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Usherin defects lead to early-onset retinal dysfunction in zebrafish

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Abstract

Mutations in *USH2A* are the most frequent cause of Usher syndrome and autosomal recessive nonsyndromic retinitis pigmentosa. To unravel the pathogenic mechanisms underlying *USH2A*-associated retinal degeneration and to evaluate future therapeutic strategies that could potentially halt the progression of this devastating disorder, an animal model is needed. The available *Ush2a* knock-out mouse model does not mimic the human phenotype, because it presents with only a mild and late-onset retinal degeneration. Using CRISPR/Cas9-technology, we introduced protein-truncating germline lesions into the zebrafish *ush2a* gene (*ush2a^{rmc1}*: c.2337_2342delinsAC; p.Cys780GlnfsTer32 and *ush2a^{b1245}*: c.15520_15523delinsTG; p.Ala5174fsTer). Homozygous mutants were viable and displayed no obvious morphological or developmental defects. Immunohistochemical analyses with antibodies recognizing the N- or C-terminal region of the

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ush2a-encoded protein, usherin, demonstrated complete absence of usherin in photoreceptors of *ush2a^{rnc1}*, but presence of the ectodomain of usherin at the periciliary membrane of *ush2a^{b1245}*-derived photoreceptors. Furthermore, defects of usherin led to a reduction in localization of USH2 complex members, whirlin and Adgrv1, at the photoreceptor periciliary membrane of both mutants. Significantly elevated levels of apoptotic photoreceptors could be observed in both mutants when kept under constant bright illumination for three days. Electroretinogram (ERG) recordings revealed a significant and similar decrease in both a- and b-wave amplitudes in *ush2a^{rnc1}* as well as *ush2a^{b1245}* larvae as compared to strain- and age-matched wild-type larvae. In conclusion, this study shows that mutant *ush2a* zebrafish models present with early-onset retinal dysfunction that is exacerbated by light exposure. These models provide a better understanding of the pathophysiology underlying *USH2A*-associated RP and a unique opportunity to evaluate future therapeutic strategies.

Keywords

Usher syndrome; usherin; *ush2a*; retinal dysfunction; zebrafish; retinitis pigmentosa

1. INTRODUCTION

Usher syndrome is a rare genetic condition characterized by hearing impairment and a progressive loss of visual function as a consequence of Retinitis Pigmentosa (RP). The latter often results in legal blindness by the sixth decade of life (Sandberg et al., 2008). Currently there are no treatments for retinal degeneration in patients with Usher syndrome, although they benefit from hearing aids or cochlear implants. Usher syndrome is classified into three types (USH1, USH2 and USH3), varying in severity of hearing impairment, age at which RP is diagnosed, and presence or absence of vestibular dysfunction (Tazetdinov et al., 2008). Approximately two-thirds of USH patients present with USH2 (Millan et al., 2011), up to 85% of whom can be explained by mutations in *USH2A* (McGee et al., 2010; Yan and Liu, 2010). Mutations in *USH2A* are also the most frequent cause of autosomal recessive RP (arRP), accounting for 7-23% of arRP cases (McGee et al., 2010). Approximately 600 different, mostly private, mutations are evenly distributed over the gene and include point-nonsense, frame-shift, splice-modulating, and missense variants⁶. However, there are a number of mutations that originate from a common ancestor and are therefore observed more frequently (Aller et al., 2010; Baux et al., 2014; Pennings et al., 2004). The two most commonly found *USH2A* mutations are c.2299delG; p.Glu767fs and c.2276G>T; p.Cys759Phe, both residing in exon 13. Bi-allelic truncating defects of *USH2A* (nonsense mutations, frameshift mutations, or mutations that affect splicing), most often result in USH2, whereas the presence of at least one hypomorphic *USH2A* allele generally results in non-syndromic arRP (Lenassi et al., 2015). The distribution of known mutations and neutral variants from the LOVD database for *USH2A* does not reveal a particular mutation tolerant or intolerant region of the gene that could pinpoint particularly important functional domains (Baux et al., 2014). Despite ongoing efforts, little is known about either the physiological role(s) of the usherin protein in photoreceptors or the pathophysiological mechanism underlying *USH2A*-associated RP (Hartong et al., 2006; McGee et al., 2010).

The *USH2A* transcript in retina consists of 72 exons and encodes a protein of 5,202 amino acids (usherin) (Adato et al., 2005; van Wijk et al., 2004). Moreover, a cochlea-specific exon has been identified between exons 70 and 71 that encodes 24 additional amino acids (Adato et al., 2005). Usherin contains an N-terminal signal peptide, a Lam-G like domain, a LamNT domain, 10 EGF-lam domains, 4 FN3 domains, two laminin G (LamG) domains, 28 FN3 domains, a transmembrane domain, and a short intracellular region with a C-terminal class I PDZ-binding motif. It is generally thought that usherin has a structural role at the periciliary region of the photoreceptor, where it is held in place via its interactions with harmonin (*USH1C* encoded), SANS (*USH1G* encoded) and whirlin (*WHRN* encoded, *USH2d*) (Chen et al., 2014; Reinert et al., 2005; Sorsuch et al., 2017; van Wijk et al., 2006; Yang et al., 2010; Zou et al., 2011). At the periciliary region, usherin possibly stabilizes the photoreceptor connecting cilium by an extracellular interaction with Adhesion G protein-coupled receptor V1 (*ADGRV1*; previously known as GPR98 or *VLGR1*) (Adato et al., 2005; Liu et al., 2007; Maerker et al., 2008; Overlack et al., 2011).

Understanding the molecular mechanisms underlying photoreceptor dysfunction in *USH2A*-associated RP and the development of treatment strategies have been severely hampered by the absence of suitable cellular or animal models mimicking the human phenotype. Although mutant mouse models are commonly used to study RP and test therapeutic strategies, for *USH* and several other types of RP the retinal phenotypes in mouse models do not mimic that of patients with defects in the orthologous genes (Slijkerman et al., 2015). In contrast, retinal dysfunction from a very young age is observed in zebrafish *USH1* gene mutants (Blanco-Sanchez et al., 2017). Zebrafish larvae lacking *Myo7aa* (*USH1b*), harmonin (*USH1c*) or *Pcdh15b* (*USH1f*) function show reduced electroretinogram (ERG) traces by 5 to 7 days post fertilization (dpf) (Phillips et al., 2011; Seiler et al., 2005; Wasfy et al., 2014). Additionally, photoreceptor-specific degeneration has been shown to occur in *ush2a* depleted morphant larvae (Ebermann et al., 2010) as well as in *myo7aa* mutant larvae exposed to elevated light levels (Wasfy et al., 2014).

