Review Article

Small fish, big prospects: using zebrafish to unravel the mechanisms of hereditary hearing loss

Barbara Vona a,*, Julia Doll b, Michaela A.H. Hofrichter b, Thomas Haaf b, Gaurav K. Varshney c, **

a Department of Otolaryngology–Head & Neck Surgery, Tübingen Hearing Research Centre, Eberhard Karls University Tübingen, Tübingen, Germany
b Institute of Human Genetics, Julius Maximilians University, Würzburg, Germany
c Institute of Human Disease Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK, United States

A B S T R A C T

Over the past decade, advancements in high-throughput sequencing have greatly enhanced our knowledge of the mutational signatures responsible for hereditary hearing loss. In its present state, the field has a largely uncensored view of protein coding changes in a growing number of genes that have been associated with hereditary hearing loss, and many more that have been proposed as candidate genes. Sequencing data can now be generated using methods that have become widespread and affordable. The greatest hurdles facing the field concern functional validation of uncharacterized genes and rapid application to human diseases, including hearing and balance disorders. To date, over 30 hearing-related disease models exist in zebrafish. New genome editing technologies, including CRISPR/Cas9 will accelerate the functional validation of hearing loss genes and variants in zebrafish. Here, we discuss current progress in the field and recent advances in genome editing approaches.

© 2020 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Contents

1. Descent into the genetics of hearing loss ................................................................. 2
2. Methodological advancements in sequencing ......................................................... 3
3. The mutational signatures of hereditary hearing loss ............................................... 4
4. Zebrafish as an animal model ................................................................................. 5
5. Random mutagenesis approaches ........................................................................ 5
6. Targeted mutagenesis approaches ........................................................................ 5
6.1. CRISPR-based approaches to study genetic variants .............................................. 6
7. Morpholino-mediated knockdown of mRNA .......................................................... 6
8. Morpholinos vs genetic mutants ............................................................................ 9
9. Zebrafish as a model to study hearing and balance disorders .................................... 9
9.1. Genetic tools to study hearing and vestibular disorders in zebrafish ....................... 10
9.2. Zebrafish disease models for hearing loss ........................................................... 10
9.2.1. Myosin 7AA (MYO7AA) ............................................................................. 10
9.2.2. Myosin 6 (MYO6) .................................................................................... 11

* Corresponding author. Department of Otolaryngology–Head & Neck Surgery, Tübingen Hearing Research Centre, Eberhard Karls University of Tübingen, 72076, Tübingen, Germany.
** Corresponding author. Institute of Human Disease Research Program, Oklahoma Medical Research Foundation, Oklahoma City, 73104, OK, United States.
E-mail addresses: barbara.vona@uni-tuebingen.de (B. Vona), Gaurav-Varshney@omrf.org (G.K. Varshney).

https://doi.org/10.1016/j.heares.2020.107906
0378-5955/© 2020 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
Hearing loss is a highly prevalent disorder, the incidence of which increases with age: At birth one to two of every 1,000 newborns are affected (Morton and Nance, 2006), yet by the eighth decade of life the disorder affects 60% of individuals (Davis, 1989). It has been long understood that hearing loss is associated with social isolation, delayed speech and language development, as well as increased risk of depression. However, the recent recognition that hearing loss is associated with dementia has recently uncovered another key risk factor for reduced quality of life (Livingston et al., 2017), and underscores the importance of understanding the molecular mechanisms of hearing loss and how they can be addressed by therapeutic treatment. In the past decade, an understanding of hidden hearing loss in seemingly normal hearing individuals has surfaced as another factor impacting quality of life. Although environmental contributions such as noise and drug exposures are long-recognized risk factors, a genetic susceptibility to hearing loss is thought to be responsible for over half of diagnoses (Morton and Nance, 2006). Particularly in developed countries, approximately 80% of congenital hearing loss is due to genetic causes and the remaining fraction is attributed to environmental factors (Shearer et al., 2017).

Genetic causes of hearing loss propagate several challenges: clinical heterogeneity manifests in the parameters used to characterize the type of hearing loss, including age-of-onset, severity, and appearance of additional clinical symptoms. Hearing loss apparent at birth is classified as congenital, whereas hearing loss with an onset before language development is called pre-lingual, and hearing loss starting after language acquisition is termed post-lingual. Presbycusis, or age-related hearing loss, is another category and is described as progressive. Hearing loss can be described as sensorineural, conductive, or mixed depending on the part of the auditory system that is affected. Severity of hearing loss is measured in decibels and is determined through a variety of physiologic and audiometric testing methods that are adapted to patient age. Hearing loss is further categorized based on the severity of the frequencies (high-, mid-, and low-frequency) that are affected. When hearing loss occurs as an isolated symptom, it is termed non-syndromic; if it presents as part of a constellation of symptoms, it is called syndromic. Adding to this complexity is the existence of syndromes that have a delayed clinical onset after hearing loss has been diagnosed; when these delays are present, it is initially impossible to make an accurate clinical diagnosis in the absence of molecular genetic data.

### 1. Descent into the genetics of hearing loss

Given the complexity of the auditory system, it can be reasoned that hundreds of genes have essential roles in the auditory pathway. Presently, over 400 genes are associated with either non-syndromic or syndromic hearing loss based on searching the term “deafness” in the Human Gene Mutation Database (HGMD, version 2019.2). Of these, roughly 130 are associated with non-syndromic hearing loss (taken from the https://hereditaryhearingloss.org/, last date accessed, September 13, 2019 and recent literature) (Fig. 1), and considerably more are associated with syndromic hearing loss. In some cases, different alleles of the same gene can underlie an autosomal dominant or recessive form of hearing loss, as well as either a syndromic or non-syndromic outcome. The most recent data from large-scale mouse phenotype screens puts the current estimate of the number of genes involved in normal hearing at roughly 1,000 (Ingham et al., 2019). Taking advantage of standardized nomenclature that has been assigned to gene loci, the Online Mendelian Inheritance in Man (https://www.omim.org/) database assigns gene locus identifiers to novel non-syndromic hearing loss genes as they are discovered depending on inheritance pattern: DFNA followed by a chronological number of discovery order denotes a locus that harbors a gene causing autosomal dominant non-syndromic hearing loss; DFNB is the locus identifier for autosomal recessive hearing loss; DFNX for X-linked; DFNY for Y-linked; and DFNM for genetic modifier loci. Other identifiers in use include AUNA for auditory neuropathy and OTSC for otosclerosis.

Gene identification in the time before the completion of the human genome project relied on large families and employed linkage studies based on microsatellite markers to map the chromosomal locus of hearing loss candidate genes. This type of analysis provided the first information about the chromosomal interval harboring the pathogenic variant, but often contained several dozens, if not hundreds, of genes. The genes in these intervals were either prioritized according to inner ear gene expression or were systematically Sanger sequenced until a putative pathogenic variant was identified. While positional gene mapping using linkage analysis has proven to be very successful, there still remain many linked deafness loci for which causative genes have not been identified. It is also worth considering that complex changes, such as copy number variation and chromosomal translocations, may be

| 9.2.3. Cadherin 23 (CDH23) | 11 |
| --- | --- |
| 9.2.4. Protocadherin related 15 (PCDH15) | 11 |
| 9.2.5. Otoferlin (OTOF) | 11 |
| 9.2.6. Deafness autosomal 5 (DFNAS) | 11 |
| 9.2.7. Granne causing head transcription factor 2 (GRHL2) | 11 |
| 9.2.8. Adenylate cyclase 1 (ADCY1) | 11 |
| 9.2.9. Immunogobulin-like domain containing receptor 1 (ILDR1) | 11 |
| 9.2.10. RHO family interacting cell polarization regulator 2 (RIPOR2) | 11 |
| 9.2.11. Transformmembrane protein 132E (TMEM132E) | 12 |
| 9.2.12. Transformation/transcription domain-associated protein (TRRAP) | 12 |
| 9.2.13. Sphingosine-1-phosphate receptor 2 (S1PR2) | 12 |
| 9.2.14. Cell division-cycle 14A (CDC14A) | 12 |
| 9.2.15. Phosphoribosyl pyrophosphate Synhetetase-1 (PRPS1) | 12 |
| 9.2.16. Methionine sulfoxide reductase B3 (MSRB3) | 12 |
missed by conventional Sanger sequencing approaches.

2. Methodological advancements in sequencing

Considering the extreme genetic heterogeneity in hereditary hearing loss, one gene in particular, GJB2 (which maps to the DFNB1 and DFNA3A loci), is involved in a disproportionate fraction of genetic diagnoses. Pathogenic variants of GJB2 are associated with both autosomal recessive and autosomal dominant forms of hearing loss, although the autosomal recessive form prevails. Systematic reviews of the literature estimate a world-wide GJB2 diagnostic yield of 17.3% (Chan and Chang, 2014).

Fig. 1. Known non-syndromic hearing loss genes arranged according to cytogenetic location. Genes following autosomal dominant (blue), recessive (red), and those following both autosomal dominant and recessive patterns of inheritance (purple) are shown. Less common genes are X-linked (orange), associated with auditory neuropathy (green), modifiers of hearing loss (pink) and autosomal dominant non-syndromic deafness and a modifier of hearing loss (turquoise). Genes that were identified using high-throughput sequencing are marked with an asterisk. Updated and adapted from (Vona et al.) (Vona et al., 2016). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
is most prevalent in Spain and Eastern Europe, where it is reported to be involved in 40–50% of genetic diagnoses (Chan and Chang, 2014). Due to the relative ease of sequencing the single coding and non-coding exons that comprise this gene, direct sequencing of GJB2 is the most logical starting point for molecular genetic testing. Exclusionary testing of GJB2 and ethnic-specific deafness-associated pathogenic variants was identified as a valuable starting strategy (albeit with a limited total diagnostic yield), before working toward positional cloning of other genes in undiagnosed patients. However, the strategy became less promising as it became clear that the sheer number of genes involved was beyond what was feasible to test in a molecular genetic diagnostic setting at that time. The combination of milestone achievements in genomics, including the completion of the Human Genome Project in 2003, and advances in bioinformatics and computational power, now permit hypothesis-free genetic analysis that may range from a subset of genes with a strong gene-disease association to all coding genes in the human genome. This has revolutionized the possibilities and approaches used in molecular genetics research and diagnostic settings.

The past decade has witnessed a transformative shift with the advent and widespread accessibility of high-throughput sequencing approaches. This is reflected by the surge of genes that have been identified using these newer sequencing approaches (Fig. 1, genes marked with asterisks, Fig. 2). The most notable outcomes from the continued development and application of these technologies is the tremendous reduction in data generation cost and subsequent increase in sequencing data output. The largest bottleneck in sequencing data generation in molecular genetic diagnostics and novel gene identification studies is removed. However, it comes at the expense of increasingly complex demands for data interpretation because of the large number of novel variants identified across many genes.

These technologies have been seamlessly integrated into the diagnostic testing setting in the form of diagnostic testing of gene panels for targeted sequencing of over 100 genes or a much boarder approach, as with exome or genome sequencing and targeted gene analysis of known or candidate genes. The advantage of the latter is that analysis of existing exome or genome datasets for newly discovered genes is possible in undiagnosed patients, which opens potential avenues for continued diagnostic analysis. This will be increasingly important, as understanding of the genes involved in hearing loss becomes more complete with rapid gene discovery.

3. The mutational signatures of hereditary hearing loss

The high prevalence of hereditary hearing loss due to a single gene like GJB2 is the exception rather than the rule. In one of the largest molecular genetic epidemiology studies of hearing impaired patients to date, DNA samples from 1,119 patients were sequenced using various versions of hearing loss gene panels (Sloan-Heggen et al., 2016). The results indicated that 39% of patients received a diagnosis that involved genetic changes in 49 genes. The most frequently implicated gene was GJB2 (involved in 22% of diagnoses), followed by STRC (16%), SLC26A4 (7%), and TECTA (5%). Although 49 genes were implicated in the diagnoses of this hearing loss cohort, nearly three-fourths of the diagnoses were attributed to pathogenic and copy number variations in only 10 genes, with the remaining 39 genes making up a very small proportion of diagnoses (Sheffield and Smith, 2019; Sloan-Heggen et al., 2016). However, individually sequencing each of the 10 most frequently involved genes from this study with an alternative widespread technique such as Sanger sequencing is unfeasible in most molecular genetic diagnostic settings.

The classification of genetic variants represents a tremendous hurdle in genetic diagnostics and novel gene discovery. The Deafness Variation Database provides the best glimpse into the molecular landscape of 152 deafness-associated genes (Azaiez et al., 2018). In a recent study that described the interpretation and prioritization of genetic variants in 152 genes implicated in non-syndromic and syndromic deafness, the authors evaluated 876,139 variants and classified >8,100 as pathogenic or likely pathogenic, >172,000 variants as benign or likely benign, and >695,000 variants as having uncertain significance. The authors also re-categorized 1,270 variants (Azaiez et al., 2018). The take-home message of this study clearly highlights two main challenges in the genetics of deafness: (1) the vast majority of variants uncovered, even in well-studied genes, are variants with unknown significance, and (2) the notion of a secure genetic analysis and diagnosis in these genes is in flux, as illustrated by the re-categorization of so many variants.

