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Review Article

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Abstract

Usher syndrome (USH) is a major cause of deaf-blindness in humans, affecting ~400,000 patients worldwide. Three clinical subtypes, USH1-3, have been defined, with 10 USH genes identified so far. In recent years, in addition to identification of new Usher genes and diagnostic tools, major progress has been made in understanding the role of Usher proteins and how they cooperate through interaction networks to ensure proper development, architecture and function of the stereociliary bundle at the apex of sensory hair cells in the inner ear. Several Usher mouse models of known human Usher genes have been characterized. These mice faithfully reproduce the auditory phenotype associated with Usher syndrome and the vestibular phenotype associated with some mutations in USH genes, particularly USH1. Interestingly, very few mouse models of Usher syndrome recapitulate the retinal phenotype associated with the disease in human.

Usher patients can benefit from hearing aids or cochlear implants, which partially alleviate auditory sensory deprivation. However, there are currently no biological treatments available for auditory or visual dysfunction in Usher patients. Development of novel therapies for Usher syndrome has sprouted over the past decade, building on recent progress in gene transfer and new gene editing tools. Promising success demonstrating recovery of hearing and balance functions have been obtained via distinct therapeutic strategies in animal models. Clinical translation to Usher patients, however, calls for further improvements and concerted efforts to overcome the challenges ahead.

1- Introduction

Usher syndrome (USH), the most common form of deaf-blindness in humans, is a rare inherited disease that is estimated to affect ~400,000 patients worldwide. Usher syndrome impacts three senses: hearing, vision and balance. Usher syndrome patients suffer from mild to severe hearing loss, combined in some cases with balance deficits, and vision loss that worsens over time leading to blindness. Helen Keller, the first deaf-blind individual to graduate with a college degree in the United States (bachelor's in art and sciences obtained with honors from Radcliff College in 1904), said: "Blindness separates people from things; deafness separates people from people"**.** This multi-sensory loss greatly affects individuals' ability to communicate and is often associated with psychological distress which can lead to social isolation, depression and decreased cognitive abilities. Clinically, Usher syndrome is classified according to the disease onset, its severity and progression. There are three types of Usher Syndrome: **USH1**, **USH2** and **USH3,** with USH1 being the most severe. Each group is associated with a specific set of genes. Many of these genes have now been identified (Table 1, Fig. 1). They are expressed in the mechano-sensitive **sensory hair cells** of the inner ear and light sensing **photoreceptor** cells in the eye. USH proteins play multiple structural and functional roles.

There are currently no biological treatments that can restore auditory or visual function in patients with Usher syndrome. For auditory dysfunction, the current therapy of choice is the use of mechanical devices like hearing aids which are commonly used by patients who have been diagnosed with USH2 and USH3. Cochlear implants (CI) are offered to children who have profound hearing loss at birth (USH1), and, as hearing loss becomes severe or profound for USH2- and USH3-affected patients. These devices when implanted early enable children born with profound hearing loss to acquire near normal speech and provide open-set speech comprehension in most patients. Similarly, patients that receive hearing aids or cochlear implants at later stages benefit from these devices which greatly improve their speech understanding, increasing their quality of life (Hartel et al. 2017a, 2017b). However, despite great technical progress in the field, these devices are unlikely to ever equal the quality of "natural" or "biological" hearing. Indeed, perceptual limitations remain that restrict patient's ability for precise discrimination of complex

sounds such as speech in noisy environments and music (Culling et al. 2012, Gifford and Revit 2010, Jiam et al. 2017).

USH1 and USH3 patients also display balance deficits that can be aggravated by the loss of vision as the disease progresses. Indeed, balance is dependent upon several sensory functions that include vision, vestibular input and proprioception. The loss of vision and hearing in USH patients thus dramatically exacerbates the balance deficits. Somewhat similar to cochlear implants, multi-electrode vestibular prosthetics are currently being developed (reviewed in Guyot and Perez Fornos 2019). Until these become available, many patients who do not respond to vestibular rehabilitation therapy are left with restricted options for the treatment of their ailment.

The need for novel therapeutic strategies to target auditory and vestibular disorders has led many teams on the path of translational research. Several strategies have been tested in animal models, including stem cell-based transplantation, drug-based pharmacological intervention, and gene transfer-based approaches, with the hopes of reaching complete hearing and/or balance rehabilitation. Here, we review progress made in the development and application of gene therapy strategies to treat inner ear sensory deficits associated with Usher syndrome. We also discuss remaining questions and challenges that will have to be addressed before such treatments make their way to the clinic.

** Insert Table 1 here*

2- The genetic landscape of Usher syndrome

Over the last two decades, the study of inherited forms of deafness and deaf-blindness has been instrumental in deciphering molecular, cellular and physiological mechanisms underlying the development and functioning of the peripheral and central auditory systems. Usher syndrome is genetically heterogeneous, associated with ten different genes (Table 1, Fig. 1). Six genes are associated with USH1, three with USH2 and one with USH3. The Usher genes encode proteins with a wide range of functions including actin-binding molecular motors (myosin VIIA: USH1B), cell adhesion molecules (cadherin 23: USH1D; protocadherin 15: USH1F; usherin: USH2A), scaffolding proteins (harmonin: USH1C; sans: USH1G; whirlin: USH2D), an adhesion G-coupled

receptor (ADGRV1: USH2C), a calcium and integrin binding protein (CIB2: USH1J) and a transmembrane protein involved in scaffolding and cellular trafficking (clarin-1: USH3A). USH proteins form complexes and function cooperatively in both the inner ear hair cells and retinal photoreceptor cells. Disabling pathogenic variants of any of the Usher genes typically cause deafness (with or without balance deficits) and blindness. It is however important to note that evidence for classification of CIB2 (USH1J) as an Usher type I gene are still missing. Unlike other USH1 genes, loss of function pathogenic variants in *CIB2* have been reported in many patients who have isolated hearing loss in absence of visual or balance deficits (Booth et al. 2018, Michel et al. 2017) (Fig. 1).

**Insert Figure 1 here*

Interestingly, pathogenic variants in Usher genes can result in multiple modes of inheritance, causing recessive and/or dominant forms of non-syndromic hearing loss (DFNB/DFNA) and Usher syndrome (USH) (Table 1). Usher syndrome is associated with loss of function mutations resulting from nonsense, frameshift, missense and splicing mutations. Less severe pathogenic variants lead to non-syndromic recessive forms of the disease with isolated hearing or vision impairments. Such occurrences have been described for six of the USH genes: *MYO7A* (DFNB2), *CDH23* (DFNB12), *USH1C (*DFNB18), *PCDH15* (DFNB23), *WHRN* (DFNB31), and *CIB2* (DFNB48) (Table 1). Heterozygous pathogenic variants have also been found to cause non-syndromic dominant forms of hearing loss (*USH1C:* Song et al. 2020, and *MYO7A*: DFNA11, OMIM 601317). Less deleterious mutations in *USH2A* or *CLRN1* were also reported to cause non-syndromic retinitis pigmentosa (RP36 and RP61, respectively) (Table 1).

In summary, identification of pathogenic variants in Usher genes along with proper understanding of the degree of their pathogenicity is essential to better predict the extent, progression and severity of the different sensory deficits that a patient might encounter.

3- Role of Usher proteins in the inner ear

In the inner ear, USH genes are expressed in sensory hair cells of the snail shaped auditory organ, the cochlea, and the five vestibular organs (Fig. 2). Usher proteins have been associated with two specialized structures: the **hair bundle**, where sound waves and/or head movements are converted into changes of membrane potentials (so called mechano-electrical transduction), and the **ribbon synapse**, site of neurotransmitter release (Fig. 2).