In this study, we generated and characterized two *ush2a* mutant zebrafish models (*ush2a^{mcl}*: c.2337_2342delinsAC; p.Cys780GlnfsTer32 and *ush2a^{b1245}*: c.15520_15523delinsTG; p.Ala5174fsTer) to study usherin function in the retina. Subsequent functional analyses showed that usherin is absent from photoreceptors in *ush2a^{mcl}*, but that the extracellular domain of usherin can still be detected at the periciliary membrane of *ush2a^{b1245}*-derived photoreceptors. The levels of usherin interaction partners *Whrna* and *Whrnb* (whirlin) are reduced at the photoreceptor periciliary membrane of homozygous *ush2a^{mcl}* larvae, whereas in homozygous *ush2a^{b1245}* larvae only the level of *Whrna* is affected. Furthermore, mutant zebrafish display elevated levels of apoptotic cells in the outer retina as compared to strain and age-matched wild-type zebrafish upon constant light rearing. We further found that ERG traces are notably attenuated in both mutants, indicating impaired outer retinal function. These mutants are the first genetic animal models for *ush2a* that present with early-onset retinal dysfunction.

2. MATERIALS AND METHODS

2.1 Zebrafish maintenance and husbandry

Experimental procedures were conducted in accordance with international and institutional guidelines (Dutch guidelines, protocol #RU-DEC 2012-301; Swiss guidelines, Veterinäramt Zürich TV4206 and University of Oregon IACUC guidelines). Wild type adult Tupfel Long fin (TLF) or Oregon AB* zebrafish were used. The zebrafish eggs were obtained from natural spawning of wild-type or mutant breeding fish. Larvae were maintained and raised by standard methods (Kimmel et al., 1995).

2.2 CRISPR/Cas9 design and microinjection

For the *ush2a^{mc1}* allele, oligos for generating guide RNAs were designed using the ZiFiT targeter software (Sander et al., 2007). Oligos were subsequently ordered from Integrated DNA Technologies. Annealing of oligos was performed in a buffer (1 M NaCl, 10 mM EDTA and 100 mM Tris-HCl pH7.5) by incubation at 90°C for four minutes, followed by a ten minute-incubation step at 70°C and gradual cooling (5°C per two minutes) to 16°C. The annealed oligos were immediately ligated into the a BsaI (New England Biolabs, #R0535S) linearized pDR271 vector (Addgene plasmid #42250) using T4 ligase (New England Biolabs, #M0202). The oligo and surrounding sequences were excised from the pDR274 vector using DraI (New England Biolabs, #R0129S). The excised DNA band (284 basepairs) was subsequently used as a template for *in vitro* transcription using the MAXIscript® T7 Transcription Kit (Ambion life technologies, #AM1314) according to the manufacturer's protocol. Obtained transcripts were purified using the MEGAClear™ Transcription Clean-Up Kit (Ambion life technologies, #AM1908). For the *ush2a^{b1245}* allele, gene specific oligos were designed by flanking the 20 bp target sequence with T7 promoter and gRNA sequence: 5'-AATTAATACGACTCACTATA-[20 bp Target Sequence]-GTTTTAGAGCTAGAAATAGC-3'. The templates for gRNA syntheses were PCR amplified using the gene specific oligo with a gRNA scaffolding primer: 5'-GATCCGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC-3'. As input 13.4 µl water, 4ul 5x Phusion DNA polymerase buffer, 0.4 µl dNTPs (10 µM), 1 µl of gene specific oligo (10 µM), 1 µl of gRNA scaffold oligo (10 µM), 0.2 µl Phusion DNA polymerase. The cycling conditions were as follows: 98°C 30 seconds, 40 cycles of 98°C 10 seconds, 60°C 10 seconds, 72°C 15 seconds and 72°C 10 minutes. PCR products were column purified and used to prepare gRNA with the Ambion T7 megascript kit (AM1344) as per manufacturer's instructions. A zebrafish codon-optimized Cas9 containing vector (pT3TS-nCas9n; Addgene plasmid #46757) was used to generate Cas9 mRNA. The vector was linearized using XbaI (NEB, #R0145S) and used as a template for an *in vitro* transcription reaction using the mMACHINE® T3 Transcription Kit (Ambion life technologies, #AM1348) according to manufacturer's instructions. Transcripts were purified using the MEGAClear™ Transcription Clean-Up Kit (#AM1908). Zebrafish embryos at a 1-cell stage were injected with 1 nl of a mixture containing gRNA (6 ng/µl), Cas9 mRNA (150 ng/µl), KCl (0.2 M) and phenol red (0.05%) using a Pneumatic PicoPump pv280 (World Precision Instruments) for the generation of the *ush2a^{mc1}* allele. To generate the *ush2a^{b1245}* allele, one-cell stage zebrafish embryos were injected with 1 nl of a mixture containing gRNA (100 ng/µl), Cas9 mRNA

(150 ng/μl), KCl (0.2 M) and phenol red (0.05%) using an MPPI-2 Pressure Injector with a BP-15 Back Pressure Unit (Applied Scientific Instrumentation, Oregon USA). After injection, embryos were raised at 28.5°C in E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄), supplemented with 0.1% (v/v) methylene blue. At 2.5 days post fertilization, part of the injected embryos were analyzed for the presence of desired mutational events. When mutations could be detected, the remainder of injected embryos was raised.

2.3 Genotyping

Genomic DNA was isolated from pools of 15 larvae after incubation in 75 μl lysis buffer (10 mM Tris HCl pH=8.2, 10 mM EDTA, 100 mM NaCl and 0.5 % SDS) supplemented with freshly added proteinase K (final concentration of 0.20 mg/ml, Invitrogen #25530049) at 55°C for two hours. The isolated genomic DNA was subsequently used as a template in a PCR. Primers used for the amplification of *ush2a* exon13 are 5'-TCCACCAACAGAATCTAAATCTTTC-3' and 5'-CTGATTTGTAAATGGTGTGGG-3' and primers used for the amplification of *ush2a* exon71 are 5'-CATGTTTTGGTTATCTGTTCTTCT-3' and 5'-GACAGCGGAATGGTGAGATAAAC-3'. The obtained amplicons were cloned into a pCR[®]4-TOPO[®] vector (Invitrogen, #450030) according to manufacturer's instructions. Individual clones were analyzed for the presence of mutational events using the ABI PRISM Big Dye Terminator Cycle Sequencing V2.0 Ready Reaction kit and the ABIPRISM 3730 DNA analyzer (Applied Biosystems).

