Cilia-Associated Genes Play Differing Roles in Aminoglycoside-Induced Hair Cell Death in Zebrafish

Tamara M. Stawicki,*† Liana Hernandez,*† Robert Esterberg,†‡ Tor Linbo,* Kelly N. Owens,*†‡ Arish N. Shah,* Nihal Thapa,* Brock Roberts,*‡ Cecilia B. Moens,§ Edwin W. Rubel,*†§ and David W. Raible*†‡

*Department of Biological Structure, †Virginia Merrill Bloedel Hearing Research Center, and ‡Department of Otolaryngology, Head and Neck Surgery, University of Washington, Seattle, Washington, 98195, and §Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington, 98109

ABSTRACT Hair cells possess a single primary cilium, called the kinocilium, early in development. While the kinocilium is lost in auditory hair cells of most species it is maintained in vestibular hair cells. It has generally been believed that the primary role of the kinocilium and cilia-associated genes in hair cells is in the establishment of the polarity of actin-based stereocilia, the hair cell mechanotransduction apparatus. Through genetic screening and testing of candidate genes in zebrafish (Danio rerio) we have found that mutations in multiple cilia genes implicated in intraflagellar transport (dync2h1, wdr35, ift88, and traf3ip), and the ciliary transition zone (cc2d2a, mks1, and cep290) lead to resistance to aminoglycoside-induced hair cell death. These genes appear to have differing roles in hair cells, as mutations in intraflagellar transport genes, but not transition zone genes, lead to defects in kinocilia formation and processes dependent upon hair cell mechanotransduction activity. These mutants highlight a novel role of cilia-associated genes in hair cells, and provide powerful tools for further study.

Cilia are microtubule-based projections located on the apical surface of cells. One class of cilia found on the majority of cells in multicellular organisms, called primary cilia, are nonmotile and important for cellular signaling (Satir and Christensen 2007; Berbari et al. 2009). Cilia are formed and maintained through regulated microtubule-dependent protein trafficking along their length, a process known as intraflagellar transport (IFT). Anterograde IFT, transport from the cell body to the ciliary tip, depends on kinesin-2 motors and the IFT-B complex of adapter proteins. Retrograde IFT, transport from the tip back to the base, depends on dynein-2 motors and the IFT-A complex of adaptor proteins (Scholey 2003; Pedersen et al. 2006). The entry and exit of protein into the cilia is further controlled by a group of proteins localized at the cilia base, in a region known as the transition zone, that act as a molecular gate (Reiter et al. 2012; Blacque and Sanders 2014).

Mutations in cilia-associated genes lead to a broad spectrum of diseases known as ciliopathies. These diseases affect a range of organ systems with different symptoms associated with each disease. In some cases, a single gene is implicated in multiple ciliopathies (Badano et al. 2006; Waters and Beales 2011). Mutations in IFT genes frequently lead to skeletal ciliopathies characterized by a shortening of bones and, in some cases, polydactyly (Huber and Cormier-Daire 2012). Mutations in transition zone genes are frequently seen in ciliopathies associated with renal disease and/or retinal degeneration (Czarnecki and Shah 2012). In a subset of cases, including those resulting in Alström Syndrome, Bardet-Biedl Syndrome, Usher Syndrome, and autosomal recessive deafness DFNB66, mutations in cilia-associated genes have been shown to cause hearing loss (Ross et al. 2005; Adams et al. 2007; Grati et al. 2015).

Hair cells, the sensory cells of the auditory and vestibular systems, contain a single primary cilium known as the kinocilium. Auditoriy hair
cells of many species lose their kinocilia during development whereas
kinocilia are maintained in vestibular hair cells (Tanaka and Smith 1978;
Lim and Anniko 1985; Ernstson and Smith 1986). The apical surfaces
of hair cells also contain actin-based stereocilia that develop in rows,
in order of ascending height. The stereocilia gate ions from the sur-
rounding medium in response to mechanical stimuli, thus activating
hair cells through a process known as mechanotransduction
(Schwander et al. 2010). The kinocilium is adjacent to the tallest stereo-
cilia row. While kinocilia may play a role in hair cell activity at early
stages (Kindt et al. 2012), mechanotransduction activity in mature hair
cells is entirely due to the stereocilia (Hudspeth and Jacobs 1979). It is
generally believed that the primary role of the kinocilium and cilia-
associated genes in auditory hair cells is in the establishment of stereo-
cilia polarity (Ross et al. 2005; Jones et al. 2008), a process also known
to be dependent upon the planar cell polarity pathway (Montcouquiol
et al. 2003; Wang et al. 2005, 2006).