Although the majority of molecular genetic diagnoses involve single nucleotide exchanges, insertion/deletion (indels), stop mutations (nonsense variants) and duplications at a single nucleotide resolution, genetic changes identifiable at lower resolutions are quite common and are involved in a significant fraction of diagnoses. The burden of copy number variation (deletion or duplication of genetic material comprising regions of 1 kilobase or larger) in the diagnostic yield of patients has proven to be substantial. Copy number variations were implicated in the molecular genetic diagnosis of approximately 18.7% patients from a large-scale study that included 686 hearing loss patients (Shearer et al., 2014). Additionally, chromosomal translocations, although apparently rare, may also contribute to the molecular spectrum of hereditary hearing loss through the interruption of critical gene sequences.

As many laboratories shift to genome sequencing technologies that provide information about non-protein coding sequences as possible contributors to inherited disease in humans, interpretation of genetic variants is likely to encounter a wave of complexity unlike we have seen before. Our current knowledge, largely restricted to coding sequences and adjacent non-coding sequences that coordinate splicing, has already uncovered hundreds of thousands of variants with uncertain significance, conflicting interpretations of pathogenicity, and many falsely classified variants and false gene-disease associations. This is a prime example of the fallout caused by post-genomics "growing pains" and is a major deterrent to the precision medicine movement, for which achieving a secure genetic diagnosis is crucial.
Although our understanding of the genes and variants involved in hearing loss has significantly advanced in the past 20 years (Vona et al., 2015), there is an urgent need to develop and utilize robust experimental systems to understand the function of newly identified genes. The last couple of decades have seen tremendous progress in identifying hearing loss genes thanks to inexpensive sequencing technologies that facilitated an increasing number of hereditary hearing loss screening projects. However, validation of genetic loci or genes that underlie the disease and determination of the pathological mechanisms of disease genes has been unacceptably slow, and has delayed progress toward the development of targeted therapies for hearing loss.

A proven approach to address these issues is to make use of animal models: mutated genes (designed to recapitulate mutations identified in human patients) are introduced into the model and the resulting phenotypes are examined. For decades, the mouse has been the most popular model organism to validate human gene function, and it has provided an invaluable tool for studying the mechanisms of hearing that would not be possible in human studies alone. However, the large-scale identification and validation of candidate genes in mice is limited by many factors including the small number of progeny, the in utero lethality of many knockouts, the labor involved in targeting the genes, and the relatively expensive husbandry.

4. Zebrafish as an animal model

The zebrafish (Danio rerio) is a small tropical fish that belongs to the teleost clade, originates in Southeast Asia and was first used to study developmental biology by George Streisinger in the early 1980s (Chakraborty et al., 2017). Zebrafish is a vertebrate model organism with several advantages over mice: zebrafish have a large number of progeny, undergo external fertilization and relatively fast embryonic development, produce transparent larvae, and are particularly suitable for high-throughput mutagenesis and drug screening approaches (Varshney et al., 2015a). As a result of the number of genetic tools and techniques available to manipulate the zebrafish genome, several large-scale mutagenesis projects have generated the second biggest mutant collection in a vertebrate model (Kettleborough et al., 2013; Varshney et al., 2013b). Three different approaches are being used to study gene function in zebrafish.

5. Random mutagenesis approaches

In the early 1990s, chemical mutagenesis using N-ethyl-N-nitrosourea (ENU) as a mutagen was used to perform large-scale random mutagenesis screens (Driever et al., 1996; Haffer et al., 1996; Solnica-Krezel et al., 1994), and later retroviruses were used for random insertional mutagenesis (Amsterdam et al., 1997; Galiano et al., 1996; Golling et al., 2002). Both screens identified thousands of mutations affecting embryonic development, including genes involved in ear development. Mutations affecting ear development were identified based on ear morphology (e.g. otolith development and the shape of the ear) and simple behavioral assays (e.g. aberrant swimming pattern) (Driever et al., 1996; Granato et al., 1996; Malicki et al., 1996; Nicolson et al., 1998; Whitfield et al., 1996). Malicki et al. characterized 20 mutants affecting ear development; identifying a variety of phenotypes such as absence of otolith, abnormal ear morphology, and abnormal otic placode development (Malicki et al., 1996). Similarly, another screen identified 58 mutants with a variety of phenotypes including abnormal development of sensory patches, otoliths, and semicircular canals (Whitfield et al., 1996). Many of these mutants with ear and otolith defects also displayed behavioral phenotypes and failed to balance properly. Another class of mutants in this screen displayed balance defects without any defects in the ear. A group of mutants show ear phenotypes as secondary phenotypes i.e. associated with a different phenotype. In another independent screen, Teresa Nicolson identified seven mutants based on abnormal swimming behavior of the larvae; these were called cycler mutants (sputnik, mariner, orbiter, mercury, gemini, skylab, astronaut, and cosmonaut). All of them had abnormal vestibular function, and none of these mutants showed any obvious morphological defects (Granato et al., 1996; Nicolson et al., 1998) (Driever et al., 1996; Haffer et al., 1996; Solnica-Krezel et al., 1994).

Over the years, many labs have identified a number of mutants affecting ear development; however, only a small number of these mutants are linked to non-syndromic hearing loss (Bang et al., 2002; Hardison et al., 2005; Nissen et al., 2003; Schibler and Malicki, 2007). Primarily, the cycler class of mutants has an association with non-syndromic hearing loss genes (Solnica et al., 2004). The Sanger Institute in the UK carried out a large scale ENU mutagenesis study to identify mutations in all protein-coding genes (Kettleborough et al., 2013), while Shawn Burgess’ and Shuo Lin’s labs used retroviruses to generate genome-wide insertional mutations (Varshney et al., 2013b). Both projects generated a large number of mutants across thousands of genes; thousands of mutants from both ENU and retroviral mutagenesis projects are currently available to the community (Kettleborough et al., 2013; Varshney et al., 2013a).

An alternative approach to random insertional mutagenesis in zebrafish involves transposable elements such as Tol2, or Ac/Ds from heterologous hosts (Varshney et al., 2015a). These transposable elements are easy to use and can carry complex DNA transgenes. The Tol2 transposon from medaka is the most popular genetic tool for creating transgenic zebrafish, and many transgenic lines expressing fluorescent proteins in the inner ear or lateral line are available to the community. A list of hearing related transgenic lines can be found in Baxendale et al. (Baxendale and Whitfield, 2016). Tol2-based gene or protein traps, used to generate conditional mutants with inactivated gene function in a tissue (spatial) or time point (temporal) specific manner are also available (Varshney et al., 2015a).

6. Targeted mutagenesis approaches

While there are a number of mutants available from both ENU and retroviral screens, mutants for all hearing loss genes are not available. Targeted mutagenesis approaches provide an opportunity to target the genes of interest at will. Moreover, mutants generated by random mutagenesis might carry additional background mutations that could complicate the phenotypic analysis (San et al., 2019). During the time when random mutagenesis-based forward genetic screens identified a number of mutants related to ear development and function, no tools existed for targeted mutagenesis in reverse genetic screens. However, the past ten years have seen an enormous development of reverse genetic tools including Zinc Finger Nucleases (ZFNs), TALENs, and CRISPR/Cas9. ZFNs and TALENs are engineered proteins, and CRISPR/Cas9 is an RNA-guided endonuclease, but all function in a similar manner by binding to the target DNA sequence and inducing a double-stranded (DSB) break. The DSB can either be repaired by the error-prone non-homologous end joining repair pathway (NHEJ) or, when a donor template is provided, by the homology-directed repair (HDR) pathway (Varshney et al., 2015a). NHEJ repair is being exploited to generate loss-of-function alleles. The CRISPR/Cas9 technique has revolutionized reverse genetics approaches because it is inexpensive, simple, easy to use, and allows multiple genes to be targeted at the same time. Multiplexing is particularly useful in
zebrafish because one-third of the genome is duplicated, and targeting two paralogs is now possible (Jao et al., 2013). CRISPR/Cas9 has two components—a guide RNA (sgRNA) that binds to the target site, and the Cas9 enzyme that induces the DSB. Any DNA sequence can be targeted using this technique, the only requirement being that a 20-nucleotide target sequence must be followed by a specific protospacer sequence motif (PAM). Different Cas9 enzymes have different PAM requirements, and the most popular Cas9 from Streptococcus pyogenes (SpCas9) requires an NNG PAM (i.e., the 20-nucleotide target sequence must be followed by NNG (e.g., AGG/C, CGG, TGG or GGG)). Cas9 from Streptococcus aureus (SaCas9) with an NNGRRT (R = A or G) PAM and Cas12a, another class of enzyme, with a TTTV PAM (V = A or C or G) have also shown to work efficiently in zebrafish (Feng et al., 2016; Moreno-Mateos et al., 2017). There are many other orthologous enzymes and engineered versions of SpCas9 that can be used as an alternate (Liu et al., 2019). Using CRISPR/Cas9 technology, mutations in more than 50 zebrafish orthologs of genes involved in human hearing loss have been generated. It is projected that this resource will accelerate the functional validation of hearing loss genes in zebrafish (Varshney et al., 2015b).

6.1. CRISPR-based approaches to study genetic variants

Since most of the mutations in hearing loss genes are point mutations, investigating their role in disease pathology has been challenging. With the advancement of CRISPR/Cas9 technology, it is possible to mimic these mutations in zebrafish if the sequences are well conserved in a targeted manner. CRISPR/Cas9 mediated knock-in approaches (via HDR with a donor template including the desired mutation) are being used successfully (Irion et al., 2014; Prykhozhij et al., 2018). In zebrafish, the HDR-based targeted knock-in approaches are more challenging than generating knockout alleles. The very first use of integrating an exogenous DNA in zebrafish was done by a TALEN-mediated knock-in approach using 900 bp long homologous arms. However, the germline transmission frequency was less than 2%, limiting its use on a large-scale (Zu et al., 2013). Chang et al. used CRISPR/Cas9 for the first time to integrate LoxP sequences using single-stranded oligonucleotide (ssODN) donor templates for HDR (Chang et al., 2013). Using the same strategy of ssODN donor template, single nucleotide changes were introduced in zebrafish genes fus (R536H) and tardo (A379T), mimicking atomyotropic lateral sclerosis (ALS)-causing missense mutations in FUS (R521H) and TARDBP (A328T) genes (Armstrong et al., 2016). Two different template sizes were tested, at 23 bp and 100 bp in length, identifying 1/46 (~2%) and 3/77 (~4%) F0 founders transmitting the mutations in the germline (Armstrong et al., 2016). In another strategy using donor oligonucleotides, an HA tag at the C9t3 locus was introduced with 1.7% germline transmission efficiency (Hruscha et al., 2013). In a different strategy, plasmids were used as a donor to correct a premature stop codon in the albino locus (slc45a2) that prematurely truncates the protein and the germline transmission efficiencies were comparable to an earlier study by Hruscha et al. (Hruscha et al., 2013; Irion et al., 2014). While many knock-in alleles have been reported mimicking disease-causing nonsense mutations in zebrafish, there is only one example related to hearing loss. A knock-in allele was generated where 670 bp of ush2a intron 40 were replaced with 557 bp of the corresponding human USH2A sequence. In patients, the c.7595–2144A>G mutation in USH2A intron 40 introduces a splice donor site resulting in the incorporation of a pseudogene predicted to terminate USH2A translation prematurely (Slijkerman et al., 2018). Over the years, several strategies are being used for knock-in of exogenous DNA or mimicking point mutations in zebrafish with low germline efficiencies. More recently, newer approaches are being developed that are more efficient and promising, but come with their own challenges (Wierson et al., 2018). Nonetheless, these new approaches need to be tested on a large-scale to effectively address the flood of variants being identified. As of today, the most common approach to study variants in zebrafish has been injecting mRNA with a specific variant into one-cell stage embryos in a null background. A wild-type mRNA should rescue the phenotype, while mRNA with a variant may not rescue the phenotype if the variant is critical to the protein function. If the phenotypes appear at later stages of animal development, then phenotype rescue or variant testing is done using a transgene. This approach is time consuming, labor-intensive, and not amenable to high-throughput testing of variants. In 2016–17, David Liu’s group at the Broad Institute repurposed the CRISPR/Cas9 based system to change the single bases at a target site (Gaudelli et al., 2017; Komor et al., 2016). Catalytically inactive Cas9 is fused with enzymes such as cytidine deaminase or adenosine base editor (a combination of wild-type TadA (tRNA specific adenosine deaminase) and evolved TadA enzyme) that can convert the C/G base pair to T/A or A/T to G/C, respectively. The conversion of the nucleotide occurs without creating a DSB and thus does not generate indels (or fewer indels) via DSB repair mechanisms. This base editing approach can be used to investigate non-pathogenic vs. non-pathogenic single nucleotide mutations, wherever a targeting vector is available, which is usually ~5 bp and PAM dependent. The PAM requirement can be relaxed by using a different enzyme recognizing a different PAM.