2.4 Transcript analysis

Pools of 15 larvae were snap frozen in liquid nitrogen and subsequently homogenized in 500 μl QIAzol (Qiagen, #79306) using a 25 gauge 16 mm needle. Total RNA was isolated using phenol:chloroform extraction and precipitated using isopropanol. Extracted total RNA was further purified and DNase treated using a NucleoSpin[®] RNA II Isolation kit (Macherey-Nagel, #740955.50, Düren, Germany) according to manufacturer's protocol. Subsequently, 0.5-1.0 μg of total RNA was used as a template for cDNA synthesis using SuperScript III RT (Life Technologies, #11755050, Carlsbad (California) - United States). An *ush2a* amplicon (1096 bp) of homozygous *rmc1* larvae was amplified using with primers located in exon11 (5'-AGCGCTGTCGGAGTCTCTTC-3') and exon14 (5'-CCATCACTGACCGGTCACAG-3'). An *ush2a* amplicon (710 bp) of homozygous *b1245* larvae was amplified using primers located in exon68 (5'-TGGACTGGAGTGGCTCTTTC-3') and exon73 (5'-GATGAGGACTTTGGAGAGACCA-3').

2.5 Antibodies and Immunohistochemistry

Dissected adult eyes (12-18 mpf) and larval zebrafish (4-6 dpf) from homozygous *ush2a^{rmc1}* and *ush2a^{b1245}* mutants and their age and strain-matched wild-type controls were cryoprotected with 10% sucrose in PBS for 30 minutes prior to embedding in OCT compound (Tissue-Tek, 4583, Sakura). After embedding, samples were slowly frozen down using melting isopentane. To assess retinal morphology, cryosections (seven μm thickness along the lens/optic nerve axis) were fixed for 10 minutes with paraformaldehyde (PFA) 4%, stained with hematoxylin and eosin and analysed on a Zeiss Axioskop light microscope. For

analysis by scanning confocal microscope, embryos were fixed in 4% PFA overnight at 4°C, washed 3x in PBS-T, dehydrated in 100% methanol, rehydrated in descending methanol series and washed several times in PBS-T before being cryoprotected in 30% sucrose for several hours at room temperature. Larvae were then embedded in molten agarose blocks, frozen, and sectioned on a cryomicrotome. For immunofluorescence on unfixed cryosections (seven µm thickness) were permeabilised for 20 minutes with 0.01% Tween20 in PBS. Sections were rinsed 3 times for 5 minutes with PBS and blocked for 1 hour with blocking buffer (10% normal goat serum and 2% bovine serum albumin in PBS). Antibodies diluted in blocking buffer were incubated overnight at 4 °C. Secondary antibodies were also diluted in blocking buffer and incubated together with DAPI (1:8000; D1306; Molecular Probes) for 1 hour. Sections were post fixed with PFA 4% for 10 minutes and mounted with Prolong Gold Anti-fade (P36930; Molecular Probes). For immunofluorescence on fixed cryosectioned tissue (16µm thickness), slides were hydrated 10 minutes in PBS-T, then immersed in Sodium Citrate solution at pH 8.5 and heated in a pressure cooker for 10 minutes. After cooling to 37°C, slides were washed twice in PBS-T, blocked in 10% NFDM in PBS-T and incubated with primary antibodies. The following primary antibodies and dilutions were used: rabbit anti-active caspase 3 (1:500; #559565; BD Pharmingen), rabbit anti-Whrn (1:300; #42690002 Cip98a; Novus Biological), rabbit anti-Whrna (1:300; #42700002 Cip98b; Novus Biological), rabbit anti-usherin-C (1:500; #27640002; Novus Biological), rabbit anti-Adgrv1 (1:1000) (Ebermann et al., 2010) and as marker for connecting cilium region in photoreceptor cells we used mouse anti-centrin (1:500; #04-1624; Millipore) or acetylated α-tubulin (Sigma T7451). Secondary antibodies (Alexa Fluor 568 goat anti-rabbit (A11011), Alexa Fluor 488 goat anti-guinea pig (A11073) and Alexa Fluor 488 goat anti-mouse (A11029)), derived from Molecular Probes, were used in a 1:800 dilution. Images were taken using a Zeiss Axio Imager fluorescence microscope equipped with an AxioCam MRC5 camera (Zeiss) or a Zeiss LSM5 Confocal. The intensity of Whrna and Whrn immunofluorescence was measured in FIJI version 1.47v⁵⁸. First, the outer segment layer was isolated based on the centrin immunostaining. Subsequently, a mask was made based on the centrin staining using the “Find Maxima” option (noise=50), and dilated five times. To find the exact location of Whrna or Whrn staining, the centrin mask and Whrn layer were combined. Find Maxima (noise=10) was used to identify the Whrn staining within the Centrin mask. The resulting mask was dilated three times and touching objects were separated using the watershed option. Subsequently, the maximum gray value of the identified regions was measured on the original image of Whrn immunofluorescence (Analyze Particles option; size=0-50, pixel circularity=0.00-1.00). Active caspase-3 labeled cells were analyzed on a Zeiss fluorescence microscope by manually counting positively stained cells in the ONL.

2.6 Constant light rearing

For constant light treatment, embryos were raised in transparent 10-cm petri dishes under normal conditions and were placed under constant light from 5-8 dpf. From 5 dpf onwards, *ush2a^{mc1}* larvae were either maintained in facility conditions of 300 lux white light in a 14/10h day/night rhythm or were exposed to continuous white light with an intensity of 3000 lux, using a 150 W LED light source (Zeiss). *ush2a^{b1245}* larvae were either maintained in facility conditions of 300 lux white light in a 14/10h day/night rhythm or were exposed to

continuous white light with an intensity of 3000 lux using two LED strips (Westek) mounted in parallel. Light input: 12V DC, transformer input 120V ~60Hz, transformer output 12V DC, 6W max. Light intensities were measured at water level using a lumino meter (Testo 540, Lenzkirch, Germany or Advanced Light Meter 840022, Sper Scientific, Arizona, USA).