However, there are reasons to believe that cilia-associated genes may
be playing additional roles in hair cells. In mice deficient in the ciliary
basal body gene responsible for Alström syndrome, Alms1, defects in
stereocilia morphology are present early in development, however,
hearing loss shows a delayed onset. This suggests the stereocilia mor-
phology defects are not solely responsible for hearing loss (Jagger
et al. 2011). Recent evidence has shown that genes that are traditionally
thought of as cilia genes can also have cellular functions independent
of the cilium. IFT genes have been implicated in protein trafficking and
cytoskeletal organization in nonciliated cells (Finetti et al. 2009; Delaval
et al. 2011; Cong et al. 2014), and mutations in cilia-associated genes
have been shown to lead to increased DNA damage (Choi et al. 2013;
Slaats et al. 2015). This leaves open possible roles for cilia-associated
genes in hair cells after the developmental loss of the kinocilium.
Indeed, a number of cilia-associated genes remain expressed in mamma-
lian auditory hair cells after kinocilium loss (Liu et al. 2008).

It is well known that aminoglycoside antibiotics cause hearing loss
and vestibular dysfunction in human patients (Moore et al. 1984; Lerner
et al. 1986). Through genetic screening for modulators of sensitivity to
aminoglycoside exposure, using the zebrafish lateral line system, we
have discovered that mutations in multiple cilia-associated genes lead
to resistance to aminoglycoside-induced hair cell death. We have pre-
viously reported that a mutation in the cilia transition zone gene
cc2d2a leads to resistance to the aminoglycoside neomycin (Owens
et al. 2008). As stereocilia morphology appears grossly normal
in these mutants, we believe that this work reveals a novel role for cilia-
associated genes in auditory hair cells. It also suggests that antibiotic resistance
may be a useful phenotype for discovering other cilia-related genes that
play a role in mature hair cells, while working toward uncovering the
full role of cilia-associated genes in regulating hair cell structure and
function.

MATERIALS AND METHODS
Animals
All experiments used 5 d post-fertilization (dpf) Danio rerio (zebrafish)
larvae, unless otherwise noted. Mutant alleles were maintained as Het-
eozygotes in the “AB wild-type strain, and experiments were carried

![Figure 1](image-url)

**Figure 1** Identification of mutations in cilia-associated genes that confer resistance to aminoglycoside-induced hair cell death. (A) Two mutations have been identified in the cc2d2a gene. One mutation (w38) (W628X; Owens et al. 2008) causes a premature stop codon in a coiled-coil (CC) domain. The other (w123) leads to the retention of the intron between exons 20 and 21 causing a frameshift. Both pink boxes are putative CC domains. (B) Top: neomycin-resistant mutant allele w46 mapped to a re-
gion of approximately 0.5 Mb on chromosome 15. The microsatellite markers used for mapping are shown, as well as the number of recombi-
nant animals at each position. Bottom: sequencing of the
cc2d2aw46 gene contained in this region identified a G to A nucleic acid change in mutants at residue 1452, leading to a premature stop codon in the N-terminus of the protein (W484X). All green boxes represent AAA domains (ATPases Associated with diverse cellular Activities). (C) Top: neomycin-resistant mutant allele w150 mapped to a region of approximately 2.3 Mb on chromosome 13. The microsatellite markers used for mapping are shown, as well as the number of recombinant animals at each position. Bottom: sequencing of the wdr35 gene contained in this region identified a T to A nucleic acid change in mutants at residue 2426, leading to a premature stop codon just upstream of the tetratricopeptide repeat TPR-Like domain (L809X). (D and E) Frameshift mutations were generated in the N-terminus of both Cep290 (D) and Mks1 (E) using genome editing techniques. The cep290 mutation was a 2 bp deletion, resulting in a frameshift and trun-
cated coding sequence after A99, whereas the mks1 mutation was a 25 bp insertion causing a frameshift and truncated coding sequence after S61. Due to the large range of sizes, the individual protein images are not to the same scale. DHC, dynein heavy chain domain.
**Table 1 Mutations in numerous cilia genes leads to resistance in neomycin-induced hair cell death**

