increase in both parameters. When BSO was administered daily antioxidants failed to increase retinal glutathione concentration or to decrease the number in TUNEL positive cells. In all groups BSO induced cataracts.
Usher Syndrome (USH) is an autosomal recessive disorder characterized by congenital sensorineural hearing loss, vestibular dysfunction, and progressive retinitis pigmentosa (RP). Three clinical types are recognized based on the severity, age of onset of RP, progression of hearing loss and extent of vestibular involvement (USH1, USH2 and USH3). Eleven loci have been mapped and nine genes identified (MYO7A for USH1B, USH1C for USH1C, CDH23 for USH1D, PCDH15 for USH1F, SANS for USH1G, USH2A for USH2A, VLGR1b for USH2C, WHRN for USH2D and USH3A for USH3). We performed haplotype, SSCP and sequencing analysis in order to define USH genetic subtypes in 23 Colombian families with USH: 9 USH1 and 14 USH2. Audiometry, campymetry, electroretinography, direct fundus and angiography were performed in order to define clinical diagnosis and obtain detailed clinical information. We defined genetic subtypes as follows: six families USH1B, one family USH1D, one family USH1F, one family USH1G, 13 families USH2A and one family USH2C. Here, we report detailed information about clinical findings in affected patients by USH subtypes. We describe in detail the evaluation of these patients and the possible phenotype associated with the USH subtypes.
Cochlear implantation for Usher syndrome type III: Finnish experience

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Purpose: Usher syndrome (USH) is the most common form of combined blindness and deafness. Usher syndrome type 3 (USH3) is the rarest subtype with progressive hearing loss and variable vestibular dysfunction. From 1995 to 2005 19 USH3 patients received cochlear implants in five different Finnish university hospitals. This study was done to evaluate the results and benefits of cochlear implantation in patients with USH3.

Methods: Saliva samples were collected to verify the USH3 mutation from these patients. The patients with cochlear implants filled three questionnaires: Glasgow Benefit Inventory (GBI), Glasgow Health Benefit Inventory (GHBI) and a questionnaire made for this study. Audiological data was collected from patient records.

Results: All the USH3 patients participating in this study had the Finnish founder mutation (p.Y176X). The preoperative hearing was 108 ± 8 dB and the preoperative aided hearing was 57 ± 17 dB (**, p<0.01, t-test). The speech discrimination scores preoperatively (5-10 years) showed a mean of 17 ± 25% and prior the surgery 4 ± 9%. Postoperative discrimination was significantly better compared to the preoperative value (52 ± 33%, ***, p<0.001). The GBI total score was 33 ± 19. Total score of GHSI data related to hearing was 59 ± 9 and vision related GHSI was 56 ± 14.

Conclusions: Cochlear implantation is beneficial to patients with USH3 and patients learn to use the implant independently, despite their visual handicap. GBI results show that cochlear implantation is as successful with USH3 patients as with other postlingually deafened patients.

Support: De Blindas Vänner Foundation, Leo, Mary och Mary-Ann Hackman Foundation, The Eye and Tissue Bank Foundation (Finland), Hope for Vision.
Prevalence of different types of Chronic Stress in individuals with Usher syndrome

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Purpose: The purpose of the study was to investigate whether people with Usher syndrome experience higher frequencies of stress than the general population. Dual sensory impairment of vision and hearing in Usher syndrome leads to a significant impact on activities and participation in society which can cause high levels of stress. The main areas affected are communication as well as Orientation and Mobility. Together these two factors cause isolation. Since both distance senses are involved, the loss of one sense cannot be compensated by the other. Thus, individuals affected are particularly at risk of developing stress.

Methods: The standardized “Trierer Inventory of Chronic Stress (TICS)” by Schulz, Schlotz and Becker was used to assess stress in 17 subjects aged between 23 and 58 (x=41 years). TICS measures the frequency of different types of chronic stress on nine different stress scales. It also calculates a total score (‘Total Score Chronic Stress’) using a screening scale. In addition, a questionnaire was developed by the author in order to examine possible causes of stress. Six hypotheses were constructed and tested using standard test theory.