2.7 Fixation and pre-embedding labeling for immunoelectron microscopy

For immunoelectron microscopy of adult zebrafish retinas, we followed the previously published protocol for pre-embedding labeling (Maerker et al., 2008; Sedmak et al., 2009; Sedmak and Wolfrum, 2010). In brief, rabbit anti-usherin C (1:500; #27640002; Novus Biological) was applied on vibratome sections of pre-fixed (4% PFA) murine eye-cups, followed by incubation with biotinylated secondary antibodies. Antibody reactions were visualized by a Vectastain ABC-Kit (Vector Laboratories) and 0.01% hydrogen peroxide to 0.05 M diaminobenzidine (DAB) solution was added. Stained retinas were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), followed by silver enhancement of DAB precipitates and post-fixation in cacodylate buffered 0.5% OsO₄ on ice. Dehydrated specimens were flat-mounted between two sheaths of ACLAR-films (Ted Pella Inc., Redding, USA) in Araldite resin. Ultrathin sections were made using a Reichert Ultracut S ultramicrotome (Leica), collected on Formvar-coated copper or nickel grids and counterstained with 2% uranyl acetate in 50% ethanol and aq. 2% lead citrate. Ultrathin sections were analysed in a Tecnai 12 BioTwin transmission electron microscope (FEI, Eindhoven, The Netherlands). Images were obtained with a CCD camera (charge-coupled-device camera; SIS MegaView3; Surface Imaging Systems, Herzogenrath, Germany) and processed with Adobe Photoshop CS (Adobe Systems).

2.8 Electroretinogram (ERG) recordings

ERG recordings were performed on isolated larval eyes (5-7 dpf) as previously described (Sirisi et al., 2014). Larvae were dark-adapted for a minimum of 30 min prior to the measurements and subsequently handled under dim red illumination. The isolated eye was positioned to face the light source. Under visual control via a standard microscope equipped with red illumination (Stemi 2000C, Zeiss, Oberkochen, Germany), the recording electrode with an opening of approximately 20 μ m at the tip was positioned at the center of the cornea. This electrode was filled with E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, and 0.33 mM MgSO₄). The electrode was positioned using a micromanipulator (M330R, World Precision Instruments Inc., Sarasota, USA). A custom-made stimulator was invoked to provide light pulses of 100 ms duration, with a light intensity of 6000 lux. It uses a ZEISS XBO 75W light source and a fast shutter (Uni-Blitz Model D122, Vincent Associates, Rochester, NY, USA) driven by a delay unit interfaced to the main ERG recording setup. Electronic signals were amplified 1000 times by a pre-amplifier (P55 A.C. Preamplifier, Astro-Med. Inc, Grass Technology) with a band pass between 0.1 and 100 Hz, digitized by DAQ Board NI PCI-6035E (National Instruments) via NI BNC-2090 accessories and displayed via a self-developed NI Labview program (Rinner et al., 2005). Statistical analysis was performed using SPSS Statistics 22 (IBM). All the experiments were performed at room temperature (~22°C).

2.9 Statistical analyses

The Graphpad Prism software (version 5.03 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com) was used to generate scatter plots, calculate mean values, and perform statistical analysis using two-tailed unpaired Student's t-tests or two-tailed Mann-Whitney tests.

3. RESULTS

3.1 Zebrafish and human usherin are highly conserved

Bioinformatic analyses of the zebrafish and human usherin protein sequence revealed that the proteins have a high degree of sequence similarity (52% identity; 68% similarity) and share a similar protein domain architecture (Fig. 1). We characterized the subcellular localization of usherin in the zebrafish retina with an antibody directed against the intracellular C-terminal region of usherin (anti-usherin-C). In adult zebrafish retina, usherin is not only present at the periciliary membrane of both cones and rods, but also apical to the cone connecting cilium (Fig. 2A, B and C; Fig. S1). Subsequent immunoelectron microscopy identified these structures as cone accessory outer segments (AOS) (Fig. 2D and E). AOS are structures that run along the photoreceptor outer segment and are predominantly found in cone photoreceptors of adult frogs and teleost fish (Januschka et al., 1987).

3.2 Generation of zebrafish *ush2a^{rnc1}* and *ush2a^{b1245}* mutants

Using CRISPR/Cas9 technology, we generated two *ush2a* mutant alleles (sequences in Fig. S3). *ush2a^{rnc1}* contains a frameshift mutation in *ush2a* exon13 (c.2337_2344delinsAC; p.Cys780GlnfsTer32), that is predicted to result in the premature termination of translation of usherin (Fig. 3A). *ush2a^{b1245}* harbors a frameshift mutation in *ush2a* exon71 (ENSDART00000086201.4; c.15520_15523delinsTG; p.Ala5174fsTer), predicted to encode an usherin protein that retains the transmembrane domain but lacks the C-terminal 62 amino acids of the intracellular region including the class I PDZ binding motif. This allele provides an opportunity to assess the functional importance of the intracellular region, including the PDZ-binding motif, relative to the ectodomain of usherin (Fig. 3A). Both homozygous mutants were viable and no abnormalities in morphology, development, or swimming behavior were observed.

3.3 *ush2a^{rnc1}* and *ush2a^{b1245}* do not affect *ush2a* pre-mRNA splicing

In patient-derived fibroblasts, the *USH2A* c.2299delG mutation results in skipping of *USH2A* exon12 and exon13 in some transcripts, or in skipping of exon13 only (Lenassi et al., 2014). Recent studies showed that CRISPR/Cas9-induced exonic lesions could also induce an (in-frame) skipping of the targeted exon thereby preventing generation of a functional knock-out model (Mou et al., 2017). If the lesion introduced in the *ush2a^{rnc1}* mutant causes an in-frame skipping of exon 13, this could potentially result in expression of a shortened usherin with residual function. Similarly, the c.15520_15523delinsTG (*ush2a^{b1245}*) mutation is predicted to disrupt an exonic splice enhancer site and potentially induce skipping of exon71, thereby also leaving the *ush2a* open reading frame intact. We, thus, analyzed *ush2a* transcripts in homozygous *ush2a^{rnc1}* and *ush2a^{b1245}* larvae to assess

whether the introduced lesions would result in alternative *ush2a* pre-mRNA splicing events. Amplicons spanning exon13 or exon71 and their surrounding exons were amplified by RT-PCR. Because no alternatively spliced *ush2a* transcripts were amplified from cDNA derived from homozygous *ush2a^{rmc1}* or *ush2a^{b1245}* larvae (Fig. 3B and C), we expect that the introduced mutations in the *rmc1* and *b1245* alleles result in premature termination of usherin translation.