| Gene               | Control 200 µM Neomycin | % HCs Remaining | Mutant 200 µM Neomycin | % HCs Remaining |
|--------------------|-------------------------|-----------------|------------------------|-----------------|
| Ciliary axoneme and intraflagellar transport |                      |                 |                        |                 |
| dyn2ch1 (w146)     | 12.13 ± 1.15            | 1.05 ± 0.33     | 8.65 ± 1.27            | 5.63 ± 1.26     |
| wdr35 (w150)       | 11.37 ± 0.92            | 1.84 ± 0.63     | 16.18 ± 0.0001         | 6.17 ± 1.41     |
| ift88 (ts288)      | 12.38 ± 1.78            | 1.17 ± 0.71     | 9.42 ± 0.0002          | 4.43 ± 1.70     |
| traf3ip1 (tp49d)   | 11.6 ± 1.43             | 1.08 ± 0.55     | 9.34 ± 0.0001          | 4.78 ± 0.69     |
| arl13b (h459Tg)   | 11.78 ± 1.54            | 1.65 ± 0.85     | 14.00 ± 0.0001         | 1.30 ± 0.37     |
| Transition zone    |                        |                 |                        |                 |
| cc2d2a (w38)       | 11.10 ± 1.72            | 1.53 ± 0.69     | 13.78 ± 0.0007         | 3.68 ± 0.47     |
| cc2d2a (w123)      | 12.78 ± 2.5             | 1.17 ± 0.38     | 9.13 ± 0.0097          | 3.05 ± 0.48     |
| mks1 (w152)        | 12.34 ± 0.86            | 1.26 ± 0.46     | 10.21 ± 0.0097         | 2.88 ± 0.65     |
| cep290 (h378)      | 12.30 ± 1.02            | 1.44 ± 0.34     | 11.70 ± 0.0678         | 2.24 ± 0.88     |

Numbers are average number of hair cells/neuromast ± SD. Significance was calculated using an ANOVA (analysis of variance) and Sidak multiple comparisons test. n = 10 for dyn2ch1, ift88, traf3ip, arl13b, cc2d2aw38, and cc2d2aw123; n = 16 for cep290; n = 31–37 for wild-type siblings, and n = 8–14 for mutants of wdr35 and mks1. HCs, hair cells.

cc2d2a and dyn2ch1 mutants can be consistently identified by their sinusoidal body shape (Owens et al. 2008; Ryan et al. 2013), whereas arl13b, ift88, and traf3ip mutants had a c-shaped body (Tsujikawa and Malicki 2004; Omori et al. 2008; Duldulao et al. 2009). For mks1w152 and wdr35w150, genotyping was used to distinguish between mutants and wild-type siblings. For the cep290h378 mutant strain, genotyping was always performed to confirm animals as wild-type siblings or mutants; however, in some experiments, animals were sorted based on body morphology. Genetic mapping used the Wik strain. Larvae were raised in embryo media (EM) consisting of 1 mM MgSO4, 150 mM K2HPO4, 42 mM Na2HPO4, 1 mM CaCl2, 500 mM KCl, 15 mM NaCl, and 714 µM NaHCO3. The University of Washington Institution Animal Care and Use Committee approved all experiments. Mutant strains used in this study are available upon request.

**Genetic screening**

F2 mutant families were generated as previously described (Owens et al. 2008). To screen F2 families, adult pairs were incrossed and 15–50 offspring per pair were treated with 200 µM neomycin at 5 dpf. Fish were screened for neomycin resistance using the vital dye DASPEI [2-(4-dimethylaminostyryl)-N-ethylpyridinium iodide] (Molecular Probes), and the numbers of animals per clutch showing either a typical or atypical neomycin response were counted. Pairs where approximately one-quarter of all animals showed an atypical response were rescanned to confirm the phenotype and then outcrossed with *AB* wild-type fish. Approximately 500 F2 families were screened with at least 4–6 pairs screened per family when possible.

**Genetic mapping**

The molecular bases of mutations first found by phenotypic screening were identified by genetic mapping using the polymorphic Wik strain. Hybrid *AB/Wik* mutant carriers were intercrossed to generate progeny for linkage marker analysis. Mutant and wild-type fish were selected based on susceptibility to 200 µM neomycin. Microsatellite markers for each chromosome (Knappik et al. 1998; Shimoda et al. 1999) were amplified by PCR and tested for cosegregation with mutant phenotypes. Pools of 20 mutants and 20 wild-type siblings were used for bulk segregant analysis. Markers cosegregating with the mutant allele were further evaluated with individual DNA from 213 mutants and 16 wild-type siblings for the dyn2ch1w146 mutation, and 508 mutants and 73 wild-type siblings for the wdr35w150 mutation. In addition to the existing microsatellite markers, candidate simple sequence repeat (SSR) marker primer pairs were generated using the Zebrafish Genome SSR search website (Massachusetts General Hospital, Charlestown MA 02129; World Wide Web URL: http://danio.mgh.harvard.edu/chrMarkers/zfssr.html). To sequence candidate genes following linkage mapping, RNA was isolated from pools of 20 wild-type sibling or mutant embryos using TRizol Reagent (Ambion), and cDNA was prepared using SuperScript III Reverse Transcriptase (Invitrogen). Genes were amplified by PCR from the resultant cDNA and then sent to Eurofins MWG Operon for sequencing.