7. Morpholino-mediated knockdown of mRNA

Since early 2000, the use of chemically synthesized morpholinos (MOs) has been a popular method among zebrafish researchers for the transient knockdown of target mRNAs to study loss-of-function phenotypes (Nasevicius and Ekker, 2000). MOs work either by binding to the translation start site to inhibit translation (ATG blocker) or by interfering with the splicing mechanism of mRNA (splice blocker). One of the reasons that MOs became the most popular antisense knockdown tool in zebrafish is their ease of delivery in early stage embryos. MOs can knockdown the expression of both maternal and zygotic transcripts to create complete loss-of-function phenotypes. Soon after, MOs became a popular tool for targeted reverse genetic studies in zebrafish. These studies made it possible to quickly and inexpensively study genes from various exome- and genome-based studies, as well as genes and variants that were identified as interesting targets by genome-wide association studies. Using MO-mediated knockdown, ~25 hearing-related genes have been studied (Table 1) and their roles in hearing and balance validated. MOs were also used to phenocopy mutants with otic defects identified in a random mutagenesis screen (Whitfield, 2002). The first hearing loss gene analyzed by MO was the DFNAS locus. In humans, DFNAS-associated hearing loss is caused by pathogenic variants in the gene GSDME that result in a non-syndromic, autosomal dominant form of progressive hearing loss. The MO-mediated knockdown of dfna5a in zebrafish showed disorganization of the developing semicircular canals (Busch-Nentwich et al., 2004).

While MOs are an effective knockdown tool, their utility can be limited by off-target toxicity and the fact that their knockdown effect is transient (typically 2–4 days post-fertilization (dpf)). The off-target toxicity can be excluded by injecting wild-type mRNA from the target gene together with MO, specific phenotypes should be rescued by wild-type mRNAs either partially or fully. As targeted mutagenesis methods like CRISPR/Cas9 and TALENs became available, a few studies demonstrated that genetic mutants did not recapitulate the MO-induced phenotype (Kok et al., 2014; Law and Sargent, 2014). For example, phenotypes induced by targeting the
| Human deafness locus | Human gene | Zebrafish gene(s) | Zebrafish model | Type of mutant | References |
|----------------------|------------|------------------|----------------|---------------|------------|
| DFNA20/26            | ACTG1      | -                | -              | -             | -          |
| DFNB44               | ADCY1      | adcy1a, adcy1b   | Yes            | Morpholino    | Santos-Cortez et al. (2014) |
| DFNX5                | AHRM1      | afmr1            | -              | -             | Zong et al. (2015) |
|                      | ATP2B2     | atp2b            | -              | -             | Smits et al. (2019) |
| DFNB93               | C4BP2      | c4bp2a, c4bp2b   | -              | -             | Schrausen et al. (2012) |
| DFNA44               | CDC50      | cdc50            | -              | -             | Modamio-Hoybjørn et al. (2007) |
| DFNB32/105           | CDC14A     | cdc14a, cdc14ab  | Yes            | Morpholino    | (Delmaghani et al., 2016; Intizad et al., 2018) |
| DFNB66               | CDH16      | cdh16            | -              | -             | Nyeegaard et al. (2015) |
| DFNB12               | CDH23      | cadherin-like 23 | Yes            | ENU           | (Bork et al., 2001; Nicolson et al., 1998; Söllner et al., 2004) |
| DFNB48, DFNB113      | CEACAM16   | sckey-11a1.7     | -              | -             | (Booth et al., 2018; Zheng et al., 2011) |
| DFNB29               | CIR2       | cib2             | Yes            | Morpholino    | Riazuddin et al. (2012) |
|                      | CLDN9      | -                | -              | -             | Sineni et al. (2019) |
| DFNB103              | CLCS6      | clcs6a, clcs6b   | -              | -             | Wilcox et al. (2001) |
| DFNA9                | COCH       | coch             | -              | -             | Seco et al. (2015) |
| DFNB1                  | COL11A1    | col11a1a, col11a1b | Yes           | Morpholino    | Riazuddin et al. (2006a) |
|                      | COL4A6     | col4a6           | -              | -             | (Chen et al., 2005; McGuir et al., 1999) |
| DFNA7                | GIPC3      | gipc3            | Yes            | CRISPR        | (Wayne et al., 2001; Schonberger et al., 2005) |
| DFNB15/72/95         | GIPC3      | gipc3            | Yes            | Morpholino    | Diaz-Horta et al. (2014) |
|                      | GAB1       | gab1             | Yes            | CRISPR        | Badereni et al. (2019) |
| DFNB91A, DFNA3A      | GJB2       | cc30.3           | Yes            | Morpholino    | (Alvarez et al., 2005; Barashkov et al., 2011; Chang-Chien et al., 2014; Kelsell et al., 1997) |
|                      | GJB3       | cc35.4           | Yes            | Morpholino    | Xia et al. (1998) |
|                      | GJB5       | cc30.3           | Yes            | Morpholino    | (Chang-Chien et al., 2014; del Castillo et al., 2002; Grif et al., 1999) |
| DFNA2B               | GPR52M     | gpr52m           | -              | -             | Yousaf et al. (2018a) |
| DFNA14               | GRAP       | grapa, grapb     | -              | -             | Li et al. (2019) |
| DFNA28               | GRHL2      | grhl2a, grhl2b   | Yes            | Tol2          | Yona et al. (2013) |
| DFNB25               | GXRCR1     | gxrcr1           | Yes            | CRISPR        | Han et al. (2011) |
| DFNB101              | GXRCR2     |                 | -              | -             | Intizad et al. (2014) |
| DFNB39               | HGF        | hgf, hgbf        | -              | -             | Schultz et al. (2009) |
| DFNA68               | HOMER2     | homer2           | -              | -             | Ataiz et al. (2015) |
| DFNA2C               | IFNR1      | cfrb2            | Yes            | Morpholino    | Gao et al. (2018) |
|                      | INSR1      | idra1, idra1b    | Yes            | Morpholino    | (Brock et al., 2011; Sang et al., 2014) |
| DFNB89               | KARS       | -                | -              | -             | Santos-Cortez et al. (2013) |
| DFNA2A               | KCNQ4      | kcnq4            | -              | -             | Kubisch et al. (1999) |
| DFNA69               | KITLG      | kitiga, kitgib   | -              | -             | Zago Seco et al. (2015) |
| DFNB66/67            | LHEP5      | lhp5a, lhp5b     | Yes            | ENU           | (Kalay et al., 2006; Obholzer et al., 2012; Shabbir et al., 2006; Tlili et al., 2005) |
| DFNA7                | LMX1A      | lmx1a            | -              | -             | (Bademci et al., 2018; Wesdorp et al., 2018b) |
| DFNB77               | LOXH1D1    | loxh1d1a, loxh1d1b | Yes           | Tol2          | Xia et al. (2017) |
|                      | LRPS5      |                  | -              | -             | (Ahmed et al., 2008b; Du et al., 2008; Erickson et al., 2017) |
| DFNB9                | MARVELD2   | marved2a, marved2b | -              | -             | Riazuddin et al. (2006a) |
| DFNA70               | MCM2       | Mcm2             | -              | -             | Gao et al. (2015) |
| DFNB97               | MET        | met              | -              | -             | Mujtaba et al. (2015) |
| DFNM1                | METTL13    | mettl13          | -              | -             | Yousaf et al. (2018b) |
| DFNA50               | MIR96      | mir96            | -              | -             | Mencia et al. (2009) |
| DFNB111              | MIRL2      | mirl2            | -              | -             | (Bademci et al., 2018; Wesdorp et al., 2018b) |
| DFNB74               | MSRB3      | msrb3            | Yes            | Morpholino    | (Ahmed et al., 2011; Shen et al., 2015) |
| DFNA4A               | MYH14      | myh14            | -              | -             | Danoody et al. (2004) |
| DFNA17               | MYH9       | myh9a, myh9b     | -              | -             | Lalwani et al. (2000) |
| DFNB3                | MYO15A     |                  | -              | -             | Wang et al. (1998) |

(continued on next page)
mRNA of the non-syndromic hearing loss gene, CDC14A, ortholog by MO-mediated knockdown were not replicated in a genetic mutant (Delmaghani et al., 2016) (Imtiaz et al., 2018). These findings led to the introduction of more stringent guidelines for MO-mediated knockdown experiments; the community now recommends using multiple MOs, or MOs in combination with other approaches such as genetic mutants or CRISPR interference-mediated knockdown (CRISPRi) (Stainier et al., 2017). Since the MO-

| Human deafness locus | Human gene | Zebrafish gene(s) | Zebrafish model | Type of mutant | References |
|----------------------|------------|-------------------|----------------|---------------|------------|
| DFN30, DFNA1 | MYO3A | myosin XVIIA | – | ENU | (Grati et al., 2016; Walsh et al., 2002) |
| DFN37, DFNA22 | MYOD1 | myosin VIIIb, myosin VIIIa | – | ENU | (Ahmed et al., 2003c; Melchionda et al., 2001; Seiler et al., 2004) |
| DFN22, DFNA11 | MYOT | myosin VIIIb, myosin VIIa | – | ENU | (Ernest et al., 2000; Liu et al., 1997a; Wasyf et al., 2014; Weil et al., 1997) |
| DFN94 | NARS2 | nars2 | – | Simon et al. (2015) |
| DFN40 | NLRP3 | – | – | Nakanishi et al. (2017) |
| DFN47 | OSBPL2 | osbpl2a, osbpl2b | – | ENU | (Thoenes et al., 2015; Wang et al., 2019; Xing et al., 2015) |
| DFN22 | OTOA | – | – | – | (Zwaenepoel et al., 2002) |
| DFN9 | OTOF | otolfin-a, otolfin-b, | – | Morpholino | (Chatterjee et al., 2015; Yasunaga et al., 1999) |
| DFN118b | OTOG | otogelin | – | ENU | (Schraders et al., 2012; Stooke-Vaughan et al., 2015) |
| DFN84 | OTCl | ototelin-like | – | Morpholino | Yariz et al. (2012) |
| DFN41 | P2RX2 | p2rx2 | – | – | Yan et al. (2013) |
| DFN23 | PCDH15 | pcdh15a, pcdh15b | – | Tol2 | (Ahmed et al., 2003a; Seiler et al., 2005) |
| DFN47 | PDE1C | pde1ca, pde1cb | – | – | Wang et al. (2018) |
| DFN57 | PDEZD7 | pdez7a, pdez7b | – | Morpholino | Ebermann et al. (2010); Schneider et al., 2009) |
| DFN59 | PFKX | dfnk59 | – | – | Delmaghani et al. (2006) |
| – | PLS1 | pls1 | – | – | Schrauwien et al. (2019) |
| DFN70 | PMRT1 | – | – | – | von Ameln et al. (2012) |
| DFNX2 (DFN3) | POU3F4 | – | – | – | de Kok et al. (1995) |
| DFN15 | POU4F3 | pou4f3 | – | – | Vahava et al. (1998) |
| DFN100 | PPP5K2 | ppp5k2 | – | – | Yousaf et al. (2018a) |
| DFN2 (DFN2) | PRPS1 | prps1a, prps1b | – | Morpholino | (DeSmidt et al., 2019; Liu et al., 2010; Pei et al., 2016) |
| DFN84, DFNA73 | PTPRQ | ptpaq | – | – | (Eisenberger et al., 2018; Schraders et al., 2010a) |
| DFN24 | RDH5 | moses a, moses b | – | – | Khan et al. (2007) |
| DFN27 | REST | rest | – | – | Nakano et al. (2018) |
| DFN100 | ROR1 | ror1 | – | – | Diaz-Horta et al. (2016) |
| DFN68 | STIP2 | stip2 | – | – | Santos-Cortez et al. (2016) |
| DFN49 | SERTPNB6 | – | – | – | Sirmaci et al. (2010) |
| DFN23 | SIX1 | six1a, six1b | – | – | Moscati et al. (2011) |
| DFN25 | SLC17A8 | slc17a8/qlia3 | – | Morpholino | (Obholzer et al., 2008; Ruel et al., 2008) |
| DFN60 | SLC22A6 | slc22a6 | – | – | Ben Said et al. (2016) |
| DFN84 | SLC26A4 | slc26a4 | – | – | Li et al. (1998) |
| DFN61 | SLC26A6 | slc26a6 | – | – | Liu et al. (2003) |
| DFN64 | SMAC/DIABLO | diabola | – | – | Cheng et al. (2011) |
| DFN4 (DFN6) | SNMX | snmx | – | – | (Huebner et al., 2011; Schraders et al., 2011) |
| – | SPATC1 | – | – | – | Morgan et al. (2019) |
| DFN115 | SPPN2 | spnn2 | – | – | Ingham et al. (2019) |
| DFN16 | STRC | stra | – | – | Verpy et al. (2001) |
| DFN76 | SYNE4 | – | – | – | Horn et al. (2013) |
| DFN86, DFNA65 | TBC1D4Z | tbc1d4z | – | – | (Azaiez et al., 2014; Rehman et al., 2014) |
| DFN51 | TIP2 | tip2a, tip2b | – | – | (Mustapha et al., 1999; Stooke-Vaughan et al., 2013; Verhoeven et al., 1998) |
| DFN87/11, DFNA36 | TMCI | tmc1 | – | – | Walsh et al. (2010) |
| DFN89 | TMEM132E | tmem132e | – | Morpholino | Kurima et al. (2002) |
| DFN6 | TMIE | tmie | – | – | Li et al. (2015) |
| DNB8/10 | TMRSS5 | tmrss3a, tmrss3b | – | ENU | (Gleason et al., 2009; Naz et al., 2002) |
| DFN56 | TNC | tenucin C | – | – | Scott et al. (2001) |
| – | TOP2B | top2b | – | – | Runge et al. (2016) |
| DFN79 | TPRI | tpri | – | – | Zhao et al. (2013) |
| DFN28 | TRIBDP | tribdp | – | – | Xia et al. (2019b) |
| – | TRRAP | trrap | – | CRISPR Morpholino | Xia et al. (2019a) |
| DFN98 | TSPPEAR | tspeara, tspearb | – | – | Delmaghani et al. (2012) |
| DFN18 | USH1C | ush1c | – | ENU | (Ahmed et al., 2002; Ouyang et al., 2002; Phillips et al., 2011) |
| DFN107 | WBP2 | wbp2 | – | – | Buniello et al. (2016) |
| DFN6/14/38 | WFS1 | wfs1a, wfs1b | – | ENU | (Bespalova et al., 2001; Young et al., 2001) |
| DFN31 | WHRN | dfnb31a, dfnb31b | – | – | Mburu et al. (2003) |
based phenotypes are dose-dependent, it is recommended to have a dose response curve, and to perform additional experiments when a dose higher than 5 ng is required to observe a phenotype. In conclusion, MOs are not recommended as a reverse genetic tool. In cases where a genetic mutant is not available, appropriate controls must be used to rule out phenotypes caused by off-target effects (Stainier et al., 2017). Alternatively, CRISPR/Cas9 is highly efficient in inducing biallelic mutations, and phenotypes can be observed in the F0 generation (akin to morphants) (Varshney et al., 2015a). Recently, a study adopted this method to screen for phenotypes in the F0 generation by utilizing multiple sgRNAs together with Cas9 (Wu et al., 2018). Furthermore, a recent publication showed that the mutagenesis efficiencies can be increased by using a dual gRNA approach where CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA) are synthesized separately and injected together with Cas9 protein (Hoshijima et al., 2019).