3.4 *ush2a^{rmc1}* leads to absence of usherin, whereas *ush2a^{b1245}* results in truncated usherin at the periciliary region

Using zebrafish-specific anti-usherin antibodies directed against the N- or C-terminus of the protein, we evaluated the presence of usherin in the retina of homozygous *ush2a^{rmc1}* and *ush2a^{b1245}* larvae and strain-matched (TLF or AB*, respectively) wild-type larvae at 5 dpf. Anti-usherin-N is directed against an amino acid sequence present at the N-terminus of usherin. In wild-type larvae, both antibodies detected usherin adjacent to the basal body and connecting cilium marker, centrin (Fig. 4). In contrast, no usherin signal was detected in the retina of *ush2a^{rmc1}* mutant larvae using either anti-usherin antibody. Also in the retina of *ush2a^{b1245}* larvae, no signal was detected when using anti-usherin-C antibodies (Fig. 4A and B). However, a signal at the photoreceptor periciliary region of homozygous *ush2a^{b1245}* larvae was obtained when using anti-usherin-N antibodies. These results suggest that the truncated usherin protein still localizes appropriately at the membrane even though it lacks the C-terminal intracellular region and the PDZ binding motif (Fig. 4A and B). We conclude that *ush2a^{rmc1}* mutants completely lack usherin at the photoreceptor periciliary region and the mutation should be considered a true null allele, whereas *ush2a^{b1245}* should be considered a hypomorphic allele with potential residual function.

3.5 Ablation of usherin affects the localization of Whrna and Whrnb in the retina

Human usherin was previously shown to interact directly with whirlin *in vitro* (van Wijk et al., 2006). The biological relevance of this interaction was confirmed by the absence of whirlin at the photoreceptor periciliary membrane of *Ush2a* knock-out mice (Yang et al., 2010). Here, we determined whether or not whirlin localization was also affected in the retina of homozygous *ush2a^{rmc1}* and *ush2a^{b1245}* zebrafish larvae (5 dpf). The zebrafish genome harbors two whirlin-encoding orthologs. *whrna* (previously known as *dfnb31a* (ENSDARG00000075362)) encodes Whrna and *whrnb* (previously known as *dfnb31b* (ENSDARG00000068166)) encodes Whrnb. Whrna was found to be located at the photoreceptor periciliary region in wild-type larvae (Fig. 5A, Fig. S2). Reduced intensity of the Whrna signal was observed in the retinas of homozygous larvae of both *ush2a* mutants, which was further confirmed by quantification of fluorescence signal intensities (Fig. 5A). The same results were observed in homozygous *ush2a^{rmc1}* larvae when an anti-Whrnb antibody was employed (Fig. 5B, Fig. S2). In contrast, the intensity of the Whrnb signal was unaltered in photoreceptors of homozygous *ush2a^{b1245}* larvae as compared to AB* wild-type larvae (Fig. 5B).

3.6 Zebrafish *ush2a* mutants exhibit elevated levels of photoreceptor apoptosis

Based on the assumption that usherin is required for long-term maintenance of photoreceptor cells, we assessed whether absence of usherin in the zebrafish retina would

lead to increased levels of apoptosis in the outer nuclear layer (ONL). Larvae were raised under a 14/10 hours light/dark cycle until 5 dpf. After 5 dpf, they were either raised for an additional 72 hours in regular facility conditions of 300 lux light intensity of a 14hr/10hr light/dark cycle, or exposed to constant light with an intensity of 3000 lux for 72 hours. A slight but significant increase in the amount of apoptotic photoreceptor cells was observed in 8 dpf *ush2a^{rmc1}* larvae raised in normal facility conditions (Fig. 6A). These results were consistent with our previously reported data on photoreceptor degeneration in larvae treated with a morpholino targeting an early splice junction in *ush2a* (Ebermann et al., 2010). Subsequently, we assessed the number of apoptotic events in *ush2a^{b1245}* mutant larvae. No significantly elevated levels of cell death in the ONL were observed when these larvae were raised under normal facility conditions (Fig. 6B). Constant light rearing with an elevated light intensity of 3000 lux significantly increased the amount of apoptotic cells in the ONL of both mutant alleles as compared to their strain-matched wild-type controls (Fig. 6A and B). Variations in the genetic backgrounds of the *rmc1* and *b1245* alleles, variances in the light sources used in the elevated light exposure assay, and other environmental differences between the two facilities where these experiments were conducted could contribute to the observed difference in the absolute number of apoptotic cells.

Because significantly elevated levels of apoptotic events were seen in the ONL of *ush2a^{rmc1}* mutant larvae when kept at facility conditions, we examined whether retinal degeneration was progressive under these conditions. Retinal histology was analyzed for both homozygous *ush2a* mutants at 5 dpf and at 18 months of age, which revealed that cellular organization and retinal lamination in mutants was indistinguishable from those in wild-type controls. Moreover, photoreceptor outer segments of both *ush2a* mutants had a normal morphology (Fig. S4). The observed lack of progressive retinal degeneration in these mutants suggests that the rate of photoreceptor apoptosis might be compensated by the rate of photoreceptor regeneration when fish are raised in low-intensity light.

3.7 *ush2a* mutants show reduced visual function

To assess whether absence of usherin has implications for visual function in the *ush2a^{rmc1}* and *ush2a^{b1245}* mutants, electroretinograms (ERGs) were recorded in 5 dpf *ush2a* mutant larvae and strain- and age-matched wild-type controls that were raised under normal facility conditions. In the zebrafish retina, a small negative a-wave generated by photoreceptor hyperpolarization is immediately followed by a large positive b-wave, generated largely by the depolarization of second- order ON-bipolar cells in response to photoreceptor hyperpolarization (Dowling, 2012). The ERG maximum b-wave amplitudes were significantly reduced in homozygous *ush2a^{rmc1}* larvae as compared to age- and strain-matched wild-type controls (Fig. 7A and 7B). The same result was observed in homozygous *ush2a^{b1245}* larvae (5 dpf) (Fig. 7C and 7D). To investigate whether the decreased inner retinal function was primarily mediated by a decrease in photoreceptor function, we recorded pharmacologically isolated a-waves by abolishing b-waves with glutamate receptor antagonists t-APB and TBOA. Maximum a-wave amplitudes in homozygous *ush2a^{rmc1}* larvae were significantly reduced compared to wild-type larvae (Fig. 7E and 7F). Again, a similar defect was detected in homozygous *ush2a^{b1245}* larvae, in which the maximum a-wave amplitudes were also significantly decreased compared to wild-type controls (Fig. 7G

and 7H). Despite the absence of detectable retinal morphological defects, early-onset retinal photoreceptor dysfunction was recorded in both *ush2a* mutants.