**CRISPR and TALEN mutagenesis**

To generate mks1 mutants, guide RNA (gRNA) was designed to two different target sites: 5'-GGAGGCCGTCTGAGTGGCTGA-3' and 5'-GTGTGATACTGAGCTGTCG-3'. Targets were selected using the design tool at http://crispr.mit.edu. Cas9 mRNA and gRNA were synthesized as previously described (Shah et al. 2015). Embryos were injected with 1 nl of a solution containing 200 ng/µl of Cas9 mRNA and 50 ng/µl each of the two gRNAs. Transmission of a genetic change at the gRNA target site was screened for by performing a PCR using primers flanking the target and running the product on a 3% lithium borate gel (Brody et al. 2004) to look for size changes. Experiments were
performed on larvae in the F3 generation containing a 25 bp insertion at the first target site (Supplemental Material, Table S1).

To generate cep290 mutants, a pair of TALENs was designed for exon 6 of the cep290 coding sequence. The first TALE binds the sequence 5’-CTGCCTCACTTCGCTCCA-3’ and the second binds the sequence 5’-TTGTCCTCCCTCCCATCA-3’. TALENS were generated as previously described (Sanjana et al. 2012). TALEN mRNA was generated and polyadenylated using the T7 ULTRA Transcription Kit (Ambion), and RNA was purified using a standard phenol-chloroform extraction. Zebrafish embryos were injected at the one-cell stage with 200 pg of each TALEN mRNA. F0 founders of fh378 were identified and F1 heterozygotes were raised to establish the mutant strain.

Neomycin treatment
Fish were treated with the designated concentration of neomycin (Sigma-Aldrich) dissolved in EM for 30 min at 28.5°C. They were then washed three times in EM, and left to recover for 1 hr. At the end of recovery, animals used for hair cell counts were fixed for immunostaining. Hair cells were counted in the OP1, M2, IO4, O2, MI2, and MI1 neuromasts (Raible and Kruse 2000). All hair cell number and neomycin response data were obtained through hair cell counts. For hair cell data presented as the percentage of controls, the average number of hair cells/neuromast was calculated for each individual fish by dividing the total number of hair cells counted per fish by the number of neuromasts analyzed. This number was then divided by the average number of hair cells/neuromast for control animals, and expressed as a percentage. Fish that were not treated with neomycin were used as controls. Animals used for genetic mapping were screened for neomycin resistance using the vital dye DASPEI (Molecular Probes). They were exposed to DASPEI at a final concentration of 0.05% for 15 min. Neuromasts SO1, SO2, IO1, IO2, IO3, IO4,

Figure 2 Mutations in intraflagellar transport (IFT) genes lead to resistance to neomycin-induced hair cell death across a range of neomycin doses. Mutants of the retrograde IFT genes dyn2ch1 (A) and wdr35 (B), as well as the anterograde IFT genes ift88 (C) and traf3p (D), show strong resistance to neomycin-induced hair cell death across a range of doses. Dose response curves for both wild-type siblings and mutants were independently normalized to either the wild-type sibling or mutant group of control fish not treated with neomycin. Data are displayed as mean ± SD. n = 10 fish for dyn2ch1, ift88, and traf3p. n = 10–20 for wdr35. *** P < 0.00001 by two-way ANOVA and Sídák multiple comparisons test. Mut, mutant; WT Sib, wild-type sibling.

Figure 3 Mutations in transition zone genes lead to resistance to neomycin-induced hair cell death across a range of neomycin doses. Mutants of the transition zone genes cc2d2a (A), mks1 (B), and cep290 (C) show moderate resistance to neomycin-induced hair cell death. Dose response curves for both wild-type siblings and mutants were independently normalized to their no neomycin control hair cell numbers. Data are displayed as mean ± SD. n = 10 fish for cc2d2a, n = 11–22 for cep290, and n = 8–14 for mks1. ** P < 0.01, *** P < 0.001, and **** P < 0.00001 by two-way ANOVA and Sídák multiple comparisons test. Mut, mutant; WT Sib, wild-type sibling.
Figure 4  cc2d2a and cep290 mutations do not show synergistic interactions. Hair cells per neuromast following 200 μM neomycin in animals with various combinations of cc2d2a and cep290 mutant alleles. There does not appear to be any genetic interaction between the two mutant alleles. + is a wild-type allele, and − is a mutant allele. n = number of fish for each group. Data are displayed as mean ± SD.

O2, M2, MI1, and MI2 were scored as previously described (Harris et al. 2003). Animals with a score of eight or higher were considered resistant.