**Insert Figure 2 here*

In recent years, in addition to identification of new Usher genes and diagnostic tools, major progress has been made in understanding the role of Usher proteins thanks to transdisciplinary and integrative experimental approaches ranging from (1) the deciphering of the biophysical properties of Usher molecules; (2) the identification of the molecular networks involving each USH protein; and (3) the elucidation of the nature and kinetics of the physiological, morphological, and molecular mechanisms underlying Usher sensory deficits (For review, see Bonnet and El-Amraoui 2012, El-Amraoui and Petit 2014, Mathur and Yang 2015). Dozens of mouse models have been characterized which faithfully reproduce the auditory, and when present, the vestibular phenotypes of USH-affected patients. These studies have established the key role of USH1 and USH2 proteins in the hair bundle (Fig. 3A, B). USH proteins cooperate and interact to form molecular complexes that ensure normal formation, organization and function of the hair bundle. During development, USH1 proteins form heteromeric structures required for the formation of transient apical interstereocilia links anchored to the core of actin filaments (Fig. 3). Together, these molecular and structural coupling events are necessary for proper formation of cohesive stereocilia and normally shaped hair bundles. At adult stages, USH1 proteins also form the core of the mechano-electrical transduction machinery, with cadherin-23 and protocadherin-15 forming the tip-link that gates the mechano-electrical transduction channel complex (Fig. 3). USH2 proteins are not necessary for stereocilia cohesiveness. Instead they are required during the later stages of bundle morphogenesis when they form the ankle link complex at the base of the stereocilia base. This structure is required during terminal stage of the hair bundles' shaping, enabling the refinement of the typical U- and V-shapes of the auditory IHCs and OHCs (Fig. 3). The USH3a protein, clarin-1,

has been shown to be play a role in mechano-electrical transduction while also acting as an essential organizer of the IHC synaptic active zone (Geng et al. 2009, 2012, Dulon et al. 2018).

**Insert Figure 3 here*

4- Role of Usher proteins in the retina

While the large majority of USH mouse models recapitulate the auditory and vestibular phenotypes of Usher patients, these models fail to reproduce the retinal phenotype. As a result, limited progress has been made elucidating the underlying mechanisms of Usher syndrome in the retina and identifying novel treatments for the retinal degeneration associated with this disease. Interdisciplinary work on the retina of several species (mice, pigs, frogs, monkeys and humans) concluded that lack of a USH1 retinal phenotype in mice is probably due to the absence in rodent photoreceptor cells of the calyceal processes, an F-actin-filled ring localized to the base of photoreceptor outer segments (Sahly et al. 2012). In primate photoreceptor cells, USH1 proteins have been proposed to operate in a similar way as in the stereocilia (Fig. 3A), forming a protein network mediating membrane-membrane coupling between the photoreceptor outer segment and the surrounding calyceal processes (Fig. 3C). Studies in the retina of *Xenopus tropicalis* further showed that the lack of a functional USH1 protein in this species causes alterations in the calyceal processes, also leading to impaired growth and organization of the newly formed disk that compose the outer segment (Fig. 3C, and Schietroma et al. 2017).

The USH2 proteins (usherin, Adgvr1, and whirlin) have been detected in a spatially restricted inner segment membrane region that surrounds the photoreceptor connecting cilium, the periciliary ridge membrane complex region where vesicles dock with the plasma membrane during translocation to the outer segment. USH2 mutant mice lacking usherin or the whirlin long isoform have been shown to display retinal abnormalities, including retinal degeneration (reviewed in Maerker et al. 2008, El-Amraoui and Petit 2014, Mathur and Yang 2015, 2019).

The function of the USH3 protein, clarin-1, in the retina is currently unknown, mostly due to the lack of appropriate USH3 animal models that mimic the human retinal phenotype and because

of multiple conflicting results regarding clarin-1 localization in photoreceptor cells, retinal interneurons and muller cells (Xu et al. 2020).

5- Therapeutic approaches for the treatment of vision loss

In recent years, a wide range of therapeutic strategies have demonstrated efficacy in preclinical studies and several have entered clinical trials aiming to improve vision in blind patients (Sahel et al. 2019). A culmination of these studies came out with the recent approval of the first commercial gene supplementation therapy targeting an inherited monogenic sensory disorder, the congenital Leber Amaurosis causing precocious vision loss. This product, LUXTURNA™ (voretigene neparvovec-rzyl; Spark Therapeutics, Inc., Philadelphia, PA), delivers a normal copy of the *RPE65* cDNA using an adeno-associated virus (AAV) to retinal cells for the treatment of biallelic *RPE65* mutation–associated retinal dystrophy (Russell et al. 2017, Maguire et al. 2019).

A great number of clinical trials have been designed to prolong retinal cell survival and halt vision decline. Usher patients have been included in some trials designed to treat late-onset progressive retinitis pigmentosa. For example, several patients with early-stage retinitis pigmentosa, including USH2 and USH3, have received implant capsules of human NT-501 cells that provide subretinal delivery of a ciliary neurotrophic factor (CNTF). This factor maintains photoreceptor cell survival, especially cones, and halts gradual vision loss (Trials #NCT00447980; #NCT01530659). This trial is now completed but the outcome of this study has not yet been published. The first Usher-dedicated gene therapy study in humans was a phase I/II clinical trial carried out using lentiviral (EIAV)-based sub-retinal delivery of the USH1B gene, *MYO7A.* This trial has been terminated due to review by the sponsor (Sanofi) of clinical development plans and priorities (Trial #NTC01505062). Another USH1B trial is ongoing using AAV-mediated delivery, vectors that are better suited to transduce photoreceptors, the primary target cells of the USH1 retinopathy (Jacobson et al. 2008; Schietroma et al. 2017). Considering the larger size of *MYO7a*, optimized dual vector systems are used to deliver the gene in two separate fragments with the possibility to reconstitute the full-length coding sequence in the target cell (reviewed in McClements and MacLaren 2017, Trapani 2019). Using different dual vectors injected in the retina to assess successful expression of a functional myosin VIIa, promising results were obtained with

hybrid vectors (Dyka et al. 2014, Trapani et al. 2014). A clinical trial is underway in Europe to supplement *MYO7A* with dual AAV vectors to target the retina of USH1B-affected patients (UshTher, Horizon 2020; Trial #NCT02065011).

AAV-mediated gene expression has been reported to be quite stable, lasting for several years in human clinical trials and in dogs (Wojno et al. 2013). However, despite the promise of gene replacement therapies, this approach is not amenable for a very large genes (> 10 kb), or which expresses multiple essential isoforms, such as *CDH23*, *USH2A*, or *GPR98/ADGVR1*. Another clinical trial, also in phase I/II (ProQR, Stellar; Trial #NCT03780257), evaluates the safety and tolerability for a mutation-specific therapeutic strategy, an intravitreal injection of an antisense oligonucleotide (ASO), QR-421a, designed to skip the mutated exon 13 of the USH2A gene. Indeed, one of the most common pathogenic variant in the large *USH2A* gene (~71 exons, 15606 bp) is localized to exon 13 in human. For this clinical trial, ASOs have been designed to enact an in-frame deletion of exon 13, thereby bypassing the mutated site. In the absence of validated animal models with this *USH2A*-mediated vision loss, efficiency of QR-421a has been tested in a zebrafish *Ush2a* model (Dona et al. 2018, Han et al. 2018).

