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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 *ush2a*-encoded protein, usherin, demonstrated complete absence of usherin in photoreceptors of $ush2a^{rmc1}$, 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 $ush2a^{rmc1}$ 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.

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

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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 variants [6]. 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-alellic 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 5202 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; Reiners et al., 2005; Sorusch 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 5–7 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*^{rmc1}: 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*^{rmc1}, 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*^{rmc1} 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*^{rmc1} 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 Bioloabs, #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 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′-GATCCGCACCGACTCGGTGCCACTTTTTCAAGTTGA TAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC-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 s, 40 cycles of 98 °C 10 s, 60 °C 10 s, 72 °C 15 s and 72 °C 10 min. 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 mMESSAGE 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*^{rmc1} allele. To generate the *ush2a*^{b1245} allele, one-cell stage zebrafish

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