**Immunohistochemistry**

Fish used for immunohistochemistry were fixed for 2 hr at room temperature in 4% paraformaldehyde. Antibody labeling was carried out as previously described (Stawicki et al. 2014). Fish used for hair cell counts were labeled with a mouse anti-parvalbumin primary antibody (Millipore, MAB1572) diluted at 1:500 in antibody block and 0.2% BSA. Fish used for the analysis of cilia morphology were fixed in Alexa Fluor 488 Phalloidin (Molecular Probes) diluted 1:100 in antibody block, incubated for 2 hr at room temperature, washed three times in EM and anesthetized with MS222 for imaging. Images were obtained and analyzed using SlideBook software on a Maria-nas spinning disk confocal system (Intelligent Imaging Innovations). For each animal, a single neuromast was imaged and analyzed. A stack of 30 1-μm optical sections was obtained and maximum projection images were analyzed. The average fluorescence intensity of the cell bodies of the neuromast was calculated and divided by the background fluorescence of the image. The fluorescence measurements of mutant neuromasts were then normalized to the average fluorescence intensity of wild-type sibling controls imaged on the same day with the same imaging parameters.

**Data availability**

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

**RESULTS**

**Mutations in cilia-associated genes lead to resistance to neomycin-induced hair cell death**

To identify novel genes involved in aminoglycoside-induced hair cell death we performed a genetic screen looking for mutants resistant to the effects of exposure to neomycin. Through this screen we identified two mutants, w46 and w123, that showed a sinusoidal body pattern similar to that previously seen in the sentinel mutant (cc2d2aw46; Owens et al. 2008). Through complementation testing we found that the w123 allele, but not the w46 allele, failed to complement cc2d2aw46. Sequencing showed that the intron between exons 20 and 21 is retained in cc2d2a cDNA isolated from the w123 mutant, leading to a frame shift in the protein (Figure 1A and Table S1).

Genetic mapping of the w46 allele localized the mutation to chromosome 15 (Figure 1B). The body morphology of w46 mutants is similar to a previously reported dyn2c1h1 mutant (Ryan et al. 2013), one of the genes in the mapped interval. Dyn2c1h1 is believed to be the motor protein responsible for retrograde IFT, the trafficking of protein from the tip to the base of cilia (Pazour et al. 1999; May et al. 2005). Sequencing of dyn2c1h1 cDNA in w46 mutants identified a premature stop codon in the 5’ region of the gene (Figure 1B and Table S1).

An additional mutant isolated in this screen, w150, had a grossly normal body morphology; genetic mapping localized the mutation to a region on Chromosome 13 containing the retrograde IFT gene wdr35 (Figure 1C). Wdr-35 is part of the IFT-A complex, a group of proteins believed to serve as adaptors between the cargo and motors in retrograde IFT (Cole 2003; Mukhopadhyay et al. 2010). Sequencing of wdr35 cDNA in the w150 mutant strain identified a premature stop codon (Figure 1C and Table S1).

As three mutations identified in our screen are all implicated in cilia function, we tested additional mutations in cilia-associated genes for resistance to neomycin. We obtained existing mutations in the cilia gene arl13b (Golling et al. 2002), and anterograde IFT genes ift88 (Tsuji-kawa and Malicki 2004) and traf3ip (Omori et al. 2008). Arl13b localizes to the cilia axonemal region and is believed to play roles in cilia formation and Sonic hedgehog signaling (Sun et al. 2004; Casparry et al. 2007; Duldulao et al. 2009; Larkins et al. 2011). Ift88 and Traf3ip are components of the IFT-B complex, the protein adaptors for anterograde IFT, and are also required for cilia formation (Pazour et al. 2000; Cole 2003; Kunitomo and Iino 2008; Berbari et al. 2011). Additionally, we used genome editing techniques to generate new mutations in the transition zone genes mks1 and cep290 (Figure 1D, E and Table S1). Mks1 and Cep290 are both part of a large complex of proteins implicated in human ciliopathies localized to the transition zone of cilia (Garcia-Gonzalo et al. 2011; Chih et al. 2012). Cep290 has also been shown to interact with Cc2d2a (Gorden et al. 2008; Garcia-Gonzalo et al. 2011), and is believed to bridge the basal body and transition zone (Yang et al. 2015).

We found that all IFT and transition zone genes tested showed resistance to neomycin-induced hair cell death. In control animals, approximately 9–14% of hair cells remained in each neuromast after treatment with 200 μM neomycin. In IFT mutants, these numbers were increased to 48–70% (Table 1), with significant protection seen across the range of neomycin doses from 50–400 μM (Figure 2). Transition zone mutants showed slightly less resistance to neomycin-induced hair cell death than IFT mutants. cc2d2a and mks1 mutants had 24–30% hair cells remaining following treatment with 200 μM neomycin, and cep290 mutants had 18% remaining (Table 1). cc2d2a and mks1 mutants additionally showed significant protection across a range of neomycin doses from 50–400 μM, whereas protection in cep290 mutants was limited to 100 and 200 μM neomycin (Figure 3). arl13b mutants failed to show any
IFT mutants, we generated death, potentially resulting in a level of resistance like that seen in gism was seen in terms of resistance to neomycin-induced hair cell protection against neomycin-induced hair cell death (Table 1). IFT mutants, but not transition zone mutants, additionally showed a decrease in control hair cell numbers as compared to their wild-type siblings (Table 1). Decreases in hair cell number have previously been shown in the inner ear of ift88 zebrafish mutants (Tsujikawa and Malicki 2004).