ASOs target RNAs, and are limited to diseases amenable to repair by blocking translation or a specific splice site. Other approaches targeting specific Usher mutations are ongoing, although their validation in appropriate animal models is still lacking. These include: (1) Translational Read-Through-Inducing Drugs (TRIDs), which targets nonsense mutations and can apply to disease genes independent of their identity. TRIDs have been assessed in the retina of *Ush1c* mice (Goldmann et al. 2010, 2011, 2012). New-generation aminoglycosides NB30, NB54 and chemical compound PTC124 were assessed for retinal toxicity and read-through efficacy of a nonsense pathogenic variant, p.Arg31*, in the *Ush1c* gene (Godmann et al. 2012). These drugs showed successful biocompatibility and led to partial recovery of harmonin expression in the treated retina, but they have not yet been assessed in the inner ear; (2) Overlack et al. (2012) explored the use of Zinc Finger nucleases (ZFNs), as a gene editing tool to target a nonsense mutation in *USH1C*. The p. p.Arg31* mutation was introduced into mouse *Ush1c* DNA. Stable p.Arg31*-*Ush1c* and control *Ush1c* cell lines were generated by transfecting plasmids into Flp in-

HEK293 cells. Homologous recombination using ZFNs targeting the non-sense pathogenic variant was validated at the genomic and protein level. This work also showed reduced toxicity in cell cultures. While double stranded breaks and homologous recombination were achieved *in vitro*, the process is expected to be significantly less efficient *in vivo* in non-dividing cells. ZFNs (such as TALENs) are now being replaced by the more performant CRISPR/Cas9 system, making it unlikely that the approach will move forward to the clinic; (3) Finally, studies in human fibroblasts using CRISPR/Cas9 recently showed successful repair of a common mutation in the USH2A gene, the c.2299delG, which accounts for 31% of all USH2 cases (Fuster-Garcia et al. 2017). In a first set of experiments, Fuster-Garcia et al. assessed *USH2A*-specific *sgRNAs* by introducing two exon 13 *USH2A* common mutations by homologous recombination in HEK cells, c.2299delG, c.2276G>T. Select *Cas9-sgRNA* plasmids demonstrated cleavage efficiency and homology-directed repair (HDR) efficiency up to 16% *in vitro*. The same approach was used to assess mutation repair of the c.2299delG pathogenic variant in human fibroblasts. HDR efficiency was low but measurable with a correction rate of 2.5% and no detectable off-target activity. With the development of new tools that allow more efficient targeting, an increase in the use of gene editing for the treatment of human disorders, including Usher syndrome, is expected in the near future.

Success of these treatment solutions in the eye has helped accelerate the development and potential efficiency of similar approaches for Usher and other deafness genes in the inner ear.

6- Gene specific mediated approaches in the inner ear for Usher syndrome

Like the eye, the inner ear offers an accessible and a relatively immune-privileged environment that is suitable for the development of potential treatment solutions. Information on genetic etiology and the uncovering of molecular disease mechanisms are helpful for choosing the most appropriate strategy to alleviate, prevent and or correct the burden of inner ear related sensory deficits (see Fig. 4). Several approaches, some of them validated for other genetic diseases, have been recently used to target different Usher genes (cf. below). These include gene replacement, or mutation specific interventions that may correct, knock-down or knockout a given gene by correcting and bypassing specific causal pathogenic variants.

6a- Gene replacement in the inner ear

The classic gene therapy strategy involves the introduction of a functional copy of the defective gene, gene supplementation or replacement. In this case, the cDNA encoding the functional gene is packaged into a vector which can be delivered directly to the inner ear. Various vectors have been assessed, including adenovirus (AdV), adeno-associated viruses (Chien et al. 2015a, 2015b, Dazert et al. 1997, Husseman and Raphael 2009, Kesser et al. 2008, Li Duan et al. 2002, Sacheli et al. 2013, Takada et al. 2015, Venail et al. 2007, Zhang and Bergelson 2005), retrovirus (Bedrosian et al. 2006), lentivirus (Bedrosian et al. 2006, Han et al. 1999, Pietola et al. 2008, Wei et al. 2013, Wang et al. 2013, Yang et al. 2013a) and Herpes Simplex virus (Chen et al. 2001, Derby et al. 1999). With their low-immunogenicity across ongoing clinical trials, in addition to established evidence of efficacy in various inner ear cell types, AAVs have become a standard vector for future gene therapy treatment in the inner ear (reviewed in Ahmed et al. 2017, Fukui and Raphael 2013, Lustig and Akil 2019, Omichi et al. 2019, Sacheli et al. 2013). Advantages and disadvantages of AdV and AAV vectors for inner ear gene therapies, as well as AAV differences in cell type efficiency rates have been the topics of many recent reviews (Ahmed et al. 2017, Devare et al. 2018, Lustig and Akil 2019, Omichi et al. 2019, Ren et al. 2019, Taiber et al. 2019).

6a.1- Single AAV gene replacement therapy

First described about 50 years ago, AAVs are single stranded, DNA viruses belonging to the family *Parvoviridae.* They have been isolated from a wide range of organisms and have not been associated with any disease. Their nomenclature (AAV2/1, AAV2/8, etc…) is such that the viral serotype – is denoted by the second number; and the first number stands for the inverted terminal repeat (ITR) type (short DNA sequences that flank the AAV genome and allow it to form concatemers in host cells). So far, almost all vectors used for gene therapy contain AAV type 2 ITRs. For the purpose of this review, we include the full nomenclature hereafter. Vector efficacy is measured by both the efficiency with which the transgene is delivered and its specificity for the target cell type (tropism). In the inner ear, whatever the causal gene, success and efficiency of

targeting hair cells depends on several parameters: viral serotype, viral purity, titer, mode of injection, and promoter used (for example, cytomegalovirus CMV, hybrid CAG, hair cell specific, etc.), along with developmental age at which the treatment is performed (Ahmed et al. 2017, Corey and Maguire Hearing Research SI 2019, Emptoz et al. 2017, Fukui and Raphael 2013, Isgrig et al. 2019, Yoshimura et al. 2019). Promising gene replacement results have been obtained using distinct AAVs to replace 4 USH genes, *USH1C, USH1G, USH2D,* and *USH3A*.

**Insert Table 2 here*

USH1C: The USH1C gene encodes for the scaffolding protein harmonin (Fig. 1). A founder mutation in *USH1C,* c216 G>A, was identified in patients of southern Louisiana (Lentz et al. 2005, 2010). This pathogenic variant, located in exon 3, creates a cryptic 5' splice site that is used preferentially over the authentic 5' splice site of exon 3. This pathogenic variant leads to translation of a truncated protein which lacks all PDZ and PST domains (see Fig. 1) necessary for the scaffolding function of harmonin. Gene replacement therapy was assessed in a knockin mouse model that reproduces the *Ush1c*. c216 G>A mutation (Lentz et al. 2010; Pan et al. 2017). Pan et al. (2017) took advantage of a newly generated synthetic vector, AAV2/Anc80L65 (Landegger et al. 2017, Zinn et al. 2015), a predicted ancestor of the widely studied AAV2/1, 2/2, 2/8, and 2/9. Analyses of ancestral sequences of viral capsid components led to creation *in silico* of evolutionary intermediates (Zinn et al. 2015), among which AAV2/Anc80L65 was found to successfully and efficiently target the sensory hair cells in neonatal mice (Landegger et al. 2017).

Pan et al. (2017) used AAV2/Anc80L65 to deliver the coding sequence for two isoforms of harmonin known to be expressed in hair cells. Harmonin-b is predominantly localized to the upper tip link density (Grillet et al. 2009, Michalski et al. 2009) and harmonin-a is associated with the ribbon synapse where it modulates voltage-dependent calcium channels (Gregory et al. 2013). Injection of AAV2/Anc80L65-CMV-harmonin-b through the round window membrane (RWM) at P1 led to expression and correct targeting of harmonin to the tip of the stereocilia, as well as to recovery of normal hair bundle morphology and functional mechanosensitivity. Importantly, the

strategy promoted hair cell survival and restored auditory sensitivity, otherwise absent (> 100 dB), to thresholds as low as 30 dB in the best performers, and ~50 dB on average with significant improvements in the lower frequencies up to 16 KHz. Addition of harmonin-a containing vectors did not lead to significant improvement of the mutated hair cells features and hearing sensitivity. As for vestibular deficits in these mice, *Ush1c* replacement therapy also enabled vestibular hair cell function and totally abolished the circling behavior in treated mice (Pan et al. 2017).