Results: Subjects in this study experienced more stress than individuals in the general population. They had significantly higher stress experiences in categories such as “Social Isolation” (p=0.023) and “Overwhelmed at Work” (p=0.023) than the standardization group. Also a significantly higher “Total Score Chronic Stress” (p=0.009) was seen in individuals with Usher syndrome.

Conclusion: The results indicate that intensive measures of rehabilitation addressing the needs of individuals with dual sensory impairment are necessary. Interventions need to be geared towards adapting the environment (especially the work environment) with special (assistive) technologies and aids as well as towards developing and improving personal competency in Orientation and Mobility, communication and Daily Living Skills. TICS is a tool assessing individual stress experiences in the general population. It is necessary to develop an instrument that identifies stress factors specific to Usher syndrome.
Usher syndrome Missense analysis (USMA): preview of a new bioinformatics tool

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Assessing the pathogenic effect of missense variants is one of the numerous challenges in human genetics. As molecular tests can be time and money consuming, or not applicable, a number of bioinformatics software has been developed to predict the impact of these variants. Although very useful, most of these tools are designed to perform predictions on all positions of every known gene, and thus, are standardised in this way. The most popular (SIFT, polyphen, AlignGVGD...) are based on sequence conservation and sophisticated algorithms and/or statistical analyses. The user is provided a score translated into an comprehensible result (e.g. tolerated, likely pathogenic…). Tested on thousands of variants, the most effective have a predictive value around 0.8.

Usher Syndrome Missense Analysis (USMA) is a web-based tool that has been designed to study missense variants (also called non-synonymous coding single nucleotide polymorphisms, nsSNPs) with the objective of providing to the biologist the most relevant information related to the variant.

This software is dedicated to Usher proteins and includes ortholog alignments as well as deeper domain alignments (analysed separately, up to 2000 sequences). For each ortholog alignment, the probability that an invariant position is functionally constraint has been calculated following the method described by Greenblatt et al. in 2003. USMA also uses the concept of Average Alignment Percentage Identity (calculated using BioPerl), that determines the conservation rate of a region. In addition, the tool includes secondary structure predictions (made using Psi-pred, using as input Multiple Protein Sequences Alignments), and re-calculated frequencies of appearance of each of the 20 amino-acids in secondary structures, based on 8,365 non-redundant 3D structures, representing 1,598,587 residues. The software also provides, when applicable, 3D models of the wild-type and mutant structures, an analysis of the potential impact of the mutation, in addition to relevant structure pictures and a fully functional Jmol applet for both models. Therefore, by manipulating the 3D structures, the user will be able to perform his own investigation.

USMA analyses the data and performs fastidious tasks (calculating conservation scores, counting the number of hydrogen bonds in the wild-type and mutant structure…), and renders the results to the user. The role of the biologist will be to provide the name of a gene and the nsSNP to study, and to interpret the results.

By compiling USMA to other clinico-biological information, the interpretation of any new missense variant in Usher genes will be greatly improved.
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**USH2A mutation profile of 19 arRP Spanish patients**

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**Introduction:** Retinitis Pigmentosa (RP) is an inherited retinal dystrophy caused by a progressive loss of photoreceptors. To date, 43 genes have been associated with RP, 25 of which are associated with autosomal recessive retinitis pigmentosa (arRP). Due to its phenotypic and genetic heterogeneity, molecular diagnosis of arRP is highly complex and time consuming. The *USH2A* gene was initially shown to be involved in Usher syndrome; however, several studies have revealed that it is also associated with 4-7% of arRP cases.

**Purpose:** The aim of this work was to screen the *USH2A* gene by direct sequencing in order to identify the causative mutations in a panel of 19 Spanish subjects affected by arRP.

**Patients:** Patients were selected for this study because they were found to carry one heterozygous mutation in the *USH2A* gene, after being tested using a specific arRP genotyping microarray.

**Results:** The screening of the *USH2A* gene in these 19 patients led to the identification of a second putative pathologic mutation in 10 cases. Most of these variants were novel: one isocoding (p.V191V), three amino acid changes (p.T1238R, p.S4420Y, p.A5102D), one deletion (c.13745delT) and three variants located at intronic positions (IVS59+2T>G, IVS38+43C>T, IVS69+25G>T).