It has previously been reported that there was a synergistic genetic interaction when cc2d2a<sup>wt/wt</sup> mutants were injected with cep290 morpholino oligonucleotides (Gorden et al. 2008). To test if similar synergism was seen in terms of resistance to neomycin-induced hair cell death, potentially resulting in a level of resistance like that seen in IFT mutants, we generated cc2d2a, cep290 double mutants. We found that the presence of either one or two copies of the other gene’s mutant allele had no additional synergistic effect on the amount of neomycin resistance seen in cc2d2a or cep290 homozygous mutants (Figure 4), or on the number of hair cells under control conditions (data not shown).

**Kinocilia morphology is disrupted in IFT but not transition zone mutants**

To test what effects cilia gene mutations had on kinocilia morphology in hair cells, we stained mutants with acetylated tubulin. It has been shown previously that zebrafish ift88 and traf3ip mutants show a loss of kinocilia (Tsujikawa and Malicki 2004; Omori et al. 2008). We confirmed these observations, and additionally saw a loss of kinocilia at 5 dpf in the retrograde IFT mutants dyn2h1 and wrd35 (Figure 5A). Hair cells in arl13b mutants maintain their kinocilia at 5 dpf, although in some cases the kinocilia appear shortened as compared to wild-type siblings (Figure 5A). These observations are in agreement with previous work that has shown kidney ciliogenesis differs between IFT and arl13b mutants (Caspy et al. 2007; Duldulao et al. 2009). In contrast, all transition zone mutants we tested showed grossly normal kinocilia morphology (Figure 5B).

**Zebrafish cilia gene mutations show a delayed effect on hair cells**

Cilia-associated genes are believed to play a role in the development of stereocilia polarity in mammalian auditory hair cells (Ross et al. 2005; Jones et al. 2008), but not vestibular hair cells (Sipe and Lu 2011). Previous studies have also shown that stereocilia morphology of lateral line hair cells is grossly normal in zebrafish ift88 mutants (Kindt et al. 2012). We examined stereocilia morphology in dyn2h1 and cc2d2a mutants using phalloidin labeling to visualize the actin-based stereocilia. In both cases we observed no obvious defects (Figure 6A), suggesting that gross defects in stereocilia formation are not the underlying cause for aminoglycoside-resistance in these mutants.

Defects in kinocilia morphology and inner ear hair cell number get worse with age in zebrafish ift88 mutants (Tsujikawa and Malicki 2004). To test if this is also true for other cilia mutants, we examined dyn2h1 mutants at additional developmental time points. While kinocilia are never observed in 5 dpf dyn2h1 mutants, grossly normal appearing kinocilia were present in a subset of 3 dpf mutants (Figure 6B). Additionally, we found that at 3 and 4 dpf, unlike at latter ages, there was no significant decrease in control hair cell numbers between dyn2h1 mutants and wild-type siblings (Figure 6C). This delayed onset in defects could be due to maternal expression of cilia-associated genes, and may explain why stereocilia are able to develop normally. Maternal expression of cilia-associated genes is frequently observed in zebrafish (Huang and Schier 2009; Duldulao et al. 2009; Cao et al. 2010). We find that dyn2h1 mRNA is present in 2–4 cell zebrafish embryos, as assayed by RT-PCR (data not shown), supporting the notion that dyn2h1 is maternally expressed.

**IFT but not transition zone mutants show decreased mechanotransduction-dependent FM1-43 and neomycin loading**

Previous mutants identified through genetic screening for resistance to neomycin-induced hair cell death have shown defects in hair cell loading of FM1-43 and aminoglycosides (Hailey et al. 2012; Stawicki et al. 2014). However, aminoglycoside loading appeared grossly normal in cc2d2aw<sup>38</sup> mutants (Owens et al. 2008). To test whether neomycin loading was altered in cilia mutants, we treated fish with neomycin that was covalently labeled with the fluorophore Texas Red (neomycin-TR),.
and quantified the fluorescent signal in the cell body of neuromast hair cells. We found significant decreases in neomycin-TR loading in all four IFT gene mutants tested, although the decrease was less dramatic in wdr35 mutants than other mutants (Figure 7A). Mutations in cc2d2a, mks1, and cep290 do not result in a significant decrease in neomycin-TR loading (Figure 7B).