USH1G: The USH1G gene encodes the scaffolding protein sans (Fig. 1). Emptoz et al. (2017) assessed gene replacement therapy targeting a mouse model of USH1G. *Ush1g*$^{-/-}$ mice display profound deafness and vestibular dysfunction. Different AAV serotypes were assessed for delivery via the RWM at P2.5: AAV2/1, AAV2/2, AAV2/5 and AAV2/8 from two vector core facilities. The AAV2/8 vector from Penn vector core facility was selected for this study. Viral transfer of *Ush1g* cDNA lead to rescue of hair bundle defects, partial restoration of hearing, from >100 dB down to ~75 dB in the low frequency region, and significant recovery of vestibular phenotypes, as shown by recovery of angular vestibular ocular reflexes (Emptoz et al. 2017).

USH2D: The USH2D gene encodes the PDZ containing protein whirlin (Fig. 1), which has been shown to be expressed at the tip, and transiently at the base, of stereocilia. Different isoforms of whirlin, long and short (Fig. 1), targeted to different regions of the hair bundle are expressed in the inner ear during development (Mathur et al. 2015, Mathur and Yang 2019). Pathogenic variants leading to a premature stop codon at the whirlin N- and C- terminal regions lead to Usher syndrome type 2D, USH2D, and non-syndromic recessive deafness, DFNB31, respectively (Mathur and Yang 2015, see Table 1). In their initial study, Chien et al. (2016) demonstrated that RWM injections of the whirlin long isoform, AAV2/8-CMV-whirlin, lead to recovery of hair bundle height, but without significant recovery of hearing, probably because of the low rate of hair cell transduction. In a subsequent study, using the same vector but through another route of gene delivery, an injection via the posterior semicircular canal, Isgrig et al. (2017) obtained a higher hair cell transduction rate in the whirler mice and a significant, albeit mild, recovery of auditory function with improved average thresholds from >100 dB in untreated mice down to 80 dB on average in injected mice, with thresholds, in the best performers of ~60 dB for 8 KHz pure tones. Recovery of

balance function was significant with reduction in circling behavior, improved swimming and rotarod performances.

USH3A: The USH3A gene encodes a tetraspan-like glycoprotein, clarin-1 (Fig. 1). Pathogenic variants in *USH3A* are associated with post-lingual progressive hearing loss and retinitis pigmentosa. Three different gene replacement studies have been published for USH3A (Table 2). Each explores the use of different mouse models: a knockout mouse harboring a transgene consisting of *Clrn1*-ITR under the control of regulatory elements to direct hair cell expression and postnatal downregulation (Geng et al. 2017), two total knockout mouse models (*Clrn1*$^{ex1-/-}$: Gyorgy et al. 2018, and *Clrn1*$^{ex4\ -/-}$: Dulon et al. 2018), and a conditional knockout mouse harboring hair cell-specific deletion of the *Clrn1* gene from postnatal stages onwards, *Clrn1*$^{ex4fl/fl}$, *Myo15*$^{cre+/-}$ mouse (Dulon et al. 2018). For these studies, different viral vectors were used: AAV2/8 (Geng et al. 2017; Dulon et al. 2018) and AAV2/9 capsid variant (AAV2/9-PhPB with CAB promoter and woodchuck hepatitis virus posttranscriptional regulatory element or WPRE) (György et al. 2018).

Dulon et al. (2018) showed that three different isoforms of *Clrn1* are expressed in the hearing organ. Clarin-1 has been localized to the hair bundle and synaptic zone in Zebrafish hair cells (Ogun and Zallochi, 2014, Gopal et al. 2015). It has been shown to play a functional role in the hair bundle mediating mechanoelectrical transduction (Geng et al. 2012; Dulon et al. 2018) and at the hair cell ribbon synapse, modulating exocytosis via interactions with L-type calcium channels and harmonin (Dulon et al. 2018). Gene replacement strategies in above-mentioned total knockout mice showed that expression of *Clrn1* variants encoding the predicted principal isoform expressed in hair cells (clarin-1 isoform 2) led to poor auditory recovery (Geng et al. 2017; Dulon et al. 2018). Variable outcome was observed by György et al. (2018) with gene replacement therapy using *Clrn1* isoform 2 on *Clrn1*$^{ex1-/-}$ with further improvement in the low frequency region. However, a similar treatment in mice with delayed onset of *Clrn1* deletion (*Clrn1*$^{ex4fl/fl}$ *Myo15*$^{cre\ +/-}$) led to significant recovery, from >100 dB of ABR thresholds in untreated mice down to wild type level thresholds in treated mice (30-40 dB) at P22-P24 (Dulon et al. 2018). Threshold shifts to 60-70 dB were observed by P60, perhaps due to low transduction efficiency in OHCs.

In these studies, hearing recovery in treated Usher mouse models was often low and incomplete, probably due to low viral transduction rates in auditory OHCs using conventional AAVs (Emptoz et al. 2017, Isgrig et al. 2017). A much better hearing improvement was obtained with the introduction of the synthetic AAVs Anc80L65, which has been shown to infect cochlear IHCs and OHCs with high efficiency (Landegger et al. 2017). Recent studies identified two other AAVs with superior infection efficiencies in both IHCs and OHCs, namely AAV2/9-PHPB (György et al. 2018, 2019, Lee et al. 2020) and the synthetic AAV2/2.7m8 (Isgrig et al. 2019). While AAV2/2.7m8 infects also inner pillar cells and inner phalangeal cells with high efficiency, it has been shown to preferentially target the cochlear hair cells compared to the vestibular hair cells (Isgrig et al. 2019). Further studies in mouse, and especially in larger animals such as non-human primates in anticipation of future translation to patients, are needed to test these new viruses. Optimization of the most efficient promoter and delivery method (semicircular canal or macular, round window approach) may help improve the degree of hearing (and balance) restoration.

6a.2- Gene replacement therapy for genes that exceed AAV packaging capacity

While extremely valuable, AAVs have a limited cloning capacity of about 4.7 Kb (see Fig. 4A). Since the two ITRs of an AAV are about 0.2-0.3 Kb total, the foreign cDNA that can be introduced between these 2 ITRs should be smaller than 4.4 Kb. While oversized cDNA can be introduced, vectors exceeding 5 kb lead to disparities in the translated products with heterogeneous length and truncation on the 5′ end (Wu et al. 2010). As seen in Table 1 and Fig. 1, several USH genes exceed AAV capacity, and for some, different isoforms are expressed. Therefore, alternative approaches are required for oversized genes and knowing where and when specific variants are expressed can be key to the design of adapted therapies. Lentiviruses and adenovirus (AdV) are attractive in part due to their large packaging capacity (up to 36 Kb for third generation AdV), but side effects due to toxicity and immune response have led to reduced interest in their use as tools for inner ear gene therapy (Hendrickx et al. 2014). Interestingly, the low viral capacity of AAVs can be overcome by the use of dual AAV vectors, which involve cloning of two separate gene coding fragments and the reconstitution of the full-length coding sequence in the target cell (reviewed in

McClements and MacLaren 2017, Trapani 2019, Reisinger 2019). In the inner ear, two recent studies validated the use of dual AAVs in mouse to target the DFNB9 gene, *Otof*, encoding otoferlin, a putative Ca^{2+} sensor key to synapse neurotransmitter release in IHCs (Akil et al. 2019; Al-Moyed et al. 2019). Upon injection of two AAVs, one with the 5′ part of *Otof* cDNA followed by a splice donor site, the second with a splice acceptor site followed by the *Otof* 3′ C-terminal region, reconstitution of otoferlin full length protein in IHCs led to durable restoration of its expression and a reversal of the deafness phenotype to wild type level. The approach, while expanding gene replacement therapy with AAVs to genes over 5 kB is still only applicable to genes under <~9kB while allowing space for split genes, promoter, poly-adenylyation signals and, depending on the approach, splice acceptors and donors. Two Usher genes *MYO7A* (USH1B) and *PCDH15* (USH1F) can be targeted for dual gene therapy. A clinical trial is underway in Europe to supplement *MYO7A* with dual AAV vectors to target the retina and vision loss of USH1B patients (UshStat, Horizon 2020, NCT02065011), but the efficiency of this approach for Usher genes in the inner ear remains to be established.