**Discussion:** The mutation screening is still in progress in those patients with no second putative pathologic mutation identified so far. However, it is possible that some patients carry the second mutation at deep intronic sequences, or that the second mutation is a large heterozygous deletion/duplication that escapes direct sequencing screening.
CDH23 compound heterozygotes: nonsyndromic deafness DFNB12 alleles are dominant to type 1 Usher syndrome USH1D alleles with respect to vestibular dysfunction and retinitis pigmentosa

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Nonsyndromic recessive hearing loss at the DFNB12 locus is associated with missense mutations of CDH23, whereas allelic truncating mutations of CDH23 (nonsense, splice-site, and frame-shift) as well as some missense mutations are associated with Usher syndrome type 1D (USH1D). The phenotypic consequence of a DFNB12 allele of CDH23 in combination with an USH1D allele of CDH23 in trans (i.e a compound heterozygote) is not known and cannot be predicted from current knowledge. In this study we screened affected singletons and families segregating nonsyndromic deafness and USH1 for mutations in CDH23 to ascertain CDH23 compound heterozygotes. We identified an individual presenting with congenital profound hearing loss affecting all frequencies. The subject has a novel CDH23 frame-shift mutation, c.8883_8884insT, in trans with a previously published CDH23 missense substitution, p.F1888S, known to be associated with nonsyndromic hearing loss. The truncating allele c.8883_8884insT is presumed to be an USH1D allele. The subject has normal vestibular function shown by caloric stimulation and video-nystagmography, and normal retinæ confirmed by funduscopy and electroretinography. One possible explanation for her phenotype is that a DFNB12 allele is phenotypically dominant over an USH1D allele in both the retina and vestibular neurosensory organs. We also report here a second family segregating four mutant alleles of CDH23. Of the four family members, two have nonsyndromic deafness and two have Usher syndrome type 1. Their observed genotypes and phenotypes are consistent with our proposed model in which hypofunctional cadherin 23 protein is sufficient to preserve retinal and vestibular function but not hearing. These observations have implications for therapy of RP in type 1 Usher syndrome subjects.
Mutational spectrum of Usher syndrome in Colombia

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Introduction: Usher Syndrome (USH) is an autosomal recessive disorder characterized by congenital sensorineural hearing loss, vestibular dysfunction, and progressive retinitis pigmentosa (RP). Three clinical types are recognized based on the severity, age of onset of RP, progression of hearing loss and extent of vestibular involvement (USH1, USH2 and USH3). Eleven loci have been mapped and nine genes identified (MYO7A for USH1B, USH1C for USH1C, CDH23 for USH1D, PCDH15 for USH1F, SANS for USH1G, USH2A for USH2A, VLGR1b for USH2C, WHRN for USH2D and USH3A for USH3).

Methods: We ascertained 73 Colombian families with USH: 45 USH1, 27 USH2 and 1 family as possible USH3. Among these families, we selected 16 for haplotype analysis using short tandem repeat polymorphic markers (STRPs) to map these families to known USH loci. Sequencing was performed to screen for mutations in USH1B, USH1D, USH1F, USH2A and USH2C, prioritizing exons known for their higher mutability. USH1G gene was fully sequenced. In the remaining 57 families, we selected the propositus (36 USH1 and 21 USH2) for mutational analysis. In USH1 propositi, we performed screening for two mutations in MYO7A and one mutation in SANS genes. For USH2 propositi, we performed single strand conformational polymorphic analysis (SSCPs) and sequencing for the usherin short isoform.

Results and Discussion: By haplotype segregation, five families mapped to USH1B, five families mapped to USH2A, and one family each mapped to USH1D, USH1F, USH1G and USH2C. These results are similar to those reported in other populations. One family USH1 and the USH3 family did not show segregation with any locus. Mutational screening identified two mutations in MYO7A gene (USH1B): p.R634X in one family and p.R2024X (novel mutation) in another family. The p.R2024X mutation was identified in a heterozygous state. We also identified a novel large deletion in SANS (USH1G). Screening of USH2A identified two mutations: c.2299delG in one family and six USH2 propositi, and p.R334W in four USH2 propositi (a higher frequency than expected). Two propositi were compound heterozygotes for the c.2299delG and p.R334W mutations. We also identified a promoter variant in USH2A that may be disease-associated. No mutations were identified in CDH23, PCDH15 and VLGR1b, suggesting a different frequency and distribution for USH mutations in the Colombian population.