8. Morpholinos vs genetic mutants

More than 50% of the zebrafish models used in hearing research have been generated using MO-mediated knockdown that only permit analysis of effects during the first 4–5 days of embryonic development and makes the study of genes involved in delayed-onset hearing loss challenging. Some studies have suggested that MO-mediated knockdown phenotypes may not be recapitulated by genetic mutants (Kok et al., 2014). The suggestion has fueled a debate about whether MO-mediated phenotypes are generated by off-target effects and strengthened the argument in favor of genetic mutants as superior. However, another study showed that the absence of phenotypes in genetic mutants could be caused by genetic compensation (Rossi et al., 2015). Since most of the mutations generated by genome editing technology are small indels that introduce mediated decay of mRNA, the decayed mRNA enters the nucleus and upregulates genes similar to ones targeted in mutants (Rossi et al., 2015). The activity of the upregulated gene compensates for the loss of the target gene and eliminates the phenotype that would otherwise be observed with loss of the target. The mechanisms of genetic compensation in mutants have been described in two recent, independent publications (El-Brolosy et al., 2019; Ma et al., 2019). These studies have identified that smaller indels cause nonsense-mediated decay of the mutant mRNA, which in turn triggers the recruitment of the COMPASS complex to the regulatory regions of closely related genes. This results in epigenetic changes that activate the expression of closely related genes. It has been suggested that genetic compensation can be avoided if RNA-less alleles (in which the promoter and first exon have been deleted), which do not create a premature termination codon are employed (El-Brolosy et al., 2019). An alternative approach could be generating missense mutations in the functional domains of the target protein that could make the protein non-functional but will not induce nonsense-mediated decay of mRNA; however, this approach has not yet been tested. It has also been shown that CRISPR/Cas9-mediated indels might induce altered mRNA processing such as exon skipping that could escape nonsense-mediated decay (Anderson et al., 2017). However, a large-scale mutant analysis showed only 4/36 alleles had altered mRNA processing, and only two of them generated in-frame mutations at low-levels, suggesting that this process may not be very common in CRISPR generated alleles but cDNA sequencing must be done as a quality control step for functional genomics studies (Samanagouda-Bhojappa et al., 2018). The gold standard approach to verify null alleles is to perform Western blot or immunohistochemistry experiments in cases where antibodies are available.

9. Zebrafish as a model to study hearing and balance disorders

Numerous studies have shown that zebrafish and mammalian genes are highly conserved, and ~82% of human disease genes listed in the OMIM database have an ortholog in zebrafish (Howe et al., 2013). Thus far, 129 loci, and 130 genes have been identified in humans that are associated with non-syndromic hearing loss as per the hereditary hearing loss database (http://hereditaryhearingloss.org). Out of 130 human hearing loss genes, 103 have a matching ortholog in zebrafish, suggesting that hearing loss genes are highly conserved in zebrafish and humans. Since ~20% of the zebrafish genome is duplicated, there are many genes with a paralog. Of 103 non-syndromic hearing loss genes, 36 have known/annotated paralogs in zebrafish. Classical forward genetic screens performed in the early 1990s for identifying hearing related mutants did not identify many genes linked to non-syndromic hearing loss genes.

Zebrafish have two related sensory organs that utilize mechanosensory hair cell receptors: the inner ear, similar to mammalian ears, is used to detect sound, gravity, and motion; and the lateral line, a fish- and amphibian-specific organ used to detect water flow over the surface of the body and the head (Elefant, 1988; Nicolson, 2005) (Fig. 3A–D). The lateral line consists of neuromasts. Each neuromast is composed of support cells surrounding hair cells. The lateral line hair cells are similar to the hair cells found in the inner ear. Their development and differentiation follow the similar developmental regime (Ghysen and Dambly-Chaudiere, 2004; Nicolson, 2005) (Fig. 3B). Many non-syndromic deafness-related genes are expressed in lateral line hair cells (Erickson and Nicolson, 2015; Matern et al., 2018), as well as the inner ear of zebrafish (Yao et al., 2019; Barta et al., 2018).

In addition, zebrafish offer unique advantages for studying defects in the inner ear. Their external fertilization and optically transparent larvae enable direct observation of the sensory epithelium of the inner ear, while observation or manipulation of sensory hair cells in mice requires the dissection of the inner ear, which is technically challenging. Unlike mice, the embryonic inner ear is accessible for imaging. As the development of zebrafish hearing and vestibular system has been covered in greater detail in many excellent reviews and research articles, we aim to summarize the important steps of inner ear and lateral line development here (Nicolson, 2005; Whitfield, 2002; Whitfield et al., 2002).

In zebrafish, the otic vesicle starts to form within 16 h post fertilization (hpf) in the form of otic placodes (which result from thickening of the ectoderm), and by 24 hpf, the anterior and posterior sensory maculae start to form at ends of the otic vesicle (Nicolson, 2005; Whitfield et al., 2002). The inner ear has multiple sensory patches required for different functions. The vestibular system is required to sense gravity and head movement, and is composed of the semicircular canal, and three macular endorgans, such as saccule, utricle, and lagena in zebrafish. These endorgans are composed of neuromast epithelium attached to specialized structures made up of mostly aggregates of calcium carbonate crystals called otoliths (Fig. 3A) (Lundberg et al., 2015). During the first seven days of embryonic development, only the saccule and utricle develop; the lagena develops at a later stage (~11 days post fertilization (dpf)) (Lu and DeSimidt, 2013). The saccule is the main hearing organ, while the utricle is important for balance, motor coordination and gravity sensing. The function of the lagena is still not clear, though some reports suggest that the lagena might also play a role in hearing function (Lu and DeSimidt, 2013; Yao et al., 2016). The hair cells begin to function by 4 dpf, and by 5 dpf, larvae can respond to vibrational reflexes and vestibular reflexes (Raible and Kruse, 2000; Metcalfe et al., 1985).
9.1. Genetic tools to study hearing and vestibular disorders in zebrafish

To investigate the roles of candidate disease genes in hearing loss, a growing number of tools including transgenic lines, antibodies, and live dyes that can label hair cells, the lateral line system, and other inner ear structures in zebrafish are available (Baxendale and Whitfield, 2016; Kindt and Sheets, 2018). A number of transgenic lines such as Tg(pou4f3:GAP-GFP) (Xiao et al., 2005) and Tg(myo6b:GFP) label hair cells (Obholzer et al., 2008), Tg(pvalb3-b:ribeyea-mCherry) labels hair cell synapses (Kindt and Sheets, 2018; Obholzer et al., 2008), Tg(atoh1a:tdTomato) labels immature hair cells (Wibowo et al., 2011) or transgenic lines that can detect neuronal activity using a genetically encoded calcium indicator (GECI), such as GCaMP6 (Lukasz and Kindt, 2018). There are many live dyes such as DASPEI (2-(4-dimethylaminostyryl)-N-ethyl-pyridinium iodide) that labels hair cells, and DiASP (4-(4-diethylaminostyryl)-N-methylpyridinium iodide) that labels hair cells, as well as afferent fibers. FM1-43, FM4-64 labels hair cells, and Yo-Pro1 can label hair cell nuclei which is useful for counting hair cells (Fig. 3 G & H). FM1-43/Yo-Pro1 labeling is being used to investigate the mechanotransduction defects in inner ear and lateral line hair cells in many zebrafish mutants. Other live dyes can also be used but FM1-43 and Yo-Pro1 are easy to use and have strong fluorescence signals. Other stains such as Phalloidin, a phalloxin from the poisonous mushroom, Amanita phalloides, binds to filamentous actin and, thus, can provide a snapshot of the size, shape or splaying of the hair bundles. Phalloidin is available coupled with different fluorophores such as FITC or TRITC and other dyes. A number of antibodies are available such as Myosin VI or Otoferlin that are used for hair cell staining, as well as Ribeye a & b for staining ribbon synapses. A list of antibodies to study hair cell development and function are described in Kindt et al. (Kindt and Sheets, 2018).

9.2. Zebrafish disease models for hearing loss

As of December 2019, only 34 models for non-syndromic hearing loss-related genes in zebrafish have been published. Twenty-five of them have been generated using MO-mediated mRNA knockdown, followed by 8 models generated by ENU mutagenesis, 3 models by Tol2 insertional mutagenesis, and only 4 models by targeted mutagenesis approaches such as CRISPR/Cas9 and TALEN. Mutants for non-syndromic hearing loss can be grouped in many categories, such as those with mutated genes affecting hair cell function, synapse function, morphogenesis of otic vesicles, and epithelial integrity. Here, we discuss selected genes from each category. Many of these genes have been discussed in reviews by Blanco-Sánchez et al. and Nicolson T. in detail (Blanco-Sanchez et al., 2017; Nicolson, 2017).

9.2.1. Myosin 7AA (MYO7AA)

Myo7aa was one of the earliest genes linked to hearing disease pathology. In humans, MYO7A has been involved in both syndromic (Usher syndrome) and non-syndromic (DFNB2, DFNA11) deafness (Bharadwaj et al., 2000; Hildebrand et al., 2010; Liu et al., 1997b; Riazuddin et al., 2008; Wei et al., 1997). In the zebrafish mutant called mariner, a circler mutant was identified with mutations in its myo7aa gene (Ernest et al., 2000). myo7aa is expressed in hair cells of the inner ear and the lateral line in zebrafish. Zebrafish mariner mutants showed abnormal stereocilia of the hair cells in the inner ear, and unbalanced swimming behavior. These phenotypes were also observed in CRISPR/Cas9-generated alleles of myo7aa (Zou et al., 2019).
9.2.2. Myosin 6 (MYO6)

Another gene from the myosin family, MYO6, is involved in both non-syndromic autosomal recessive (DFNB37) and autosomal dominant (DFNA22) deafness (Ahmed et al., 2003b; Melchionda et al., 2001). In zebrafish, myo6a and myo6b are both paralogs, but only myo6b is expressed in sensory hair cells of the inner ear and lateral line. One of the mutants (satellite) from earlier forward genetic screens with vestibular defects was later mapped to the myo6b gene (Seiler et al., 2004). myo6b mutants have abnormal stereocilia growth and showed spaying of the hair bundles and vestibular defects, and were unresponsive to startle stimuli (Seiler et al., 2004).

9.2.3. Cadherin 23 (CDH23)

Mutations in CDH23 cause the DFNB12 form of hearing loss and are also involved in Usher syndrome type 1D (Bork et al., 2001; Hilgert et al., 2009). In zebrafish, the circler mutant sputnik was linked to mutations in the cdh23 gene (Nicolson et al., 1998; Söllner et al., 2004). cdh23 mRNA is expressed in the developing inner ear, and later in the neuroepithelium of the inner ear and lateral line neuromasts. The Cdh23 protein localizes near the tip of hair bundles and the tip links required for sound detection (which are absent in the mutants). cdh23 mutants do not respond to startle stimuli and have abnormal swimming behavior.

9.2.4. Protocadherin related 15 (PCDH15)

Mutations in the PCDH15 gene cause Usher syndrome type 1F and the DFNB23 form of non-syndromic hearing loss (Ahmed et al., 2003a, 2008a). In zebrafish, the orbiter mutant from the forward genetic screen showed vestibular dysfunction, failed to respond to startle stimuli and lacked hair cell microphonic potentials (Seiler et al., 2005). The gene responsible for the orbiter phenotype was later mapped to the pcdh15 gene. There are two paralogs of pcdh15 in zebrafish, pcdh15a, and pcdh15b. The hearing phenotypes were determined to be caused by mutations in the pcdh15a gene.