6b- Gene size independent strategies for USH genes

In contrast to gene replacement, mutation repair-based approaches are designed to correct a given pathogenic variant, without altering the whole gene structure. One big advantage is that the reconstituted coding sequences and all related regulatory elements remain intact, thus preserving physiological gene expression levels in the appropriate cell type. These molecular approaches make use of small molecules, drugs, short single stranded oligonucleotides or genome editing tools (ZFNs, TALENs, and particularly CRISPR/Cas9). Some of these tools have been successfully used to assess repair of Usher and/or deafness genes with promising results.

6b.1- Small molecules and oligonucleotides targeting transcription and/or translation

USH3A: In the search for small molecules, Alagramam et al. (2016) used a cell-based high-throughput screening for the identification of small molecules capable of stabilizing $CLRN1^{N48K}$. The *CLRN* missense pathogenic variant, pN48K, has been shown to lead to reduced expression

and mislocalization of the CLRN1^{N48K} protein. A lead molecule was identified (BF844) that acted as a proteasome inhibitor and increased the stability of the protein via modulation of HSP90 and HSP60. Injected intraperitoneally with dose escalation starting at 10 mg/Kg at P10, BF844 successfully protected *Clrn1*^{N48K} knockin mice from progressive hearing loss. In the absence of an *Ush3a* model for vision loss, possible efficiency of this compound to mitigate vision loss remains to be established.

USH1C: Lentz et al. (2013) developed the antisense oligonucleotides strategy to target the Acadian *USH1C* mutation. For this mutation, different ASOs were designed aiming to inhibit the cryptic splice site abnormally created by the 216 G>A pathogenic variant and restore expression of the correct normal *RNA*. Systemic neonatal injections of the most efficient ASOs (selected *in vitro*) led to recovery of full length harmonin expression in the *Ush1c c.216G>A* mice, recovery of hair bundle morphology and restoration of auditory function as assessed by ABR and balance behavior as assessed by analysis of open field behavior (Lentz et al. 2013).

6b.2- Genome editing in the inner ear

The advent of novel Cas9 enzymes (high fidelity and short versions with distinct PAM recognition sites) is likely to further expands the targeting range of Cas9 and increases specificity of gene editing (György et al. 2019). Excellent and promising results were recently obtained using cationic-lipid (Gao et al. 2018) and AAV -mediated (György et al. 2019) delivery of CRISPR-Cas9 and gene-specific single guide RNA (sgRNA) components to disrupt the mutated *Tmc1* allele in the *Beethoven* (*Bth*) mouse, a model for DFNA36 dominant form of hearing loss in humans. Lipid-mediated Cas9/sgRNA delivery in the cochlea of *Tmc1*$^{Bth/WT}$ mice resulted in modest improvement in hearing thresholds 4 weeks after treatment (Gao et al. 2018). Hearing preservation was modest and not sustained even at low frequencies, which was ascribed to insufficient number of edited hair cells, and/or lack of specificity of the Cas9 enzyme that led to unwanted inactivation of the WT *TMC1* allele (a difference of only one base pair from the normal allele) (Gao et al. 2018). The recent use of a *Staphylococcus aureus* Cas9 (SaCas9-KKH), a shorter variant that fit into a single AAV, significantly improved the outcome of allele-specific genome editing strategies for dominant

hearing loss. Interestingly, the PAM sequence of this Cas9 variant “NNNRRT” has been used to select efficient sgRNAs that were proven, *in vitro* and i*n vivo*, to specifically induce indels only in *Tmc1*$^{Bth/WT}$, but not in *Tmc1*$^{WT/WT}$ edited cells. The AAV-mediated delivery of SaCas9-KKH/sgRNA complex successfully resulted in prevention of deafness in *Tmc1* $^{Bth\ /WT}$ mice for up to one year (György et al. 2019). While further work is necessary to test and validate such approach for *USH* genes, CRISPR-Cas9 now offers a wide range of tools with increased targeting specificity (Doudna and Charpentier, 2014, Lino et al. 2018). Such tools can be used to disrupt dominant mutations or correct recessive mutations via HDR or base editing. In many instances, however, use of larger variants of Cas9 will be necessary that will require different delivery vehicles, such as non-viral delivery (e.g. liposomes, gold particles), viral vectors with large cargo capacity such as adenovirus and lentivirus or the use of dual AAV vectors to package the enzyme along with the gRNA and when required donor DNA (for HDR) (reviewed in Knott and Doudna, 2018, Lino et al. 2018). While challenging, dual AAV vectors have been successfully used *in vivo* for genome editing of other genes with split CRISPR base editors (Truong et al. 2015; Winter et al. 2019; Lim et al. 2020). Such advances, along with the continuing development of new and improved gene editing tools and new delivery methods bring genome editing to the forefront as potential therapeutic for the treatment of Usher syndrome.

7- From bench to bedside: promises and challenges for Usher syndrome therapies

Over the next decade, novel tools validated for the treatment of Usher syndrome, whether the auditory, balance or retinal phenotype, will most likely find their way to the clinic.

7a- Pre- *versus* Post-natal time window of treatment efficacy in Humans

Except for USH3 patients, deafness is congenital in the prevalent forms of Usher syndrome, USH1 and USH2. While the mice are and will continue to be very instrumental to understand deafness disease mechanisms, the onset of hearing and range of sound frequency perception differ between mice and humans. The mouse inner ear is functionally immature at birth with hearing onset only during the second week of postnatal life. Conversely, the inner ear in human is

completely functional before birth, having reached its final maturation *in utero* at ~20 weeks of gestation (Hepper and Shahidullah 1994). Similarly, while in the mouse, cells of the prosensory domain exit the cell cycle around embryonic day 12 (E12), in human, cells cycle exit is already taking place at Week 7 (W7, post conception) (Roccio et al. 2018). In human fetuses, MYO7A-positive hair cells are observed in the cochlear duct by W11, while they are observed around E13 in the mouse. Are these cells still present and viable at birth in Usher syndrome patients? Unfortunately, no current technology allows the visualization of sensory cells along the auditory organ of a newborn infant.

Therefore, questions remain as to how present successes in animal models could translate to viable treatments in patients. Is fetal-oriented medicine for hearing an attainable option? If so, how applicable will this be? While USH1-affected patients are often diagnosed within the first couple of years, USH2 and USH3 patients are typically diagnosed at later ages, pre-pubertal or older. Would therapies delivered in older patient be capable of arresting the progression of the disease or better restore and correct previous defects? Variability of beneficial outcomes between different genes in related animal models suggest that translatability should be studied on case by case basis.

7b- Efficacy, safety, and delivery of Usher genes therapeutics

In anticipation of clinical translation, further studies are required to determine suitable methods for controlled, safe and efficient delivery in the inner ear and targeting of sensory hair cells at different time points. Considering the importance of tonotopic information for sound processing, the extent and rate of hair cell viral transduction is of paramount importance to restore hearing over the entire frequency range in human. More than 100 AAV serotypes have been identified and engineered to improve their transduction to target tissue. A series of viruses targeting hair cells have been validated in mice (reviewed in Corey and Maguire, Hearing Research, Special Issue, 2020). Tropism, however, is known to differ in different species, and a viral vector validated in mice may not be efficient in human. Studies in large animal models, such as non-human primates, with fetal hearing onset and a comparable acoustic range and sensitivity to that of humans, will be

necessary to enable safe and effective validation of deafness genes therapeutic options, prior to translation into clinic.