The loading of aminoglycosides into hair cells requires mechanotransduction activity (Gale et al. 2001; Marcotti et al. 2005; Alharazneh et al. 2011). To begin understanding why aminoglycoside uptake was diminished in IFT mutants, we examined the rapid uptake of the vital dye FM1-43. Previous work has shown that rapid uptake of FM-143 also requires mechanotransduction activity (Seiler and Nicolson 1999; Gale et al. 2001; Meyers et al. 2003). We found that mutations in both anterograde IFT genes ift88 and traf3p, as well as the retrograde IFT motor gene dynch21, lead to a significant decrease in FM1-43 loading; however, a mutation in the retrograde IFT adaptor gene wdr35 had no effect on FM1-43 loading (Figure 7C). Mutations in the transition zone genes cc2d2a, mks1, and cep290 also had no effect on FM1-43 loading (Figure 7D) as predicted from the neo-TR results (Figure 7B). These results show that while both IFT and transition zone genes play a role in aminoglycoside-induced hair cell death, their mechanisms of action in hair cells differ.

**DISCUSSION**

Cilia-associated genes have long been believed to primarily function in hair cells during development. Here, we have shown that multiple classes of cilia-associated genes play a role modulating the sensitivity of hair cells to aminoglycosides. Mutations in the anterograde IFT adaptor molecule genes ift88 and traf3p, as well as the gene for the retrograde IFT motor dynch21, all lead to a decrease in control hair cell numbers, a strong resistance to neomycin-induced hair cell death, loss of kinocilia, and a decrease in hair cell loading of both FM1-43 and neomycin-TR, two processes known to be mechanotransduction-dependent. The later suggesting defects in mechanotransduction itself. A mutation in the retrograde IFT adaptor gene, wdr35, also caused a decrease in control hair cell numbers, a loss of kinocilia, and a comparable level of protection against neomycin-induced hair cell death as other IFT mutants. However, uptake of FM1-43 was not decreased in this mutant and the decrease in neomycin-TR loading was not as striking. There is precedence for dynch21 mutants showing different phenotypes than mutants in IFT-A complex genes (Ocbina et al. 2011; Liem et al. 2012), and this may be due to cargo differences between the different IFT-A adaptor molecules (Mukhopadhyay et al. 2010).

Mutations in the transition zone genes cc2d2a, mks1, and cep290 lead to significant, but much more moderate, resistance in neomycin-induced hair cell death. These mutants did not have significant defects on control hair cell number, kinocilia morphology, or uptake of FM1-43 and neomycin-TR. Cc2d2a and Mks1 are believed to be in a complex at the cilia transition zone and to function similarly (Williams et al. 2011; Garcia-Gonzalo et al. 2011; Chih et al. 2012). In agreement with this observation, we see nearly identical levels of protection against neomycin-induced hair cell death in these two mutants, and a similar slight but insignificant reduction in neomycin-TR loading into hair cells. Cep290 has also been shown to interact with Cc2d2a (Gorden et al. 2008; Garcia-Gonzalo et al. 2011); however, it is believed to occupy a different region of the transition zone (Yang et al. 2015). The neomycin resistance we see in cep290 mutants is not as strong as the resistance seen in cc2d2a or mks1 mutants, suggesting that cep290 functions slightly differently than the other two transition zone genes.

There have been conflicting reports on the role of transition zone genes in cilia formation. Cep290, Cc2d2a, and Mks1 have been implicated in ciliogenesis of cultured cells and a subset of ciliated tissues in vivo (Dawe et al. 2007; Tallila et al. 2008; Kim et al. 2008; Weatherbee et al. 2009; Garcia-Gonzalo et al. 2011). However, cilia formation has been shown to be unaffected in zebrafish injected with cep290 antisense morpholino oligonucleotides (Sayer et al. 2006). Cc2d2a zebrafish mutants (Bachmann-Gagescu et al. 2011), and the hair cells of Mks1 mutant mice (Cui et al. 2011). Here we show that these genes are also not required for ciliogenesis in zebrafish hair cells. The tissue-specific
role of transition zone genes in ciliogenesis highlights that this process is not uniform across cell types.