9.2.5. Otoferlin (OTOF)

Mutations in the human OTOF gene cause the DFNB9 form of non-syndromic hearing loss. There are two copies of the otof gene (otofa and otob) in zebrafish. mRNA from both otofa and otob is expressed in the otic placodes within 3 dpf, and by 5 dpf only otob mRNA was detected in the lateral line hair cells (Chatterjee et al., 2015). Knockdown of otoferlin genes causes hearing and balance dysfunction, and otoferlin morphant animals did not have inflated swim bladders. Otof mRNA from mouse was able to rescue the morphant phenotype in zebrafish, showing the conserved role of OTOF across the species (Chatterjee et al., 2015).

9.2.6. Deafness autosomal 5 (DFNA5)

The autosomal dominant form of hearing loss DFNA5 is caused by mutations in the GDSCME gene in humans (Van Laer et al., 1998). In zebrafish, the dfna5 gene is duplicated with two paralogs: dfna5a and dfna5b. dfna5b mRNA is expressed ubiquitously in early-stage embryos, but is later restricted to inner ear tissues (Busch-Nentwich et al., 2004). MO-mediated knockdown of dfna5b mRNA causes abnormal semicircular canals, and a higher dose of MO also causes malformed ventral jaw development to impaired pharyngeal cartilage development. The malformed jaw phenotype was not recapitulated in the genetic mutant generated by CRISPR/Cas9 (Varshney et al., 2015b). The Dfna5 KO mouse does not display impaired facial cartilage differentiation as well (Op de Beeck et al., 2011). Absence of cartilage differentiation phenotypes both in zebrafish genetic mutant and mouse KO could be due to genetic compensation.

9.2.7. Grnaihead like transcription factor 2 (GRHL2)

Mutations in the GRHL2 gene are responsible for the DFNA28 form of non-syndromic hearing loss in humans (Vona et al., 2013). Due to duplication of the zebrafish genome, grhl2 has two paralogs: grhl2a and grhl2b. grhl2b mutants have smaller or missing otoliths (Han et al., 2011). Human mRNA overexpression in the mutants rescues the phenotypes, indicating a conserved role of grhl2 in inner ear development in vertebrates. However, the phenotype failed to rescue when human mRNA with a pathogenic variant was injected suggesting a pathogenic nature of the variant (Han et al., 2011).

9.2.8. Adenylate cyclase 1 (ADCY1)

ADCY1 is a member of the adenylate cyclase group of enzymes that catalyze the conversion of ATP to cyclic AMP (cAMP). Production of cAMP is important for mechanotransduction in the inner ear (Santos-Cortez et al., 2014). Mutations in ADCY1 cause autosomal recessive non-syndromic hearing loss DFNB44. A nonsense mutation in ADCY1 was first identified in a consanguineous family from Pakistan. ADCY1 is duplicated in zebrafish with two copies adcy1a, and adcy1b. The function of adcy1 genes in zebrafish was investigated by MO-mediated mRNA knockdown. Morphant animals lack smaller eyes, small brain, and observed behavioral defects. adcy1b morphant animals did not respond to acoustic startle stimuli, while adcy1a morphants have normal response similar to wild-type suggesting adcy1b is contributing to the hearing impairment (Santos-Cortez et al., 2014).

9.2.9. Immunoglobulin-like domain containing receptor 1 (ILDR1)

The Immunoglobulin-Like Domain Containing Receptor 1 (ILDR1) gene encodes an immunoglobulin-like domain-containing protein that functions as a multimeric receptor. Mutations in the ILDR1 gene cause non-syndromic DFNB42, and patients show bilateral non-progressive moderate-to-severe sensorineural hearing loss (Borck et al., 2011). Zebrafish has two copies of ildr1, ildr1a, and ildr1b. Based on in situ hybridization, only ildr1b has otic tissue-related expression. ildr1b mRNA localizes to the otic vesicle, and the posterior lateral line primordium at 24–36 hpf, and the expression became more prominent in the anterior lateral line and inner ear (Borck et al., 2011; Sang et al., 2014). MO-mediated knockdown of ildr1b exerts delays in the development of the semicircular canals in the zebrafish inner ear, a reduced number of neuromasts and defective hearing and aberrant swimming pattern (Sang et al., 2014). The morphant embryos showed downregulation of the atp1b2b gene that encodes ATPase Na+/K+/transporting subunit beta 2, and the overexpression of atp1b2b partially rescued the inner ear phenotypes. The disruption in posterior lateral line primordium migration is due to attenuated FG signaling pathway, cxcr4b, and cxcr7b expression (Sang et al., 2014).

9.2.10. RHO family interacting cell polarization regulator 2 (RIPOR2)

RIPOR2 encodes an atypical inhibitor of the small G protein RhoA. RIPOR2 is a component of hair cell stereocilia, and mutations in this gene cause autosomal recessive deafness 104 (DFNB104). Diaz-Horta et al. identified mutations in six members of a consanguineous Turkish family (Diaz-Horta et al., 2014). The mutation caused an in-frame skipping of exon 3. Zebrafish ortholog ripor2 mRNA expression was detected in the otic vesicle of 3 dpf zebrafish (Diaz-Horta et al., 2014). To investigate the function of ripor2 in zebrafish, two different splice site blocking MOs were used to reduce the protein expression. Both morphant animals showed reduced numbers of saccular hair cells and fewer lateral line neuromasts, and did not show balance abnormalities. Furthermore, auditory function in morphants was detected by recording...
microphonic potentials from hair cells, and the morphants had weaker microphonic responses than the wild-type animals (Diaz-Horta et al., 2014).

9.2.11. Transmembrane protein 132E (TMEM132E)

TMEM132E encodes for transmembrane protein 132e and mutations in this gene cause autosomal recessive deafness 99 (DFNB99) (Li et al., 2015). Homozygous missense mutations in TMEM132E were first identified in two siblings from consanguineous Chinese parents. Tmem132e is highly expressed in hair cells of the mouse cochlea. Functional studies were performed in zebrafish using MO-mediated knockdown of tmem132e mRNA. Hair cell development was monitored using cationic dye 4-Di-2-AS capable of penetrating into hair cells through mechanotransduction channels, therefore, serving as an indicator of mechanotransduction function of hair cells. Hair cells of the inner ear and the lateral line in morphant animals did not label with 4-Di-2-AS dye indicating non-functional hair cells. Co-injection of wild-type human TMEM132E mRNA together with MO rescued the phenotype. Scanning electron microscopy revealed fewer and shorter kinocilia and stereocilia in neuromasts in morphant animals. The morphant larvae showed delayed startle response, and significant reduction in microphonic potential. Together these data suggest non-functional mechanotransduction in hair cells (Li et al., 2015).

9.2.12. Transformation/transcription domain-associated protein (TRRAP)

TRRAP encodes a multi-domain protein kinase that functions as a cofactor in the histone acetylation process. Whole-exome sequencing identified a variant in the TRRAP gene that was associated with autosomal dominant deafness in a three generation Chinese family (Xia et al., 2019a). Later, three different variants were identified from molecular genetic testing of 66 sporadic cases of hearing loss. Functional studies for TRRAP were carried out in zebrafish using both MO-mediated knockdown and Knockout animals. Zebrafish has a single trrap gene, and the loss of trrap function resulted in fewer and smaller neuromasts in the lateral line, and the number of hair cells and supporting cells were reduced in both knockdown and Knockout animals. Scanning electron microscopy revealed that both kinocilia and stereocilia on hair cells per neuromast were shortened and were fewer in both mutants and morphants. Furthermore, hearing ability was measured by acoustic and c-shape startle response testing, and both knockdown and Knockout animals had reduced responses compared to the wild-type animals, confirming the role of trrap in hearing (Xia et al., 2019a).

9.2.13. Sphingosine-1-phosphate receptor 2 (SIPR2)

SIPR2 is a member of the sphingosine-1-phosphate receptor class of transmembrane G protein-coupled sphingolipid receptors. Mutations in the human SIPR2 gene cause non-syndromic autosomal recessive hearing loss DFNB68 (Santos-Cortez et al., 2016). The expression of sipr2 mRNA in zebrafish was investigated by whole-mount in situ hybridization. sipr2 mRNA expression is seen in somites, in the encephalic region at 24 hpf, and later in the lateral line and midbrain/hindbrain boundary at 48 hpf stage (Hu et al., 2013). MO-mediated knockdown of sipr2 led to abnormal semicircular canals and otoliths, abnormal deposition of neuromasts in the lateral line, and fewer hair cells. Morphant animals also showed downregulation of many hearing-related genes. Morphant animals showed an increased number of apoptotic hair cells and dead hair cells as stained by apoptotic marker Hoechst 33324 (Hu et al., 2015).

9.2.14. Cell division-cycle 14A (CDC14A)

CDC14A encodes a dual specificity protein tyrosine phosphatase. Pathogenic mutations in CDC14A are associated with autosomal recessive hearing loss (DFNB32). Biallelic nonsense mutations in CDC14A were identified in consanguineous Iranian families affected by severe or profound congenital deafness (Delmaghani et al., 2006; Imtiaz et al., 2018). In zebrafish, the cdca4a gene has two paralogs, cdc14aa, and cdc14ab. It has been shown that cdc14a plays an important role in ciliogenesis in Kupffer’s vesicle, and other ciliated organs (Clement et al., 2012). Based on this observation, Delmaghani et al. performed MO-mediated knockdown of the cdca4a gene in zebrafish to investigate its role in hearing. The morphant animals showed shortening of kinocilia in inner ear hair cells (Delmaghani et al., 2006). Subsequently, the shortening of kinocilia phenotype was not recapitulated in genetic mutants generated by CRISPR/Cas9 in another independent study (Imtiaz et al., 2018). The cdca4a mutant is predicted to ablate phosphate activity, and did not show any difference in startle response, or mechanotransduction by uptake of Yo-Pro1 live dye compared to control animals. It is possible that the discrepancy in the phenotypes between MO-mediated knockdown and genetic mutant could be due to genetic compensation (Rossi et al., 2015).

9.2.15. Phosphoribosyl pyrophosphate Synethetase-1 (PRPS1)

PRPS1 encodes for phosphoribosyl pyrophosphate synthetase-1, an important enzyme in nucleotide biosynthesis, and pathogenic mutations in PRPS1 have been implicated in X-linked non-syndromic sensorineural hearing loss (DFNX1/DFN2), as well as other diseases such as Charcot-Marie-Tooth disease-5, and ARTS syndrome. Presently, more than 25 pathogenic mutations have been identified (Liu et al., 2010). The function of prps1 was studied in zebrafish by two different groups using genetic mutants and MO-mediated knockdown (DeSmidt et al., 2019; Pei et al., 2016). Zebrafish has two paralogs, prps1a, and prps1b. The prps1a mutants showed pigmentation defects, small eyes, and a reduced number of neuromast hair cells, whereas prps1b mutants showed no overt phenotype. However, prps1a;prps1b double mutants displayed more severe defects in the eye, pigmentation, and the neuromast hair cells (Pei et al., 2016). MO-mediated knockdown of either prps1a or prps1b led to small otic vesicles, fewer hair cells in the inner ear, and reduced microphonic potential than the control animals. Taken together, loss of prps1a and prps1b functions lead to sensorineural hearing loss in zebrafish (DeSmidt et al., 2019; Pei et al., 2016).

9.2.16. Methionine sulfoxide reductase B3 (MSRB3)

MSRB3 encodes for methionine sulfoxide reductase that catalyzes the reduction of oxidized methionine in damaged proteins (Ahmed et al., 2011). Mutations in MSRB3 cause autosomal recessive hearing loss, DFNB74, two homozygous mutations were reported in 8 consanguineous families from Pakistan. These mutations led to truncation of the MSRB3 gene, suggesting its essential role in hearing. The function of msrb3 was also investigated in zebrafish using MO-mediated knockdown of msrb3 mRNA (Shen et al., 2015). Msrb3 mRNA expressed broadly at 12 hpf, and by 26 hpf, it was highly expressed in the inner ear. MO-mediated knockdown animals displayed abnormal otoliths, semicircular canals, as well as fewer and disordered neuromasts in the lateral line. Phalloidin staining showed shorter and thinner stereocilia in the inner ear, and scanning electron microscopy revealed damaged kinocilia in lateral line. Morphant embryos showed either reduced or no startle response than the control zebrafish (Shen et al., 2015). While there are only a limited number of zebrafish models that exist for non-syndromic hearing loss, the number of disease models are expected to rise with the advancement of CRISPR/Cas9-targeted mutagenesis approaches. This is expected to subsequently accelerate our ability to unravel the functional mechanisms for genes.
involved in hearing loss.

10. Conclusions

With the major advancements in sequencing technologies made in the past decade, there is an unprecedented opportunity to exploit animal models to explore gene-disease associations and uncover the molecular mechanisms involved in human deafness. Over the past two decades, zebrafish has become a popular vertebrate model organism for human disease. Development of new genetic tools and techniques is fueling the growth of disease models, including those for hearing and balance disorders. Until 2013, the majority of the mutants available for studies have been generated by random mutagenesis because systematic targeted mutagenesis was not possible; this limited functional genomic studies of hearing loss genes. The development of targeted mutagenesis methods such as TALENs and CRISPR/Cas9 made it possible to generate gene knockouts at will. CRISPR/Cas9 technology was quickly and widely adopted due to its simplicity and cost-effective nature. It also allows for simultaneous targeting of more than one gene, making it possible to target two paralogs simultaneously in zebrafish. With the widespread availability of these newer genetic methods, it is now possible to validate human hearing loss genes in zebrafish in a targeted manner in order to understand their functional mechanisms a vertebrate organism.