4. DISCUSSION

In this study we generated and characterized two zebrafish *ush2a* mutant models, *ush2a^{rmc1}* and *ush2a^{b1245}*, in which different domains of usherin were ablated. No signal was detected in photoreceptors of either *ush2a^{rmc1}* or *ush2a^{b1245}* larvae when using an antibody targeting the intracellular C-terminal region. In contrast, truncated usherin was detected at the photoreceptor periciliary region of homozygous *ush2a^{b1245}* larvae when using an N-terminal usherin antibody, but not in *ush2a^{rmc1}*. Absence of full length usherin resulted in reduced whirlin levels at the photoreceptor periciliary region. Furthermore, the *rmc1* allele showed mild levels of photoreceptor degeneration at 8 dpf raised under normal facility conditions, whereas constant light rearing from 5-8 dpf with a light intensity of 3000 lux exacerbated photoreceptor degeneration in both mutants. Finally, both models presented with early-onset retinal dysfunction, as evidenced by significantly reduced ERG a- and b-wave responses recorded at 5 dpf. After complete phenotypic evaluation of both alleles we conclude that the intracellular region of usherin is the most critical region in usherin required for visual function in zebrafish larvae.

Prior to this study, several USH mouse models were generated, which all presented with early-onset hearing defects, with or without vestibular dysfunction, closely resembling human inner ear USH defects (Slijkerman et al., 2017). These models have contributed significantly to understanding the molecular pathology underlying USH-associated hearing loss (Schwander et al., 2009; Tian et al., 2010; Yang et al., 2010). Unfortunately, these mouse models only sporadically recapitulated retinal degeneration seen in USH patients. A spontaneous mutant mouse model, *Kunming*, was described for Usher syndrome type IIa (Yao et al., 2016). This model shows a rapid, early-onset retinal degeneration, but contains mutations in two genes known to be involved in inherited retinal dystrophies: *Ush2a* and *Pde6b*. A targeted *Ush2a* knock-out mouse demonstrates only mild retinal degeneration with late age of onset (Liu et al., 2007). A likely explanation for the discrepancy in phenotypic outcome between mice and humans lies within the anatomical differences between human and mouse photoreceptor cells. The most prominent subcellular locations in human photoreceptor cells where USH1 and USH2 proteins have been found are the calyceal processes and the periciliary membrane (Maerker et al., 2008; May-Simera et al., 2017; Sahly et al., 2012; Sorousch et al., 2017). In rodents, photoreceptor calyceal processes and periciliary membranes are absent or underdeveloped, respectively, compared to humans. In contrast, these structures are well-developed and present in frogs and teleost fish (Slijkerman et al., 2015). Zebrafish mutants or morphants for USH1-associated genes *myo7a* (Ernest et al., 2000; Wasfy et al., 2014), *cdh23* (Glover et al., 2012; Sollner et al., 2004), *pcdh15* (Seiler et al., 2005) and *ush1c* (Blanco-Sanchez et al., 2014; Phillips et al., 2011) have been described in the literature, most of them showing early-onset retinal dysfunction (Blanco-Sanchez et al., 2017). Furthermore, we showed that morpholino-induced knock-down of *ush2a* expression induces photoreceptor cell death in zebrafish larvae (Ebermann et al., 2010). In this study we generated and characterized two zebrafish mutant models that will

allow us to continue to investigate the molecular pathology underlying *ush2a*-associated retinal dysfunction and future therapeutic strategies.

Although the anatomical similarities between human and zebrafish photoreceptor cells are numerous, some differences have also been noted. Cone photoreceptors of adult teleost fish contain a cilium-like structure with a 9+0 arrangement of microtubule-doublers. This structure, the accessory outer segment (AOS), protrudes from the connecting cilium and projects apically alongside the outer segment, eventually associating with the retinal pigment epithelium (RPE) (Hodel et al., 2014). The function of the AOS is still unclear, but previous reports have suggested that the AOS provides structural support to the outer segment (Yacob et al., 1977) and that it is involved in exchange of metabolites between RPE and cones (Burnside et al., 1993; Hodel et al., 2014; Yacob et al., 1977). Moreover, in response to changes in ambient light conditions and to circadian signals during light-to-dark adaptation of the teleost retina, reversible retinomotor movements take place that result in contraction and elongation of rods and cones to expose the outer segment optimally to incoming light (Hodel et al., 2006; Wagner et al., 1983). It has been postulated that by anchoring the cones to the pigment epithelium, the AOS may preserve the highly organized arrangement of the various types of cones in the fish retina (Yacob et al., 1977). Previous experiments showed that the USH1b protein, myosin VIIa, is localized in the cone AOS (Hodel et al., 2014), and we now discovered that usherin is present in these structures as well. It has been shown that myosin VIIa and usherin, together with Adgrv1 and whirlin, physically interact to form the ankle-link complex in cochlear hair cells (Michalski et al., 2007). It is therefore tempting to speculate that within the AOS, myosin VIIa and usherin also form a complex that could be of importance for alignment and connection of the AOS to cone outer segments.

In humans, usherin and the other known USH2 proteins, whirlin and ADGRV1, form a dynamic protein complex that is mainly mediated by PDZ-PBM-based interactions (van Wijk et al., 2006; Yang et al., 2010). Previous research using knockout mice showed that usherin, whirlin, and Adgrv1 are mutually dependent on each other for their localization at the photoreceptor periciliary membrane (Chen et al., 2014; Yang et al., 2010). Whereas *ush2a* and *adgrv1* are present as single copy genes, there are two zebrafish whirlin orthologs (*whrna* and *whrnb*). In larvae of the *ush2a^{rmc1}* allele, in which no usherin could be detected, we observed reduced levels of both Whrna and Whrnb at the periciliary membrane. In *ush2a^{b1245}* larvae, however, Whrna is significantly reduced at the periciliary membrane, whereas Whrnb appears unaffected. These observations fit a model in which the ectodomains of usherin and Adgrv1 interact while the intracellular regions of both proteins are anchored by whirlin (Fig. 8), as was previously proposed by Maerker and co-workers (Maerker et al., 2008; Soroush et al., 2017). Based on the results of the current study, Whrna seems to bind the intracellular region of usherin preferentially, whereas Whrnb predominantly associates with the intracellular region of Adgrv1. Complete absence of usherin (*rmc1* allele) results in absence of both Whrna and Whrnb at the periciliary membrane and probably the complete USH2 complex including Adgrv1, similar to what was observed in the *Ush2a* knockout mouse (Yang et al., 2010). Immunohistochemical analyses using anti-Adgrv1 antibodies corroborated this hypothesis (Fig. S5). Absence of the C-terminal cytoplasmic region of usherin (*b1245* allele) apparently affects the association with only the intracellular binding partner Whrna, leaving the remainder of the USH2 complex

(Adgrv1 and Whrn) intact (Fig. 8; Fig. S5). Thus, absence of zebrafish usherin affects localization of interaction partner whirlin. The observed differences in localization of both whirlin co-orthologs in the *rnc1* and *b1245* alleles suggest that Whrn and Whrn have high binding-affinities for usherin and Adgrv1, respectively, and that they most probably have acquired divergent functions during evolution, consistent with the subfunctionalization hypothesis (Force et al., 1999).