While we have shown novel roles for cilia-associated genes in hair cells through this work, a full understanding of the functions of these genes in hair cells remains to be elucidated. IFT genes ifi88, traf3ip, and dyn2h1 appear to be important for hair cell mechanotransduction activity. We believe that their role in aminoglycoside-induced hair cell death is therefore through regulating the uptake of aminoglycosides into hair cells. While all three mutants lack kinocilia, we do not believe the kinocilia itself is responsible for this defect because wdr35 mutants lack kinocilia but still show normal FM1-43 loading. A recent study has shown that IFT88 is important for the trafficking of Cadherin 23 and Harmonin (Blanco-Sánchez et al. 2014), molecules important for hair cell mechanotransduction activity (Di Palma et al. 2001; Söllner et al. 2004; Grillet et al. 2009). Hair cells contain microtubule tracks throughout their cytosol that extend into the actin-rich cuticular plate in the apical region of the cell (Jaeger et al. 1994). Therefore, in addition to anterograde IFT proteins’ role in kinociliary trafficking, they may move gene products important for mechanotransduction along microtubule tracks in the cytoplasm of hair cells to the apical region. As there is turnover of proteins at the tips of stereocilia (Zhang et al. 2012), and stereocilia tip link proteins Cadherin 23 and Protocadherin 15 are known to be rapidly replaced after damage (Zhao et al. 1996; Indzhylkilian et al. 2013), retrograde IFT genes may also play a role in clearing protein from the apical region.

wdr35 mutants show comparable protection to neomycin exposure as other IFT mutants, but no changes in hair cell loading of FM1-43, and a reduced effect on neomycin loading. These observations suggest that wdr35 functions via a distinct mechanism in aminoglycoside-induced hair cell death compared to other IFT genes. Wdr35 has been shown to play a role in mitochondria cell death signaling in cultured cells (Fan et al. 2012), and mitochondria cell death signaling is believed to be involved in aminoglycoside-induced hair cell death (Cunningham et al. 2004; Matsui et al. 2004; Owens et al. 2007; Coffin et al. 2013). It is currently unclear how wdr35 influences mitochondria cell death signaling; however, one possibility is through a role in mitochondria trafficking. IFT genes have been shown to be important for cytoplasmic microtubule morphology and dynamics (Cong et al. 2014; Bizet et al. 2015), and mitochondria are known to traffic along microtubules (Friede and Ho 1977; Frederick and Shaw 2007). An early step in aminoglycoside-induced cell death is the transfer of Ca^{2+} from the endoplasmic reticulum to the mitochondria (Esterberg et al. 2014). This step may be altered if mitochondria are mislocalized, preventing cell death from being initiated.

Finally, transition zone gene mutants appear to function distinctly from IFT mutants in aminoglycoside-induced hair cell death as they do not show as robust protection, and do not show a significant decrease in FM1-43 or aminoglycoside loading. For cc2d2a and cep290, n = 37 wild-type siblings and 10 mutants. (D) Mutations in the transition zone genes cc2d2a (w38, P = 0.4273, w123, P = 0.7372), cep290 (P = 0.6925), and mks1 (P = 0.7851) do not show any decrease in FM1-43 loading. For cc2d2a and cep290, n = 12–18 fish. For mks1, n = 36 wild-type siblings and 12 mutants. Data are displayed as mean ± SD, showing % of fluorescence intensity in the cell bodies of neuromast hair cells of mutants as compared to the average fluorescence intensity of wild-type siblings imaged at the same time. Statistics are calculated using Student’s or Welch’s t-test comparing mutants to wild-type siblings. Mut, mutant; WT Sib, wild-type sibling.

**Figure 7** Intraflagellar transport (IFT) but not transition zone mutants show defects in neomycin and FM1-43 uptake. (A) Mutations in all four IFT genes cause a significant decrease in neomycin-Texas Red (neo-TR) loading. * P = 0.0152, **** P < 0.00001. For dyn2h1, ifi88, and traf3ip, n = 14–20 fish. For wdr35, n = 38 wild-type siblings and 10 mutants. (B) Mutations in the transition zone genes cc2d2a (w38, P = 0.1414, w123, P = 0.1191), cep290 (P = 0.1414), and mks1 (P = 0.0885) do not show a significant decrease in neo-TR loading. For cc2d2a and cep290, n = 14–21 fish. For mks1, n = 38 wild-type siblings and 14 mutants. (C) Mutations in dyn2h1, ifi88, and traf3ip, but not wdr35 (P = 0.8434), cause a significant decrease in rapid FM1-43 loading. * P = 0.0164, **** P < 0.00001. For dyn2h1, ifi88, and traf3ip, n = 14–20 fish. For wdr35, n = 37 wild-type siblings and 10 mutants. (D) Mutations in the transition zone genes cc2d2a (w38, P = 0.4273, w123, P = 0.7372), cep290 (P = 0.6925), and mks1 (P = 0.7851) do not show any decrease in FM1-43 loading. For cc2d2a and cep290, n = 12–18 fish. For mks1, n = 36 wild-type siblings and 12 mutants. Data are displayed as mean ± SD, showing % of fluorescence intensity in the cell bodies of neuromast hair cells of mutants as compared to the average fluorescence intensity of wild-type siblings imaged at the same time. Statistics are calculated using Student’s or Welch’s t-test comparing mutants to wild-type siblings. Mut, mutant; WT Sib, wild-type sibling.

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