Conclusion: We were able to define the genetic subtype in 23 Colombian families and identified five different mutations (two novel) and one possible mutation. The p.R334W in USH2A is the second most frequent mutation in the Columbian USH2A population.
Nº 23
Implication of GPR98 in Usher syndrome type 2

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Three genes are known to be involved in Usher Syndrome type 2: USH2A, GPR98 and WHRN. USH2A has been the first described gene and remains the most implicated.

Today, more than 230 possibly pathogenic variants have been identified in this gene. On the other hand, few pathogenic variants have been published for GPR98 and WHRN. The purpose of this study was to analyse patients who did not carry any mutation in USH2A. To complete this work, we have implemented in our laboratory the direct sequencing of these two underrated genes. To be as exhaustive as possible, each described exon and its intronic flanking regions of GPR98 and WHRN (i.e. 90 exons for GRP98 and 12 for WHRN) is analysed by PCR-sequencing. Each new variation is stored in USHVaM (laboratory database) to be studied and characterized.

At this date, 21 variants were observed in WHRN, in 31 patients, but none of them can be considered as pathogenic. For GPR98, one 116 variants in 21 patients were identified. Seventy-seven variants were already described either in literature or in central core databases. Twenty-three others were novel but in silico studies and controls screening provided evidence for non-pathogenicity.

The remaining 16 variants have been extensively studied and are composed of 1 intronic and 4 exonic small deletions, 2 small duplications and 9 substitutions. Each of these alterations was studied with in silico software to guide us for the ex vivo splicing experiments. This work allowed the characterization of 6 frameshift mutations and 1 nonsense leading to PTCs, 3 splice mutations, and 5 missense variants susceptible to alter the protein structure.

Finally, our USH2 cohort consists in 154 patients. One hundred thirty eight (89.6 %) carry 1 or 2 probably pathogenic mutations in USH2A, none in WHRN and 8 (5.6 %) in GPR98.
Large genomic rearrangements in the Usher genes

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Usher syndrome is both clinically and genetically heterogeneous. Three clinical subtypes are defined in respect to the vestibular dysfunction and the degree of hearing loss and, at this date, 9 genes are known to be responsible. For molecular diagnosis, we have developed a strategy to prioritize genes to be sequenced. Depending on the molecular findings, a patient can be analysed in up to 9 genes (exons + flanking intronic regions). More than 220 USH families have been analysed so far. We identified the pathogenic mutations in most cases; however, 15% of our cohort carries either one or no mutation in any of the genes. The absence of mutation can sign the presence of large rearrangements that remain undetected by sequencing. Semi-quantitative multiplex assays, as well as MLPA kit designed for PCDH15, allowed to detect 12 large genomic rearrangements in 11 families and to identify the breakpoints for 6 of them. Recently our laboratory has developed a high density microarray analysis using the Roche Nimblegen technology. This customised CGH microarray chip enables the detection of copy number variants (CNVs) in the 9 Usher genes and facilitates the selection of PCR primers in close vicinity to the identified breakpoints. We could detect 6 additional deletions/duplications in 7 new families and characterize breakpoints by sequencing junction fragments. This study shows that large genomic rearrangements are implicated in at least 8 % of the Usher cases involving so far MYO7A (4 cases), PCDH15 (6 cases), CDH23 (1 case) and USH2A (7 cases). Study of patients susceptible to carry deletions or duplications in GPR98 is in progress.

Screening for large genomic rearrangements is mandatory for efficient Usher molecular diagnosis.
Mutation screening of the USH2A gene in Spanish patients with Usher syndrome type II

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Usher Syndrome type II (USH2) is an autosomal recessive disorder, characterized by moderate to severe hearing impairment, normal vestibular function and progressive visual impairment due to retinitis pigmentosa. Among the three genes implicated, mutations in the USH2A gene account for 75-80% of the USH2 cases. To identify the genetic cause of the disease and determine the frequency of USH2A mutations in our cohort of 89 unrelated USH2 Spanish patients, we carried out a mutation screening by direct sequencing of the 72 coding exons of this gene. The screening is still in progress, but up to date, mutations have been identified in 48 out of the 89 cases studied. 29 of these mutations are novel: 4 nonsense, 8 frame shift, 15 missense and 2 putative splice-site mutations. The results provide a wide spectrum of USH2A mutations in Spanish families with Usher Syndrome type II.
A new member of the USH protein network: USH1G protein SANS interacts with Myomegalin in mammalian photoreceptor cells