The presence of usherin at the periciliary region suggests a role in providing the connecting cilium with structural support. It has been proposed that the periciliary membrane is physically connected to the connecting cilium by means of interactions between the ectodomains of usherin and Adgrv1 (Fig. 8) (Maerker et al., 2008; Soroush et al., 2017). The photoreceptor apical inner segment and periciliary region are also thought to be involved in docking of trans-Golgi-derived vesicles that contain cargo essential for outer segment formation, maintenance, and function (Falk et al., 2015). The USH protein complex, including usherin, has been proposed to play a role in this process (Maerker et al., 2008). Based on our functional and immunohistochemical data, we propose a dual role for usherin in zebrafish photoreceptors: providing the connecting cilium with (ectodomain-mediated) structural support and participating in vesicle docking and/or signaling mediated by the intracellular region. Moreover, reduced retinal function and increased cell death in the retinal outer nuclear layer combined with the localization of truncated usherin at the periciliary membrane of the *ush2a*^{b1245} mutant, point towards a critical role for the intracellular region of usherin in retinal function, a significant finding for future therapeutic interventions.

The first clinical sign of retinal dysfunction in patients with Usher syndrome is typically night blindness, indicative of rod dysfunction. In zebrafish, rod photoreceptors contribute little to visual function before about 14 dpf (Bilotta et al., 2001; Moyano et al., 2013). In both *ush2a* mutants, however, we observed impaired ERGs at 5 dpf, which is suggestive of early-onset cone dysfunction. Although ERG data of pre-symptomatic patients are typically not available, a recent study demonstrated markedly reduced ERG responses for both rods and cones in adolescent Usher syndrome type 2a patients (Sengillo et al., 2017), consistent with our observations of cone defects by the onset of vision in zebrafish *ush2a* mutants. Furthermore, *USH2A*-associated retinal degeneration in humans has a slow, progressive pathology. Zebrafish, unlike humans, have the ability to regenerate retinal cells lost to injury or disease. Steady cell proliferation from stem-like populations in the ciliary marginal zone persists throughout adult stages (Wan et al., 2016). Acute damage to the retina triggers the reprogramming of Müller glial cells into retinal progenitor cells that are able to differentiate into all major types of retinal neurons including photoreceptors (Wan and Goldman, 2016). Either or both of these regenerative pathways could explain the finding that *ush2a* mutants showed no progressive loss of retinal photoreceptor cells (Fig. S4) under normal light conditions, although increased levels of photoreceptor apoptosis and impaired visual function were observed within the first week of life when larvae were challenged by exposure to constant illumination with 3000 lux of white light. These observations suggest that sustained exposure to higher light levels throughout life might disrupt the homeostasis between slow degeneration and steady repopulation by regeneration in these mutants. Importantly these observations may have significant implications for light exposure and the use of sunglasses or high-energy wavelength filtering lenses as treatments in Usher

syndrome patients. In conclusion, we have shown that zebrafish *ush2a* mutants show an early onset retinal dysfunction, mainly in the absence of photoreceptor degeneration. Not only will these mutants avail our studies of the retinal function of usherin, but the quantified phenotypes of USH2 protein mislocalization, defects in visual function and increased photoreceptor degeneration in elevated light conditions in *ush2a* mutants can all be used as measures for evaluation of therapeutic strategies that are currently being developed for the future treatment of *USH2A*-associated retinal degeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- *ush2a* zebrafish models present with early-onset retinal dysfunction
- Retinal dysfunction is observed mainly in the absence of photoreceptor degeneration
- Exposure to constant bright illumination induces photoreceptor apoptosis
- Whrna and Whrnb have high binding-affinities for usherin and Adgrv1, respectively
- The intracellular region of usherin has a critical role in retinal function

Figure 1. Schematic presentation of zebrafish usherin

Motif alignment of human and zebrafish usherin. Both proteins have an identical predicted domain architecture. EGF-lam, laminin-type epidermal growth factor-like domain; LamG, laminin G domain; LamG-like, LamG-like jellyroll fold domain; LamNT, laminin N-terminal domain; FN3, fibronectin type 3 domain.

Figure 2. Usherin localizes at the periciliary membrane and accessory outer segments of adult zebrafish photoreceptor cells

(A) Retinal sections of wild-type adult zebrafish are labeled for usherin (green signal) and the connecting cilium marker centrin (magenta signal). (B) Usherin labeling at the periciliary region of rod photoreceptors is present adjacent to the connecting cilium marked by centrin (indicated by an arrow head). (C) In cones, usherin is also detected at the periciliary region (arrow heads) as well as apical to the connecting cilium marked by centrin labeling (arrows). Nuclei were stained with DAPI (blue signal). (D, E) Electron microscopy images of adult zebrafish retinas show that usherin localizes at the periciliary membrane (arrowheads) as well as in the accessory outer segments (AOS; arrow) of cone photoreceptors. Scale bars in A: 10 μm ; B: 3 μm ; C: 3 μm ; D: 1 μm E: 0.1 μm . CC: connecting cilium, COS: cone outer segment, CP: calyceal processes, ONL: outer nuclear layer, OS: outer segment, ROS: rod outer segment, RPE: retinal pigment epithelium.

Figure 3. *ush2a* transcript analyses in homozygous *ush2a*^{rmc1} and *ush2a*^{b1245} mutant zebrafish (A) The locations of the mutations in *ush2a*^{rmc1} and *ush2a*^{b1245} are schematically depicted. (B) RT-PCR analyses of *ush2a* transcripts derived from homozygous *ush2a*^{rmc1} or (C) homozygous *ush2a*^{b1245} larvae (5 dpf) provided no indications for an effect of the introduced lesions on pre-mRNA splicing. WT: wild-type, Mut: homozygous mutant, mQ: milliQ water.