In spite of exciting developments in the genome editing field, it remains to be seen how CRISPR-based base editing technologies will accelerate the generation of pathogenic variants in zebrafish, and how effectively they can be applied to recapitulate the variants that have been identified in humans with hereditary hearing loss. There is also a need to develop more innovative approaches to understand the role of multiple genes, epigenetics, and the environment in disease pathology. Zebrafish will be important in overcoming these challenges because of its ability to generate a large number of animals and facilitate observation of minor effects and low penetrance phenotypes. Although zebrafish are phylogenetically distant from mammals, its distinct features as a vertebrate model organism and the fact that more than 90% of human hearing-related genes have orthologs in zebrafish make it an ideal model in which to study hearing loss. While zebrafish offers many advantages over other model organisms such as mouse to model hearing loss, lack of an auditory organ such as the cochlea makes it harder to recapitulate all hearing functions from such animals.

Funding

This work was supported by intramural funding from the University of Tübingen (2545-1-0) to BV, as well as NIH/COBRE GM103636 (Project 3), and the Presbyterian Health Foundation Grant to GKV.

Acknowledgements and author contributions

We thank Sheng-Jia Lin, Cassidy Petree, and Rachel Smith for their help in generating Fig. 3. BV and GKV reviewed the literature, wrote the manuscript and generated figures. JD and MAHH generated a table and figure, reviewed and edited the manuscript. TH provided supervision, reviewed and edited the manuscript. All authors have read and approve of the final article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heares.2020.107906.

References

Abe, S., Katagiri, T., Saito-Hisamino, A., Usami, S.-L., Inoue, Y., Tsunoda, T., Nakamura, Y., 2003. Identification of CRMY as a candidate responsible for nonsyndromic deafness, through cDNA microarray analysis of human cochlear and vestibular tissues. Am. J. Hum. Genet. 72, 73–82.

Ahmed, Z.M., Riazuddin, S., Aye, S., Ali, R.A., Venselaar, H., Anwar, S., Belenkyeva, P.P., Qasim, M.A., Friedman, T.B., 2008a. Gene structure and mutant alleles of PCDH15: nonsyndromic deafness DFNB23 and type 1 Usher syndrome. Hum. Genet. 124, 215–223.

Ahmed, Z.M., Smith, T.N., Riazuddin, S., Makishima, T., Ghosh, M., Bokhari, S., Morell, R.J., Deshmukh, D., Casvantes, J.A., Steinberg, A.J., Riazuddin, S., Friedman, T.B., Wilcox, E.R., 2002. Nonsyndromic recessive deafness DFNB18 and Usher syndrome type IC are allelic mutations of USH2C. Hum. Genet. 110, 527–531.

Ahmed, Z.M., Riazuddin, S., Ahmad, J., Bernstein, S.L., Guo, Y., Sabar, M.F., Sieving, P., Riazuddin, S., Friedman, T.B., Belyantseva, I.A., Wilcox, E.R., 2003a. PCDH15 is expressed in the neuroepithelium of the eye and ear and mutant alleles are responsible for both USH1F and DFNB23. Hum. Mol. Genet. 12, 3215–3223.

Ahmed, Z.M., Morell, R.J., Riazuddin, S., Croppman, A., Shaukat, S., Ahmad, M.M., Mohiddin, S.A., Fananapazir, L., Caruso, R.C., Husnain, T., Khan, S.N., Griffith, A.J., Friedman, T.B., Wilcox, E.R., 2003b. Mutations of MYO6 are associated with recessive deafness, DFNB37. Am. J. Hum. Genet. 72, 1315–1322.

Ahmed, Z.M., Morell, R.J., Riazuddin, S., Ahmad, M.M., Mohiddin, S.A., Fananapazir, L., Caruso, R.C., Husnain, T., Khan, S.N., Riazuddin, S., Griffith, A.J., Friedman, T.B., Wilcox, E.R., 2003c. Mutations of MYO6 are associated with recessive deafness, DFNB37. Am. J. Hum. Genet. 72, 1315–1322.

Ahmed, Z.M., Yousaf, R., Lee, B.C., Khan, S.N., Lee, S., Lee, K., Husnain, T., Rehman, A.U., Bonneux, S., Ansar, M., Ahmad, W., Leal, S.M., Gladyshev, V.N., Belenkyeva, I.A., Venselaar, H., Riazuddin, S., Friedman, T.B., 2014. Functional null mutations of MSRB3 encoding methionine sulfoxide reductase 3e are associated with human deafness DFNB74. Am. J. Hum. Genet. 88, 19–29.

Ahmed, Z.M., Masmoudi, S., Kalay, E., Belyantseva, I.A., Mosrati, M.A., Collin, R.W., Riazuddin, S., Hmani-Aifa, M., Venselaar, H., Kawar, M.N., Tili, A., van der Zwaag, B., Khan, S.Y., Ayadi, L., Riazuddin, S.A., Morell, R.J., Griffith, A.J., Charfedine, I., Caylan, R., Oostrik, J., Maroo, R., Horvath, J., Belyantseva, I.A., Grif

References (Continued)

Ahmed, Z.M., Smith, T.N., Friedman, T.B., Wilcox, E.R., 2003b. Mutations of MYO6 are associated with recessive deafness, DFNB37. Am. J. Hum. Genet. 72, 1315–1322.

Ahmed, Z.M., Yousaf, R., Lee, B.C., Khan, S.N., Lee, S., Lee, K., Husnain, T., Rehman, A.U., Bonneux, S., Ansar, M., Ahmad, W., Leal, S.M., Gladyshev, V.N., Belenkyeva, I.A., Venselaar, H., Riazuddin, S., Friedman, T.B., 2014. Functional null mutations of MSRB3 encoding methionine sulfoxide reductase 3e are associated with human deafness DFNB74. Am. J. Hum. Genet. 88, 19–29.

Ahmed, Z.M., Masmoudi, S., Kalay, E., Belyantseva, I.A., Mosrati, M.A., Collin, R.W., Riazuddin, S., Hmani-Aifa, M., Venselaar, H., Kawar, M.N., Tili, A., van der Zwaag, B., Khan, S.Y., Ayadi, L., Riazuddin, S.A., Morell, R.J., Griffith, A.J., Charfedine, I., Caylan, R., Oostrik, J., Maroo, R., Horvath, J., Belyantseva, I.A., Grif

References (Continued)

Ahmed, Z.M., Smith, T.N., Friedman, T.B., Wilcox, E.R., 2003b. Mutations of MYO6 are associated with recessive deafness, DFNB37. Am. J. Hum. Genet. 72, 1315–1322.

Ahmed, Z.M., Yousaf, R., Lee, B.C., Khan, S.N., Lee, S., Lee, K., Husnain, T., Rehman, A.U., Bonneux, S., Ansar, M., Ahmad, W., Leal, S.M., Gladyshev, V.N., Belenkyeva, I.A., Venselaar, H., Riazuddin, S., Friedman, T.B., 2014. Functional null mutations of MSRB3 encoding methionine sulfoxide reductase 3e are associated with human deafness DFNB74. Am. J. Hum. Genet. 88, 19–29.

Ahmed, Z.M., Masmoudi, S., Kalay, E., Belyantseva, I.A., Mosrati, M.A., Collin, R.W., Riazuddin, S., Hmani-Aifa, M., Venselaar, H., Kawar, M.N., Tili, A., van der Zwaag, B., Khan, S.Y., Ayadi, L., Riazuddin, S.A., Morell, R.J., Griffith, A.J., Charfedine, I., Caylan, R., Oostrik, J., Maroo, R., Horvath, J., Belyantseva, I.A., Grif
Borck, G., Ur Rehman, A., Lee, K., Pogoda, H.M., Kakar, N., von Ameln, S., Grillet, N., Charizopoulou, N., Lelli, A., Schraders, M., Ray, K., Hildebrand, M.S., Ramesh, A., Bharadwaj, A.K., Kasztejna, J.P., Huq, S., Berson, E.L., Dryja, T.P., 2000. Evaluation of Ben Said, M., Grati, M.h., Ishimoto, T., Zou, B., Chakchouk, I., Ma, Q., Yao, Q., Chang, N., Sun, C., Gao, L., Zhu, D., Xu, X., Zhu, X., Xiong, J.W., Xi, J.J., 2013. Genome editing with RNA-guided Cas9 nuclease in zebra fish embryos. Cell Res. 23, 685–698.

Borck, G., Ur Rehman, A., Lee, K., Pogoda, H.M., Kakar, N., von Ameln, S., Grillet, N., Charizopoulou, N., Lelli, A., Schraders, M., Ray, K., Hildebrand, M.S., Ramesh, A., Bharadwaj, A.K., Kasztejna, J.P., Huq, S., Berson, E.L., Dryja, T.P., 2000. Evaluation of the deafness gene in X-linked mixed deafness and mutations in the POU domain gene POU5F1B cause X-linked DFNB14 auditory neuropathy. Hum. Genet. 107, 88–99.

Booth, T.K., Kahrizi, K., Najmabadi, H., Azaizeh, H., Smith, R.J., 2018. New gene, new phenotype: splice-altering variants in cause recessive non-syndromic hearing impairment. J. Med. Genet. 55, 555–560.

Booth, T.K., Firestein, J.A., Zilberstein, Z., Huygen, P.L.M., Eudy, J., Kenyon, J., Hoover, D., Hildebrand, M.S., Smith, K.R., Bahlo, M., Kim, S., Lepage, M., Lemke, H., 2007. Mutations in the Wolfram syndrome 1 gene (WFS1) are a common cause of low frequency sensorineural hearing loss. Hum. Mol. Genet. 16, 2501–2508.

Bortolini, A., Kuhn, S., Berenson, E.L., Divya, T.P., 2000. Evaluation of the myosin VIIA gene and visual function in patients with Usher syndrome type I. Exp. Eye Res. 71, 173–181.

Borroni, E., Delmaghani, S., Aghaie, A., Bouyacoub, Y., El Hachmi, H., Bonnet, C., Riahi, Z., Charencon, S., Perfettini, L.J., Hardelin, J.-P., Perochon, M., Lebreton, C., Lebrun, C., Azinger, B., Claret, A., Clement, A., Solnica-Krezel, L., Gould, K.L., 2012. Functional redundancy between E53.1 and E53.2 of zebrafish harmonin and sans usher syndrome proteins. Cell Rep. 25, 1281 e4.

Borst, J.K., Yuan, H., Marcotti, W., Steel, K.P., 2016. Wbp2 is required for normal hearing: rescue of the balance and hearing phenotype with full-length and truncated forms of mouse otoferin. Mol. Cell Biol. 35, 1043–1054.

Brackmann, H.D., Wilson, J., 1989. Atlas of the brainstem and cranial nerves. Philadelphia: Saunders.

Bredberg, M., Rossin, A., Kraner, K.O., Polomeno, R., Ramesh, A., Schloss, M., Srisailapathy, C.R., Oostrik, J., Admiraal, R.J., Neely, H.R., Latoche, J.R., Smith, R.J., Muller, U., Beutler, B., 2008. A catechol-O-methyltransferase that is required for normal glutamatergic synapses in the cochlea and is crucial for hearing. EMBO Mol. Med. 1, 275–288.

Brenton, R., Challet, E., 2015. The reeler model of mouse hearing. Nat. Rev. Neurosci. 16, 228–236.

Bretschneider, M., de Kok, Y.J., van der Maarel, S.M., Bitner-Glindzicz, M., Huber, I., Monaco, A.P., Scholz, M., Fehling, P., 1997. Effect of mutations in the fibroblast growth factor 2 gene on the development of the zebrafish inner ear. J. Neurosci. Methods 74, 187–191.

Bretschneider, M., de Kok, Y.J., van der Maarel, S.M., Bitner-Glindzicz, M., Huber, I., Monaco, A.P., Scholz, M., Fehling, P., 1997. Effect of mutations in the fibroblast growth factor 2 gene on the development of the zebrafish inner ear. J. Neurosci. Methods 74, 187–191.

Bretschneider, M., de Kok, Y.J., van der Maarel, S.M., Bitner-Glindzicz, M., Huber, I., Monaco, A.P., Scholz, M., Fehling, P., 1997. Effect of mutations in the fibroblast growth factor 2 gene on the development of the zebrafish inner ear. J. Neurosci. Methods 74, 187–191.

Bretschneider, M., de Kok, Y.J., van der Maarel, S.M., Bitner-Glindzicz, M., Huber, I., Monaco, A.P., Scholz, M., Fehling, P., 1997. Effect of mutations in the fibroblast growth factor 2 gene on the development of the zebrafish inner ear. J. Neurosci. Methods 74, 187–191.
Nurnberg, G., Nurnberg, P., Ruland, R., Westerfield, M., Benzing, T., Bolz, H.J., 2010. PDZD7 is a modifier of retinal disease and a contributor to digenic Usher syndrome. Am. J. Hum. Genet. 87, 367–382.

Eisenberger, T., Di Donato, N., Becker, C., Delle Vedove, A., Neuhaus, C., Nurnberg, C., Toliat, M., Nurnberg, P., Mürbe, D., Bolz, H.J., 2018. A C-terminal nonsense mutation links PTPRQ with autosomal-dominant hearing loss, DFNA73. Genet. Med. 20, 1404–1411.