Regarding gene delivery, minimally- and non-invasive local delivery methods are currently being developed to treat inner ear disorders. Because only a limited volume of solution can be delivered to the inner ear, concentrated compounds or high titer viral vectors are required to achieve qualitative transduction of sensory cells from the basal high frequency turn to the apical low frequency turn of the cochlea. The lack of undesired expression of the virus in unwanted cellular targets also needs to be assessed and evaluated for toxicity. Finally, considering the long human lifespan, will a single injection during childhood be sufficient to ensure longstanding therapy effect? If not, how many repeated injections will be required to maintain the therapeutic benefit, and if so, would repeated injection exacerbate the risk of toxicity and immune reaction?

8- Concluding remarks and outlook

Successful outcomes in retinal treatment is likely to facilitate translation of these novel therapies to the inner ear. Interestingly, most of the gene therapy reports in mice showed optimal recovery of hearing sensitivity in lower frequencies. Because cochlear implants expand along the higher frequency region of the hearing organ, the combination of such a device with gene therapy may be the first step in the development and assessment of biological therapies for hearing loss. Gene and drug delivery to the inner ear remains technically challenging. While similar approaches as cochlear implantation may be envisaged, biological treatments have to be applied in an optimal manner that will preserve the delicate, often already compromised, structures that are embedded within the inner ear. Innovative surgical technologies are thus necessary to deliver newly developed therapies in a safe and efficacious manner. New developments in the field of viral-mediated targeting (e.g. new and more efficient AAVs) and gene editing (e.g. improved Cas9 variants, increased homologous directed repair efficiency) will provide opportunities to optimize gene replacement and/or repair. Building upon precise knowledge of a given gene, determination of the pathogenic variant and natural history of the disease, it is now possible to design and evaluate the best and most efficient therapeutic strategy for patients. However, important

considerations need to be addressed before any of these therapies can be applied to patients. One major question is whether the new therapies will be used to complement or replace current hearing devices, if clinically proven to outperform hearing prostheses. Realistic endpoints will have to be determined and carefully discussed with patients, particularly when such therapies may not restore function but rather halt progression of the disease. Nevertheless, science has now opened new doors for the treatment of genetic hearing loss and Usher syndrome. Transfer of this knowledge to the clinic with require concerted efforts by scientists in diverse fields such as virology, immunology, molecular and cell biology and physiology along with audiologists and physicians.

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Figures and Legends :

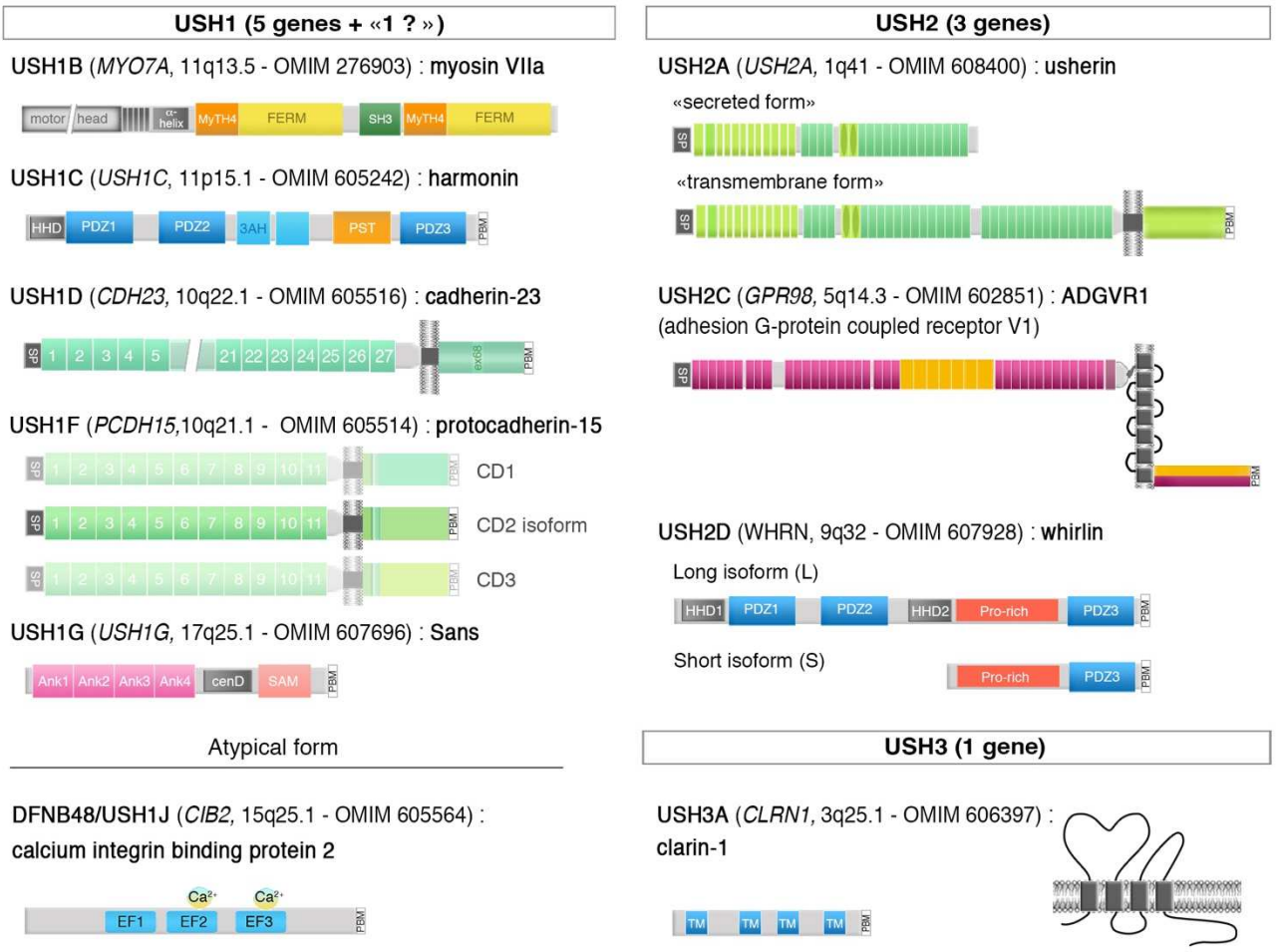

Figure 1: Domain structure of Usher proteins. Five USH1 (myosin VIIa, harmonin, cadherin-23, protocadherin-15, and Sans), a putative USH1 (CIB2), three USH2 (usherin, ADGRV1, whirlin), and one USH3a (clarin-1) proteins are indicated. Many of the USH proteins exist in multiple isoforms. There are three isoforms of protocadherin-15 with distinct cytodomains (CD); There are two isoforms of usherin, one is secreted, and one includes a cytoplasmic transmembrane domain; Two whirlin isoforms exist in the ear, the short and long isoform. A third isoform, not shown here, is also expressed in the retina (Mathur and Yang, 2019). *CIB2 is referred to as atypical USH as, unlike for all other 5 USH1 genes, several loss of function pathogenic variants in the CIB2 gene in both humans and mice were shown to cause the DFNB48 non-syndromic hearing loss, without loss of balance or vision.