Figure 4. Localization of usherin in frontal cryosections of wild-type and mutant zebrafish retinas (5 dpf)

Zebrafish-specific anti-usherin-C (A) or anti-usherin-N (B) antibodies are employed (red signal). (A) In *ush2a^{mc1}* as well as *ush2a^{b1245}* homozygous mutant larvae no signal was detected with the anti-usherin-C antibody, whereas in both TLF and AB* wild-type controls usherin was present adjacent to the connecting cilium marked by centrin (green signal). (B) No signal was detected in the retina of homozygous *ush2a^{mc1}* mutants using the anti-usherin-N antibody, however, a signal at the photoreceptor periciliary region was observed in

the retina of homozygous *ush2a^{b1245}* larvae when this antibody was used. In wild-type controls, the anti-usherin-N antibody also detected usherin at the photoreceptor periciliary region. (n= 35 larvae, from 5 biological replicates with TLF and *ush2a^{mc1}* larvae, n= 64, from 8 biological replicates with AB* and *ush2a^{b1245}* larvae). Nuclei are stained with DAPI (blue signal). Scale bars: 10 μ m.

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eyes and n=6 TLF larvae eyes, $p<0.05$ and n=10 *ush2a^{b1245}* and n=9 AB* eyes, $p<0.01$, two-tailed unpaired Student's *t*-test). **(B)** Whrnbl labeling was reduced in *ush2a^{rmc1}* mutants, but unaltered in *ush2a^{b1245}* mutants compared to corresponding wild-types (n=35 larvae from 5 biological replicates with *ush2a^{rmc1}* mutant larvae and TLF larvae, and n=54 larvae from 7 biological replicates with *ush2a^{b1245}* mutant larvae and AB* larvae). Intensity of Whrnbl labeling (red signal) was significantly reduced in *ush2a^{rmc1}* larvae as compared to wild-type (TLF) larvae (n=5 *ush2a^{rmc1}* eyes and n=6 TLF eyes, $p<0.01$, two-tailed unpaired Student's *t*-test). In *ush2a^{b1245}* larvae, the intensity of Whrnbl labeling appeared unaltered as compared to wild-type (AB*) larvae (n=27 eyes for both *ush2a^{b1245}* and AB* wild-type; $p=0.57$, two-tailed unpaired Student's *t*-test). Nuclei were stained with DAPI (blue signal) and anti-centrin (green signal) was used as a marker for the connecting cilium. Scale bars: 10 μm , dpf: days post fertilization, a.u.: arbitrary units, * indicates $p<0.05$, ** indicates $p<0.01$, and ns: not significant.

Figure 6. Quantification of apoptotic events in the ONL of zebrafish *ush2a* mutants (8 dpf)
Homozygous *ush2a^{rmc1}* and *ush2a^{b1245}* larvae were exposed either to 300 lux in a (14/10h) day/night rhythm or to continuous light at 3000 lux from 5-8 dpf. Individual dots represent the number of apoptotic cells in the ONL per eye. Strain matched wild-type TLF or AB* zebrafish were used as controls. **(A)** TLF and *ush2a^{rmc1}* larvae kept at 300 lux with a day/night cycle showed on average 0.2 (± 0.1; 30 eyes) and 0.8 (± 0.2; 28 eyes) apoptotic cells per eye, respectively ($p < 0.05$). When kept under 3000 lux of continuous light, 1.7 (± 2.0; 34 eyes) and 5.1 (± 4.5; 28 eyes) apoptotic cells per eye were observed in TLF and *ush2a^{rmc1}*, respectively ($p < 0.01$). **(B)** AB* and *ush2a^{b1245}* larvae maintained at 300 lux with a day/night cycle showed on average 2.5 (± 1.9; 28 eyes) versus 3.8 (± 2.3; 16 eyes) apoptotic cells per eye, respectively (n.s.). When kept under 3000 lux of continuous light, on average 4.2 (± 1.7; 23 eyes) versus 24.8 (± 7.9; 18 eyes) apoptotic cells per eye were observed in AB* and *ush2a^{b1245}*, respectively ($p < 0.0001$). Each point represents the number of apoptotic cells in the ONL per eye and the standard error of the mean are given as error bars. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.0001$, n.s.: not significant (two-tailed Mann-Whitney test); ONL: outer nuclear layer; dpf: days post fertilization.

Figure 7. *ush2a* mutants show reduced retinal function

Average ERG traces from a cohort of homozygous *ush2a^{rmc1}* and *ush2a^{b1245}* larvae (5 dpf) with age- and strain-matched wild-type controls are depicted. Averages of the maximum ERG amplitudes are plotted as bar graphs $\pm$ standard deviation. (A-D) Normalized b-wave amplitudes recorded in both mutants are significantly reduced compared to ERG-traces from wild-type controls (n=72 TLF and n=75 *ush2a^{rmc1}*, from 3 biological replicates, $p < 0.001$, two-tailed unpaired Student's *t*-test, and n=20 *ush2a^{b1245}* larvae and n=18 wild-type larvae, from 3 biological replicates, $p < 0.01$, two-tailed unpaired Student's *t*-test). (E-H) In both

ush2a mutants, the a-wave amplitudes are significantly reduced as compared to strain-matched wild-type controls (n=10 for TLF and *ush2a^{mc1}*, from 2 biological replicates, $p<0.05$, two-tailed unpaired Student's *t*-test, and n=19 for AB* and n=23 for *ush2a^{b1245}*, from 2 biological replicates, $p<0.001$, two-tailed unpaired Student's *t*-test). * indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.001$.

Figure 8. Schematic model of molecular consequences for other USH2 proteins in *ush2a* mutants

The ectodomain of wild-type usherin associates with the ectodomain of Adgrv1 (indicated by dashed lines). Whrna and Whrnbl bind via a PDZ-PBM-mediated interaction to usherin and Adgrv1, respectively. The *ush2a^{rmc1}* allele results in the complete absence of usherin at the periciliary region. As a consequence, localization of Adgrv1, Whrna and Whrnbl at the periciliary membrane is also affected. The *ush2a^{b1245}* allele, however, generates a truncated usherin protein that lacks only the C-terminal 62 amino acids and that is still located at the proper subcellular location in the photoreceptor cell. Because Whrna is mislocalized in this mutant and Whrnbl localization is largely unaffected, we propose a model in which Whrna has a higher binding affinity for usherin, and Whrnbl a higher binding affinity for Adgrv1. IS: inner segment, OS: outer segment, PBM: PDZ-binding motif.