El-Brosy, M.A., Kontarakis, Z., Rossi, A., Kuenne, C., Gunther, S., Lifton, P., Vasa-Pawelke, J., 2013. Variable hearing impairment in a DFNB2 family with a novel MYOA missense mutation. Clin. Genet. 77, 563–571.

Hilgert, N., Smith, R.J., Van Camp, G., 2009. Forty-six genes causing nonsyndromic hearing impairment but few genes should be analyzed in DNA diagnostics? Mutat. Res. 681, 189–196.

Horn, H.F., Brownstein, Z., Lenz, D.R., Chakravarti, A., Drumm, D.A., Dagan-Rosenthal, O., Friedman, L.M., Roux, K.J., Kozlov, S., Jeang, K.-T., Frydman, M., Burke, S.T., Stewart, C.L., Avraham, X.B., 2013. The LINC complex is essential for hearing. J. Clin. Invest. 123, 740–750.

Hosjihama, K., Jurynec, M.J., Klatt Shaw, D., Jacobo, M.B., Mehlke, B.A., Grunwald, D.J., 2019. Highly efficient CRISPR-cas9-based methods for generating deletion and POI embryos that lack gene function in zebrafish. Dev. Cell 51, 654–665 e4.

Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berchtold, C., Muffato, M., Collins, J.E., Humphrey, S., McLaren, K., Matthews, L., McLaren, S., Sealy, I., Caccamo, M., Churcher, C., Scott, C., Barrett, J.C., Koch, R., Rauch, C.J., White, S., Chow, W., Kilian, B., Quintais, L.T., Guerra-Assuncao, J.A., Zhou, Y., Gu, Y., Yen, J., Vogel, J.H., Eyre, T., Redmond, S., Banerjee, R., Chi, J., Fu, B., Langleye, E., Maguire, S.F., Laid, G.K., Lloyd, K., Kenyon, E., Donaldson, S., Sehna, H., Almeida-King, J., Loveland, J., Trenovan, S., Jones, M., Quail, M., Willey, D., Hunt, A., Burton, J., Sims, J., McMay, K., Plumb, B., Davis, J., Clee, C., Oliver, K., Clark, R., Riddle, C., Elliott, D., Threadgold, G., Harden, G., Ware, D., Begum, S., Mortimore, B., Kerry, G., Heath, P., Philpott, F., Tracey, A., Corby, N., Dunn, M., Johnson, C., Wexler, L., Clark, P., Flik, G., Wood, C., Park, K., Kollmer, L., Nagayama, K., Lloyd, C., Kour, T., Henry, X., Gielis, M., Benzing, T., Harley, J., Koltanowski, J., Völkel, L., Beasley, H., Henderson, C., Cordogan, D., Auger, K., Wright, D., Collins, J., Raisen, C., Dray, S., Leung, K., Rogers, L., Perk, C., Lemmon, L., Tindal, R., Gudberth, S., Gilderspeck, R., Griffiths, C., Manthorpe, D., Nicholson, S., Black, L., et al., 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature 496, 498–503.

Hruscha, A., Kravitz, P., Rechenberg, A., Heinrich, V., Hecht, J., Haass, C., Schmid, B., 2013. Efficient CRISPR/Cas9 genome editing with low off-target effects in zebrafish. Development 140, 4982–4987.

Hu, Z.Y., Zhang, Q.Y., Qin, W., Tong, J.W., Zhao, Q., Han, Y., Meng, J., Zhang, J.P., 2013. Gene miles-apart is required for formation of otic vesicle and hair cells in zebrafish. Cell Death Dis. 4, e900.

Hubner, A.K., Gandia, M., Frommolt, P., Maak, A., Wicklein, E.M., Thiele, H., Altmüller, J., Wagner, F., Vinuela, A., Aguilar, L., Moreno, F., Maier, H., Rau, I., Giebel, S., Nürnberg, P., Nürnberg, G., Nürnberg, G., Kloth, M., Furutani-Seiki, M., Kelsh, R.N., Mullins, M.C., Odenthal, J., Nüsslein-Volhard, C., 1996. Genes controlling inner ear development are required for auditory processing. Acta Biol. Hung. 39, 251–270.
Starnawski, A., Hedemand, A., Buniello, A., Niola, F., Overgaard, M.T., Leal, S.M., Ahmad, W., Wilkman, F.P., Petersen, K.B., Kruger, D.G., Oostrik, J., Kremer, H., Hamond, A., Funch-Nielsen, T., Steen, K.D., A., D., 2015. A novel locus harbouring a functional CD64 nonsense mutation identified in a large Danish family with nonsyndromic hearing impairment. PLoS Genet. 11, e1005386.

Oblozil, Z., San, B., Vona et al. / Hearing Research 397 (2020) 107906 18

Oblozil, Z., Sanci, C., Trapani, J.G., Mo, W., Nechiporuk, A., Busch-Nentwich, E., Seiler, C., Sidi, S., Sollner, C., Duncan, R.N., Boehrland, A., Nicolson, T., 2008. Vescular glutamate transporter 3 is required for synaptic transmission in zebrafish hair cells. J. Neurosci. 28, 2110–2118.

Ogata, K., Weigert, C., Van der Meer, A., C, Van Deventer, V.F.M., Timmermans, J.P., Van, L., A., 2011. The DFNAS1 gene, responsible for hearing loss and involvement in cancer, encodes a novel apoptosis-inducing protein. Eur. J. Hum. Genet. 19, 956–973.

Ouyang, X.M., Xia, X.J., Yerup, E., Du, L.L., Pandya, A., Petit, C., Balkany, T., Nance, W.E., Liu, X.Z., 2002. Mutations in the alternatively spliced exons of USH1C cause non-syndromic recessive deafness. Hum. Genet. 111, 26–30.

Pei, W., Xu, L., Varshney, G.K., Carrington, B., Bishop, K., Jones, K., Huang, S.C., Iol, T., Pretorious, P.D., Beil, A., Schimenti, L.A., Knod, K.S., Sood, R., Burgess, S.M., 2016. Additive reductions in zebrafish PRPS1 activity result in a spectrum of deficiencies modeling several human PRPS1-associated diseases. Sci. Rep. 6, 30488.

Phillips, J.B., Blanco-Sanchez, B., Lentz, J.J., Tallafuss, A., Khanobdee, K., Sampath, S., Robertson, N.G., Lu, L., Heller, S., Merchant, S.N., Eavey, R.D., McKenna, M., Riazuddin, S., Belyantseva, I.A., Giese, A.P., Lee, K., Indzhykulian, A.A., Ouyang, X.M., Xia, X.J., Verpy, E., Du, L.L., Pandya, A., Petit, C., Balkany, T., Nance, W.E., Liu, X.Z., 2002. Mutations in the alternatively spliced exons of Usher syndrome type 1J and nonsyndromic deafness DFNB48. Nat. Genet. 44, 388.

Pretorius, P.R., Beirl, A., Schimmenti, L.A., Kindt, K.S., Sood, R., Burgess, S.M., Washington Center for Mendelian, G., Nickerson, D.A., Shendure, J., Khan, A.A., Basra, M.A., Wasif, N., Ayub, M., Ansar, M., Ahmad, W., Wikman, F.P., Petersen, K.B., Crüger, D.G., Oostrik, J., Kremer, H., 2012. Alterations of the CIB2 calcium- and integrin-binding protein cause Usher syndrome type 1A. Hum. Mutat. 29, 502.

Pretorius, P.R., Beirl, A., Schimmenti, L.A., Kindt, K.S., Sood, R., Burgess, S.M., Washington Center for Mendelian, G., Nickerson, D.A., Shendure, J., Khan, A.A., Basra, M.A., Wasif, N., Ayub, M., Ansar, M., Ahmad, W., Wikman, F.P., Petersen, K.B., Crüger, D.G., Oostrik, J., Kremer, H., 2012. Alterations of the CIB2 calcium- and integrin-binding protein cause Usher syndrome type 1A. Hum. Mutat. 29, 502.

Rao, R., Varshney, G.K., Carrington, B., Bishop, K., Jones, K., Huang, S.C., Iol, T., Pretorious, P.D., Beil, A., Schimenti, L.A., Knod, K.S., Sood, R., Burgess, S.M., 2016. Additive reductions in zebrafish PRPS1 activity result in a spectrum of deficiencies modeling several human PRPS1-associated diseases. Sci. Rep. 6, 30488.

Rao, R., Varshney, G.K., Carrington, B., Bishop, K., Jones, K., Huang, S.C., Iol, T., Pretorious, P.D., Beil, A., Schimenti, L.A., Knod, K.S., Sood, R., Burgess, S.M., 2016. Additive reductions in zebrafish PRPS1 activity result in a spectrum of deficiencies modeling several human PRPS1-associated diseases. Sci. Rep. 6, 30488.

Rao, R., Varshney, G.K., Carrington, B., Bishop, K., Jones, K., Huang, S.C., Iol, T., Pretorious, P.D., Beil, A., Schimenti, L.A., Knod, K.S., Sood, R., Burgess, S.M., 2016. Additive reductions in zebrafish PRPS1 activity result in a spectrum of deficiencies modeling several human PRPS1-associated diseases. Sci. Rep. 6, 30488.

Rao, R., Varshney, G.K., Carrington, B., Bishop, K., Jones, K., Huang, S.C., Iol, T., Pretorious, P.D., Beil, A., Schimenti, L.A., Knod, K.S., Sood, R., Burgess, S.M., 2016. Additive reductions in zebrafish PRPS1 activity result in a spectrum of deficiencies modeling several human PRPS1-associated diseases. Sci. Rep. 6, 30488.

Rao, R., Varshney, G.K., Carrington, B., Bishop, K., Jones, K., Huang, S.C., Iol, T., Pretorious, P.D., Beil, A., Schimenti, L.A., Knod, K.S., Sood, R., Burgess, S.M., 2016. Additive reductions in zebrafish PRPS1 activity result in a spectrum of deficiencies modeling several human PRPS1-associated diseases. Sci. Rep. 6, 30488.

Rao, R., Varshney, G.K., Carrington, B., Bishop, K., Jones, K., Huang, S.C., Iol, T., Pretorious, P.D., Beil, A., Schimenti, L.A., Knod, K.S., Sood, R., Burgess, S.M., 2016. Additive reductions in zebrafish PRPS1 activity result in a spectrum of deficiencies modeling several human PRPS1-associated diseases. Sci. Rep. 6, 30488.

Rao, R., Varshney, G.K., Carrington, B., Bishop, K., Jones, K., Huang, S.C., Iol, T., Pretorious, P.D., Beil, A., Schimenti, L.A., Knod, K.S., Sood, R., Burgess, S.M., 2016. Additive reductions in zebrafish PRPS1 activity result in a spectrum of deficiencies modeling several human PRPS1-associated diseases. Sci. Rep. 6, 30488.

Rao, R., Varshney, G.K., Carrington, B., Bishop, K., Jones, K., Huang, S.C., Iol, T., Pretorious, P.D., Beil, A., Schimenti, L.A., Knod, K.S., Sood, R., Burgess, S.M., 2016. Additive reductions in zebrafish PRPS1 activity result in a spectrum of deficiencies modeling several human PRPS1-associated diseases. Sci. Rep. 6, 30488.

Rao, R., Varshney, G.K., Carrington, B., Bishop, K., Jones, K., Huang, S.C., Iol, T., Pretorious, P.D., Beil, A., Schimenti, L.A., Knod, K.S., Sood, R., Burgess, S.M., 2016. Additive reductions in zebrafish PRPS1 activity result in a spectrum of deficiencies modeling several human PRPS1-associated diseases. Sci. Rep. 6, 30488.

Rao, R., Varshney, G.K., Carrington, B., Bishop, K., Jones, K., Huang, S.C., Iol, T., Pretorious, P.D., Beil, A., Schimenti, L.A., Knod, K.S., Sood, R., Burgess, S.M., 2016. Additive reductions in zebrafish PRPS1 activity result in a spectrum of deficiencies modeling several human PRPS1-associated diseases. Sci. Rep. 6, 30488.

Rao, R., Varshney, G.K., Carrington, B., Bishop, K., Jones, K., Huang, S.C., Iol, T., Pretorious, P.D., Beil, A., Schimenti, L.A., Knod, K.S., Sood, R., Burgess, S.M., 2016. Additive reductions in zebrafish PRPS1 activity result in a spectrum of deficiencies modeling several human PRPS1-associated diseases. Sci. Rep. 6, 30488.
impairment locus heterogeneity and identification of PS1 as a new autosomal dominant gene in Hungarian Roma. Eur. J. Hum. Genet. 27, 89–94.

Scharf, M., Tejedor-Agulló, A., Takahashida, M.A., Picher, M.M., Sommen, M., Soeco, C.Z., Oonk, A.M., Dominguez-Ruiz, M., Draaisma, J.M., Gandia, M., Oonk, A., Neveling, K., Kunst, H.P., Hoefloot, L.H., del Castillo, I., Pennings, R.J., Kremers, H., Adriaas, R.J., Schraders, M., 2015. Psychological impairment and vestibular dysfunction caused by a homozygous nonsense mutation in Clic5. Eur. J. Hum. Genet. 23, 615–623.