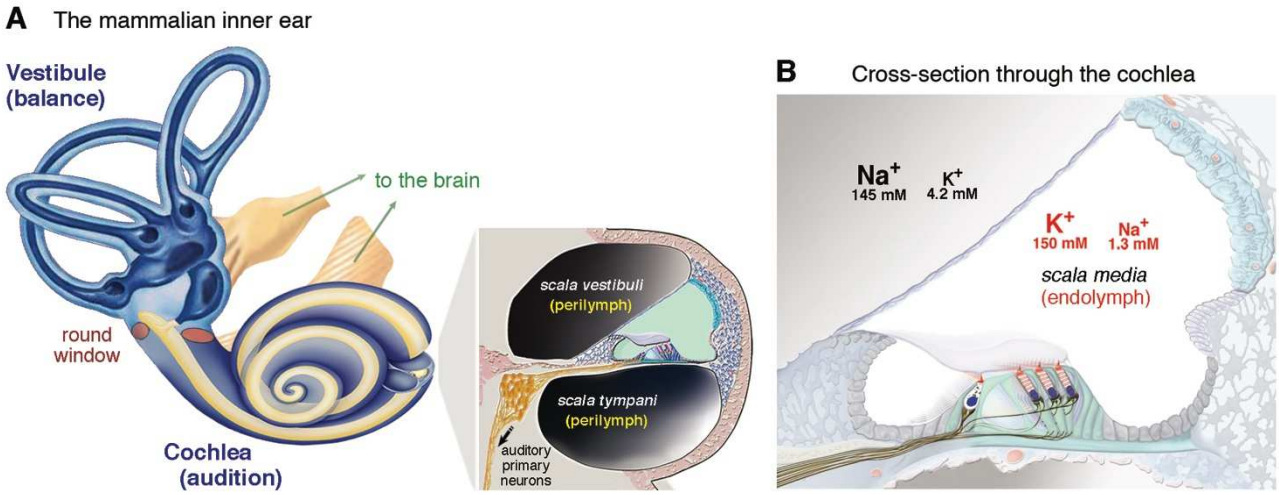

Figure 2: The mammalian inner ear, and cross section of the snail-coiled cochlea illustrating the organization of the auditory organ), with the hair cells and associated cochlear ganglion neurons. The mammalian inner ear consists of the vestibule (balance organs), which detect linear and angular accelerations, and the cochlea, the hearing organ, which detects sound waves (A). The cochlea is made up of three fluid-filled compartments with differing ionic composition: the *scala vestibuli* and the *scala tympani*, both filled with perilymph, and the *scala media*, filled with endolymph (A,B). The *scala media* houses the auditory sensory epithelium, the organ of Corti, consisting of one row of inner hair cells (IHCs), three rows of OHCs, and various types of supporting cells (B).

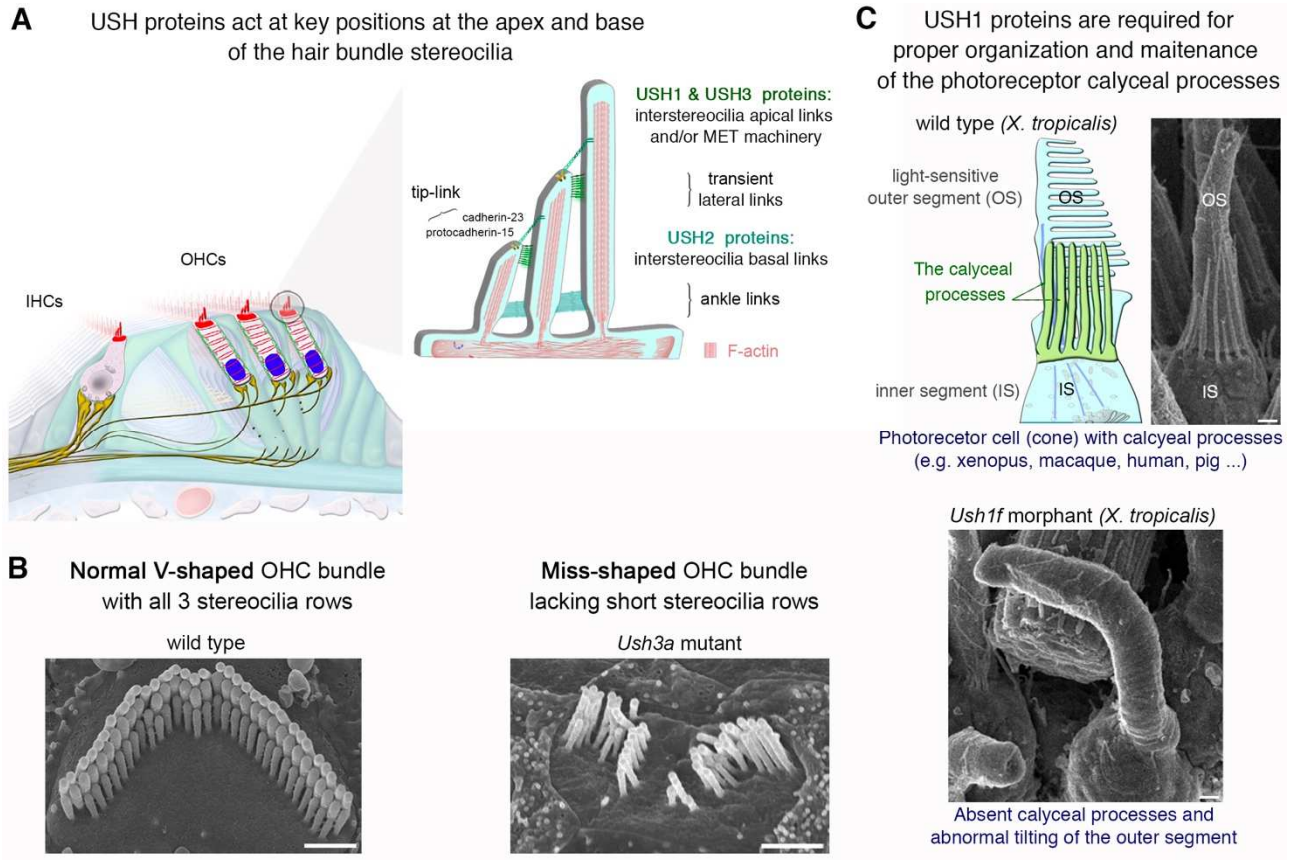

Figure 3: USH proteins properly shape the developing hair bundle, the sound-receptive structure of hair cells. (A) The stereocilia of the hair bundle are connected to each other by different subsets of fibrous intersterocilia links varying overtime, with some also connecting the stereocilia to the kinocilium. Shown here is a differentiating hair bundle (around P8 in mouse) illustrating the position of the early transient lateral links, the ankle links, and the tip-link. Note that *cis*-homodimers of cadherin-23 interact in *trans* with *cis*-homodimers of protocadherin-15 to form the upper and lower parts of the tip-link, respectively. At the upper extremity of the tip-link, cadherin-23 is connected to the actin core of the stereocilia through interactions with USH1 proteins: harmonin b, Sans and/or myosin VIIa. (B) In the absence of Usher proteins, the normal development and proper shaping of the hair bundle are impaired, as shown in the absence of the USH3a protein, clarin-1 (adapted from Dulon et al. 2018). IHC: inner hair cell, OHC, outer hair cell. (C) Amphibian photoreceptor cone cells with well-developed calyceal processes (green in the schematic representation), F-actin based microvilli surrounding the base of the light-sensitive outer segment. Upon protocadherin-15 (USH1F) knockdown in *Xenopus tropicalis,* the maintenance of the calyceal processes is impaired, which

affects the morphogenesis of the photoreceptor outer segment displaying abnormal tilting (modified from Schietroma et al. 2017). bar = 1 µm.