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Purpose: The human Usher syndrome (USH) is the most common form of combined deaf-blindness. Usher type I (USH1), the most severe form, is characterized by profound congenital deafness, constant vestibular dysfunction and pre-pubertal onset of retinitis pigmentosa. USH1G encodes the SANS (scaffold protein containing ankyrin repeats and SAM domain) protein which contains putative protein-binding motifs. Recent studies revealed SANS scaffolding function in the protein interactome related to USH through its interactions with myosin VIIa (USH1B), harmonin (USH1C) and whirlin (USH2D). The aim of the study was to further enlighten SANS cellular functions by identifying novel interaction partners of SANS.

Methods: Y2H screens of retinal cDNA libraries were performed with the central domain of SANS. Expression analyses were assessed by RT-PCR and Western blots. Protein-protein interactions were validated by GST pull-down and additional cell based assays. Indirect immunofluorescence assessed co-localization of identified partners in cell culture and mouse, macaque as well as human retinas.

Results: The phosphodiesterase 4D interacting protein Myomegalin (PDE4DIP) was identified and confirmed as an interaction partner of the USH1G protein SANS. Myomegalin, a recently identified Golgi and centrosome associated protein, was expressed in a variety of tissues including the murine, macaque and human retina. In retinas, Myomegalin was localized in the RPE, photoreceptors, inner nuclear layer and ganglion cell layer. Co-localization of SANS and Myomegalin was found in the ciliary region and the inner segment of photoreceptor cells. Co-localization of both interacting partners at centrosomes and spindle poles of cultured cells strengthen their microtubule association.

Conclusions: Myomegalin directly interacts with the USH1G protein SANS and integrates Myomegalin in the multifunctional protein interactome related to USH. Localization of both proteins at microtubule enriched compartments suggests a microtubule-associated function. A role of the SANS-Myomegalin complex in microtubule-dependent inner segment cargo transport towards the ciliary apparatus of photoreceptor cells is proposed. Any defect in the interaction of proteins may lead to the disruption of the whole Usher protein network and may result in the degeneration of neuronal retina, the clinical phenotype in the retina of USH1 patients.

Supports: FAUN; FFB; DFG
Nº 27
Searching for the components of the Dachsous pathway in Drosophila and their orthologs in mouse

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Dachsous (Ds) protein belongs to the cadherin superfamily of adhesion proteins in Drosophila melanogaster. It is a protocadherin with 27 tandem arranged cadherin motifs in its extracellular region, a transmembrane domain and a unique cytoplasmic domain. In humans, two related genes have been identified - DCHS1 and DCHS2 - with a sequence homology of 26% and 28%, respectively. However, Ds also shares main structural homology with Cadherin 23.

Ds is required for several processes in the development of Drosophila, including cell proliferation control, planar cell polarity (PCP) and the proximo-distal patterning of appendages. Interestingly, defects in these processes are associated to mutations of the Wnt, TGFβ or Hedgehog signalling pathways, suggesting a modulation of these signals by Ds. The molecular components involved in such interaction are unknown.

Cadherin 23 is required for hair cell development in the inner ear. Mutant mice for cadherin 23 show hearing impairment due to hair cell malformations and mutant alleles of the gene are associated with Usher syndrome type 1 (USH1D).

Making use of the advantages of Drosophila as a model organism, we aim to:

1) Identify the components of Ds pathway in Drosophila. For such purpose we use two different approaches:
   a. A yeast-two-hybrid assay, using Ds as bait.
   b. Genetic analysis in different developmental processes, that require tissue polarization, during embryonic stages.

2) Identify the ortholog of Ds in mice. For such purpose we generate transgenic flies overexpressing Dchs1, Dchs2 and Cdh23 in order to rescue the Ds mutant phenotypes.

Due to the proposed parallelism between Ds and Cdh23, we expect that the identification of novel components of Ds pathway can also provide new insights about the molecular mechanism of Cdh23 and, therefore, new information for a better diagnosis of Usher syndrome.