Scott, H.S., Kudoh, J., Wattenhofer, M., Shibuya, K., Berry, A., Chrast, R., Guipponi, M., Slijkerman, R., Goloborodko, A., Broekman, S., de Vrieze, E., Hetterschijt, L., Sloan-Heggen, C.M., Bierer, A.O., Shearer, A.E., Kolbe, D.L., Nishimura, C.J., Frees, K.L., Ali, G., Ansar, M., Ghosh, M., Wilcox, E.R., Ahmad, W., Merlino, G., Leal, S.M., Riazuddin, S., Friedman, T.B., Morell, R.J., 2009. Nonsyndromic mutations of Clic5 are associated with nonsyndromic hearing loss, DFNB39. Am. J. Hum. Genet. 85, 1950–1954.

Shah, M.I., Ahmed, Z.M., Khan, S.Y., Riazuddin, S., Waryah, A.M., Khan, S.N., Seiler, C., Finger-Baier, K.C., Rinner, O., Makhankov, Y.V., Schwarz, H., Neuhauss, S.C., 2015. Mutations in alpha-tectorin gene cause autosomal dominant non-syndromic deafness at the DFNB16 locus. Nat. Genet. 29, 345–350.

Shankland, C.J., Renier, J., Botto, D.L., Pericak-Vance, M.A., Towbin, R.J., 1999. New mutation in DFNA5 associated with a mutation in DFNA5. Nat. Genet. 20, 194–197.

Shattuck, D.Y.R., Raz, E. Lawson, N.D., Eícker, S.C., Burdine, R.D., Eisens, J., Ingham, P.W., Schulte-Merker, S., Yelon, D., Weinstein, B.M., Mullins, M.C., Wilson, S.W., Ramakrishnan, L., Amacher, S.L., Neuhaus, S.C.F., Meng, A., Neely, D., Han, N., Panula, P., Ylikoski, R., Lunt, A.C., 2017. Guidelines for morpholine usage in zebrafish. PLoS Genet. 13, e1007000.

Stokeo-Vaughan, G.A., Obholzer, N.D., Baxendeg, S., Msegag, S.G., Whitfield, T.T., 2015. Otolith tethering in the zebrafish otic vesicle requires Otoegin and alpha-tectorin. Development 142, 3481–3494.

Thoenes, M., Zimmermann, U., Ebermann, I., Ptok, M., Lewis, M.A., Thiele, H., Morlot, S., Hess, M.M., Gal, A., Eisenberger, T., Bergmann, C., Nürnberg, G., Nürnberg, P., Steel, K.P., Knipper, M., Bolz, H.J., 2015. OSBPL2 encodes a protein of inner and outer hair cell stereocilia and is mutated in autosomal dominant hearing loss (DFNA67). Orphanet J. Rare Dis. 10, 15.

Tili, A., Mannikko, M., Charfedine, I., Lahmar, I., Ben Amor, M., Driss, N., Al-Akkoka, L., Dirira, M., Masmoudi, S., Aujadi, H., 2005. A novel autosomal recessive non-syndromic deafness locus, DFNB66, maps to chromosome 6p21:22–23.1 in a large Tunisian consanguineous family. Hum. Hered. 60, 123–128.

Vahava, G., Morell, R., Lynch, E.D., Weiss, S., Kagan, M.E., Abitun, N., Morrow, J.E., Le, M.K., Skvorsak, A.B., Morton, C.C., Blumenfeld, A., Miller, D., Friedman, T.B., King, M.C., Avarham, K.B., 1998. Mutation in transcription factor Pou4f3 associated with inherited progressive hearing loss in humans. Science 280, 752–754.

Van Laar, H., Huizing, E.H., Verstreken, M., van zuilen, D., Wauters, J.J.C., Bossuyt, P.J., Van de Heyning, P., McGuirt, W.T., Smith, R.J., Willems, P.J., Legan, P.K., Richardton, P.G., Van Camp, G., 1998. Nonsyndromic hearing impairment is associated with a mutation in DFNAS. Nat. Genet. 20, 194–197.

van Wijk, E., Krieger, E., Kempenner, M.H., De Leenerh, E.M., Huygen, P.L., Cremers, C.W., Cremers, F.P., Kemper, H., 2003. A mutation in the gamma actin 1 (ACTG1) gene causes autosomal dominant hearing loss (DFNA20/26). J. Med. Genet. 40, 878–884.

Vaske, K.H., Sood, R., Burgsma, S., 2015. Understanding and editing the zebrafish genome. Adv. Genet. 92, 1–52.

Vaske, K.H., Huang, H., Zhang, S., Lu, J., Gildea, D.E., Yang, Z., Wolffberg, T.G., Liu, K., Burgsma, S., 2014. RNA interference using the zebrafish larval eye in vivo: a gene-based, searchable collection of zebrafish mutations generated by DNA insertion. Nucleic Acids Res. 41, D681–D686.

Vaske, K.H., Pui, W., LaFayette, M.C., Idol, J., Xu, L., Gallardo, V., Carrington, B., Bishop, K., Jones, M., Li, M., Harper, U., Huang, S.C., Prakash, A., Chen, W., Sood, R., Ledin, J., Burgsma, S.M., 2015. High-throughput gene targeting and phenotyping in zebrafish using CRISPR/Cas9. Genome Res. 25, 1030–1042.

Vaske, K.H., Lu, J., Gildea, D.E., Huang, H., Liu, W., Zhang, S., Huang, S.C., Schoenfeld, D., Pho, N.H., Casero, D., Hiraie, T., Mosbrook-Davis, D., Zhang, S., Jao, L.E., Zhang, B., Woods, I.G., Zimmerman, S., Schier, A.F., Wolffberg, T.G., Pellegrini, M., Burgsma, S.M., Lin, S., 2013b. A large-scale zebrafish gene knockout resource for the genome-wide study of gene function. Genome Res. 23, 1733–1735.

Verhoeven, K., Van Laar, L., Kirschhofer, K., Legan, P.K., Hughes, D.C., Schatteman, I., Van de Heyning, P., Van Wijk, E., Van Hauve, M., Capic, P., Chen, A., Smith, R.J., Somers, R., Officer, E.E., Van de Heyning, P., Richardton, G.P., Wachtler, F., Kerburching, W.J., Willemen, P.V., Pui, W., Legan, P.K., Verstreken, M., 2014. Mutations in the human alpha-tectorin gene cause autosomal dominant non-syndromic hearing impairment. Nat. Genet. 46, 60–62.

Verpy, E., Masmoudi, S., Zwaenepoel, I., Lebovici, M., Hutchin, T.P., Del Castillo, I., Nouaille, S., Blanchard, L., Saine, P., Popot, J.L., Moreno, F., Mueller, R.F., Petit, C., 2001. Mutations in a new gene encoding a protein of the hair bundle cause nonsyndromic deafness at the DFNB16 locus. Nat. Genet. 29, 345–349.

von Ameln, S., Wang, G., Boulouiz, R., Rutherford, S., Smith, C.M., Li, Y., Pogoda, H.-M., Nürnberg, G., Stiller, B., Börck, G., Hongs, J.G., Goodyear, R.J., Abidi, O., Nürnberg, P., Hofmann, K., Richardton, G.P., Hammerschmidt, M., Moser, T., Wollnik, B., Koehler, C.M., Tettelt, M.A., Bader, A., Kubsch, C., Hohenthal, C., Mutat. in PNP71, encoding mitochondrial RNA-import protein RNPase, causes hereditary hearing loss. Am. J. Hum. Genet. 91, 919–927.

Vona, B., Nanda, I., Neuner, C., Muller, H., Taaf, H., 2013. Confirmation of GRHL2 as the gene for the DFNA28 locus. Am. J. Med. Genet. 161A, 2090–2095.

Vona, B., Nanda, I., Hofrichter, M.A., Shehata-Dieter, W., Taaf, H., 2015. Non-syndromic hearing loss gene identification: a brief history and glimpse into the future. Mol. Cell. Probes 29, 260–270.

Vonk, M.H., Richter, M.A.H., Crosby, A.H., Nanda, I., Haaf, T., 2016. Genetic elucidation of nonsyndromic hearing loss in the high-throughput sequencing era. In: Vona, B., Haaf, T. (Eds.), Genetics of Deafness, vol. 20. Monogr Hum. Genet. Basel, Karger, pp. 56–72. https://doi.org/10.1159/000445153.

Walsh, T., Walsh, V., Vreugde, S., Hertzano, R., Shahin, H., Haika, S., Lee, M.K., Maman, K., King, M.C., Avarham, K.B., 2002. From flies’ eyes to our ears: mutations in a human class III myosin cause progressive nonsyndromic hearing loss DFNB6. Proc. Natl. Acad. Sci. U. S. A 99, 7128–7132.
Wibowo, I., Pinto-Teixeira, F., Satou, C., Higashijima, S., Lopez-Schier, H., 2011. A rapid method for systematic identification of zebrafish mutants affecting development of the zebrafish inner ear. Dev. Cell 21, 766.

Wu, R.S., Lam II, Clay, H., Duong, D.N., Deo, R.C., Coughlin, S.R., 2018. A rapid method for systematic identification of zebrafish mutants affecting development of the zebrafish inner ear. Dev. Cell 21, 766.

Xia, J.H., Liu, C.Y., Tang, B.S., Pan, Q., Huang, L., Dai, H.P., Zhang, B.R., Xie, W., Hu, D.X., Shuai, K., Zhou, Y., Zhang, J., Qi, H., Lai, L., Chen, Z., Yan, H., Zhao, S., Chen, X., Gao, F., Wang, Y., Yan, J., Zhou, Y., Zhao, Z., 2017. Development of the zebrafish model organoid system and their applications. Nat. Methods 14, 399.

Wilcox, E.R., Burton, Q.L., Naz, S., Riazuddin, S., Smith, T.N., Ploplis, B., Belyantseva, I., Wierson, W.A., Welker, J.M., Almeida, M.P., Mann, C.M., Webster, D.A., Weiss, T.J., 2015. Identification of OSBPL2 as a novel candidate gene for progressive nonsyndromic hearing loss by whole-exome sequencing. Genet. Med. 17, 45--51.

Yan, D., Zhu, Y., Walsh, T., Xie, D., Yuan, H., Sirmaci, A., Fujiwaka, T., Wong, A.C., Lathrop, M., Richardson, G., Petit, C., 2002. Otoancorin, an inner ear protein otogelin-like, causes moderate sensorineural hearing loss. Am. J. Hum. Genet. 70, 529--540.

Yousaf, R., Gu, C., Ahmed, Z.M., Khan, S.N., Friedman, T.B., Riazuddin, S., Shears, S.B., Riazuddin, S., 2018a. Mutations in Diphosphoinositol-Pentakisphosphate Kinase 3 (PLKIP) are associated with hearing loss in man and mouse. PLoS Genet. 14, e1007297.

Yousaf, R., Gu, C., Ahmed, Z.M., Friedman, T.B., Riazuddin, S., Shears, S.B., Riazuddin, S., 2018b. Modifier variant of MTM1 suppresses human GAR1-associated profound deafness. J. Clin. Invest. 128, 1509--1522.

Zaro, S., Cerrado, C., Scarfi, L., Liu, X., Liao, S., Liu, X.Z., Pennings, R.J., 2018. A dominant variant in the PDE1C gene is associated with nonsyndromic hearing loss. Hum. Genet. 137, 437--446.

Zaslaw, A., Rice, D., Zhang, F., Li, Z., Meltzer, D., DeLuca, A.P., Jang, Q., 2011. Analysis of unconventional myosin MYO15 mutations with human nonsyndromic deafness DFNB3. Science 280, 1447--1450.

Zhang, J., Millar, J., 2001. Mutations in the gamma-actin gene cause autosomal-recessive hereditary hearing loss. Hum. Mutat. 38, 1421--1428.

Zhang, Z., Tong, X., Wang, Z., Du, L., Yang, J., Wang, X., Li, Y., Cao, X., 2015. A novel association of OSBP2L with progressive nonsyndromic hearing loss. Nat. Genet. 47, 1152--1157.

Zheng, J., Miller, K.K., Yang, T., Hildebrand, M.S., Shears, S.B., deGroot, J., Riazuddin, S., 2018a. Mutations in Diphosphoinositol-Pentakisphosphate Kinase 3 (PLKIP) are associated with hearing loss in man and mouse. PLoS Genet. 14, e1007297.

Zheng, J., Miller, K.K., Yang, T., Hildebrand, M.S., Shears, S.B., deGroot, J., Riazuddin, S., 2018b. Modifier variant of MTM1 suppresses human GAR1-associated profound deafness. J. Clin. Invest. 128, 1509--1522.

Zhu, M., Yang, T., Wei, S., DeWan, A.T., Morell, R.J., Elfenbein, J.L., Fisher, R.A., Wilcox, E.R., Burton, Q.L., Naz, S., Riazuddin, S., 2015. Mutations in apoptosis-regulating genes causes autosomal dominant non-syndromic hearing loss. Clin. Genet. 90, 308--311.

Zou, B., Desmitta, A.A., Mittal, R., Yan, D., Richmond, M., Tekin, M., Liu, X., Lu, L., 2019. The generation of zebrafish mariner model using the CRISPR/Cas9 system. Anat Rec (Hoboken). https://doi.org/10.1002/ar.24331.