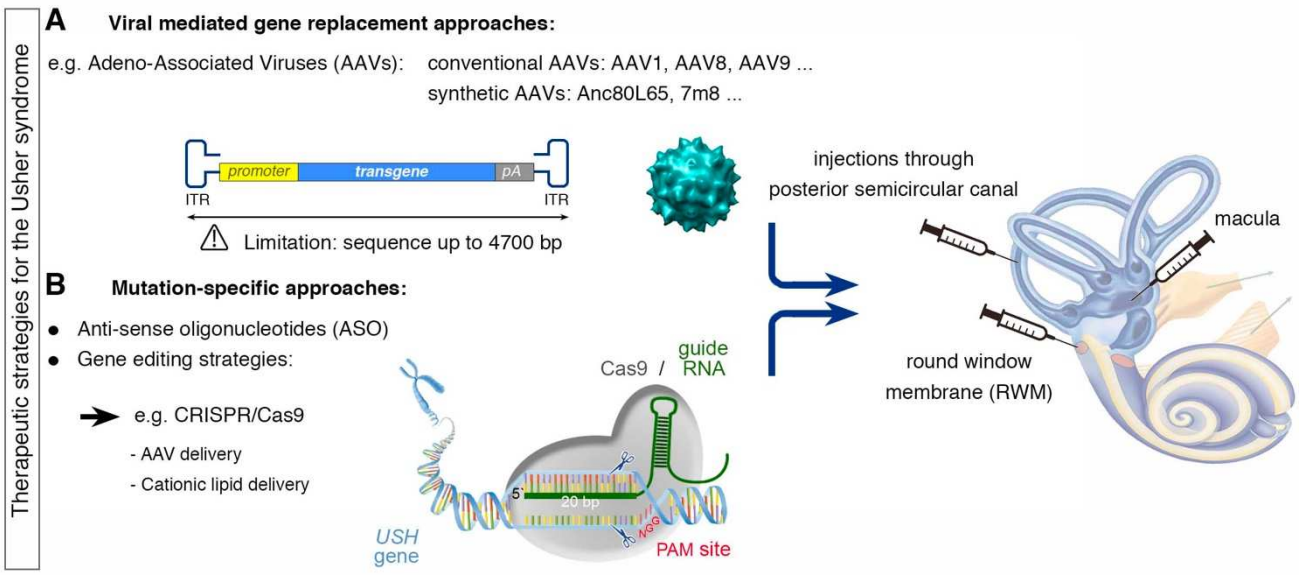

Figure 4. Various therapeutic approaches are used for the Usher syndrome. (A) Viral mediated approaches to rescue hearing and balance deficits make use of distinct conventional and synthetic adeno-associated virus (AAV) vectors, which can be delivered through different routes of administration. One major disadvantage of AAVs, however, is their low viral capacity, limited to ~ 4.7-5 kilobases, including promoter region, the transgene, and polyA 3`region, between the two inverted terminal repeats (ITRs). Out of the 10 *USH* cDNAs, only 5 can fit into AAVs: *USH1C, USH1G, CIB2, WHRN, and CLRN.* (B) Mutation specific approaches can be used that are not restricted by the size of the targeted gene. These include anti-sense oligonucleotide (ASO) or gene editing strategies, notably CRISPR/Cas9. As illustrated, guide RNAs are designed to target a specific region of the DNA that includes nuclease specific PAM motifs. Guide RNAs direct Cas9 endonucleases to the specific PAM motifs and cleaves or edits the targeted site. Different CRISPR tools have been engineered that use different PAM motif and Cas9 endonucleases. These nucleases can generate double stranded or single stranded DNA cleavage; they can disable cleavage of one DNA strand or allow base editing with the use of a dead Cas9. Gene alteration, repression, activation and correction can now be precisely designed with the use of these new tools (see Knott and Doudna, 2018).

		Hearing Loss (HL)	Vestibular dysfunction	Retinitis pigmentosa (RP)	Other disease (OMIM numbers)
USH1		**Congenital, and profound**	**Bilateral vestibular areflexia**	**Prepubertal onset**	**Non syndromic HL**
Genes (OMIM number)	*USH1B* (276900)	Myosin VIIa, an actin-based motor protein ***ORF** = 6645 bp*, 2215 aa, 254 kDa			DFNB2 (600060) DFNA11 (601317)
	USH1C (605242)	Harmonin, a PDZ-domain scaffolding protein (long isoform b3, ***ORF** = 2697 bp*, 899 aa, 98 kDa			DFNB18 (602092)
	USH1D (601067)	Cadherin-23, adhesion molecule (upper tip-link region) ***ORF** = 10 062 bp*, 3354 aa, 369 kDa			DFNB12 (601386)
	USH1F (602083)	Protocadherin-15, adhesion molecule (lower tip-link region) ***ORF** = 5865 bp*, 1955 aa, 216kDa			DFNB23 (609533)
	USH1G (607696)	Sans, an ankyrin and SAM-domain containing scaffolding protein ***ORF** = 1383 bp*, 461 aa, 51 kDa			
	USH1J (614869)	CIB2, calcium and integrin binding family member 2 ***ORF** = 561 bp*, 187 aa, 21 kDa			DFNB48 (609439)
USH2		**Congenital, and moderate**	**Normal**	**Postpubertal onset**	Non-syndromic RP or HL
	USH2A (608400)	Usherin, laminin- and fibronectin-domain containing protein ***ORF** = 15 606 bp*, 5202 aa, 575 kDa)			RP36 (613809)
	USH2C (605472)	ADGRV1, Adhesion G-protein coupled receptor V1 ***ORF** = 18 918 bp*, 6306 aa, 693 kDa)			
	USH2D (611383)	Whirlin, a PDZ-domain scaffolding protein (long isoform ***ORF** = 2721 bp*, 907 aa, 96 kDa)			DFNB31 (HL) (607084)
USH3		**Postlingual, and progressive**	**Variable**	**Variable onset and severity**	
	USH3A (276902)	Clarin-1, a tetraspan-like glycoprotein (***ORF** = 696 bp*, 232 aa, 25 kDa)			RP61 (614180)

Table 1: Usher syndrome subtypes: clinical features and genes involved. Pathogenic variants in USH genes can lead to either syndromic or non-syndromic hearing or vision loss (refer to indicated OMIM numbers for detailed description of clinical features, pathogenic variants and related references*). Abbrev.* ORF, open reading frame, denoting the gene size, without 5' UTR and the 3' region with the poly-adenylyation site.

	MOUSE MODEL	THERAPEUTIC APPROACH	DELIVERY	REFERENCE
Usher syndrome type I				
USH 1C	*Ush1c c.216G>A* Knockin mice (Acadian mutation)	Gene replacement (AAV2/Anc80L65)	RWM injection (P1, P10)	Pan et al. 2017
		Antisense Oligonucleotides (ASO-29)	Intra-peritoneal injection (P3, P5, P10, P13, P16)	Lentz et al. 2013
			Intra-amniotic injection (E13-E13.5)	Depreux et al. 2016
USH 1G	*Ush1g*$^{-/-}$ mice	Gene replacement (AAV2/1, 2/2, 2/5, 2/8)	RWM injection (P2.5)	Emptoz et al. 2017
Usher syndrome type II				
USH 2D	*Whirler* mice	Gene replacement (AAV2/8)	RWM injection (P1-P5)	Chien et al. 2016
			Posterior semicircular canal injection (P4)	Isgrig et al. 2017
Usher syndrome type III				
USH 3	*Clrn1*$^{-/-}$ *and* KO-TgAC1 mice	Gene replacement (AAV2/2, 2/8)	RWM injection (P1-P3)	Geng et al. 2017
	Clrn1 $^{ex4fl/fl}$ *Myo15-Cre*$^{+/-}$ mice	Gene replacement (AAV2/8)	RWM injection (P1-P3)	Dulon et al. 2018
	Clrn1$^{-/-}$ mice	Gene replacement (AAV2/9.PHP.B)	RWM injection (P0-P1; P30)	György et al. 2018

Table 2: List of gene replacement and mutation specific-based therapeutic strategies carried out in the inner ear of Usher mouse models. RWM, round window membrane.