The zebrafish *merovingian* mutant reveals a role for pH regulation in hair cell toxicity and function

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ABSTRACT
Control of the extracellular environment of inner ear hair cells by ionic transporters is crucial for hair cell function. In addition to inner ear hair cells, aquatic vertebrates have hair cells on the surface of their body in the lateral line system. The ionic environment of these cells also appears to be regulated, although the mechanisms of this regulation are less understood than those of the mammalian inner ear. We identified the *merovingian* mutant through genetic screening in zebrafish for genes involved in drug-induced hair cell death. Mutants show complete resistance to neomycin-induced hair cell death and partial resistance to cisplatin-induced hair cell death. This resistance is probably due to impaired drug uptake as a result of reduced mechanotransduction ability, suggesting that the mutants have defects in hair cell function independent of drug treatment. Through genetic mapping we found that *merovingian* mutants contain a mutation in the transcription factor *gcm2*. This gene is important for the production of ionocytes, which are cells crucial for whole body pH regulation in fish. We found that *merovingian* mutants showed an acidified extracellular environment in the vicinity of both inner ear and lateral line hair cells. We believe that this acidified extracellular environment is responsible for the defects seen in hair cells of *merovingian* mutants, and that these mutants would serve as a valuable model for further study of the role of pH in hair cell function.

KEY WORDS: Aminoglycosides, Cisplatin, Hair cells, H⁺-ATPase, Ototoxicity, pH

INTRODUCTION
Hearing loss is currently the most prevalent sensory disorder; about 10% of adults and 35% of people over 65 suffer from hearing impairment (Davis, 1989; Ries, 1994). The inner ear is highly sensitive to damage, and numerous genetic mutations and environmental insults lead to hearing loss (Dror and Avraham, 2009; Rybak and Ramkumar, 2007; Sliwinska-Kowalska and Davis, 2012). The inner ear is enriched in ionic transporters also highly expressed in the kidney, such as the H⁺-ATPases and Cl⁻/HCO₃⁻ exchangers (Lang et al., 2007), suggesting a role for ionic homeostasis in the functioning of the audiovestibular system. Active pH regulation in the inner ear is suggested by studies showing altered pH of endolymph and the endolymphatic sac following treatment with carbonic anhydrase or H⁺-ATPase inhibitors (Coulouigner et al., 2000; Sterkers et al., 1984). Additionally, mutations in H⁺-ATPase transporter subunits cause hearing loss in the human disease distal renal tubular acidosis (dRTA) and in mouse models of this disease (Hennings et al., 2012; Karet et al., 1999; Norgett et al., 2012; Smith et al., 2000).

Aquatic vertebrates also control the ionic environment of hair cells of the lateral line system. Lateral line hair cells are located on the surface of the animal, with apical structures protruding into the water enclosed in a gelatinous matrix called the cupula. The ionic environment of the cupula differs from the surrounding water, suggesting active ionic regulation (McGlone et al., 1979; Russell and Sellick, 1976). However, the mechanisms of this regulation are not known. Ionic homeostasis is a particular challenge for freshwater fish, due to ion loss by diffusion into their environment (Dymowska et al., 2012). To combat this problem, fish use specialized cells enriched in ionic transporters called ionocytes (Evans et al., 2005; Hwang and Lee, 2007). It is believed that the gills and the associated ionocytes are the primary site of osmoregulation in fish rather than the kidneys (Evans et al., 2005). One type of ionocyte, the H⁺-ATPase-rich ionocyte, expresses high levels of the H⁺-ATPase transporter and the Cl⁻/HCO₃⁻ exchanger SLC4A1B, and contributes to pH regulation (Lee et al., 2011; Lin et al., 2006).

Hair cells of the lateral line are susceptible to the same ototoxic drugs as mammalian inner ear hair cells, including aminoglycoside antibiotics and chemotherapeutics (Harris et al., 2003; Ou et al., 2007; Ton and Parng, 2005). We have used the zebrafish lateral line system to screen for genes involved in aminoglycoside toxicity (Owens et al., 2008). In this report we show that that the *merovingian* (mero) mutant is resistant to both neomycin- and cisplatin-induced hair cell death due to impaired uptake of these toxicants into hair cells. The gene responsible for the defects in *merovingian* mutants is *gcm2*, a transcription factor important for the generation of H⁺-ATPase-rich ionocytes (Chang et al., 2009). We show that *merovingian* mutants have an acidified extracellular environment in the vicinity of hair cells of both the lateral line and inner ear. Thus, the *merovingian* mutant and zebrafish lateral line might be useful model systems to assess the role of pH regulation in hair cell function.

RESULTS
*merovingian* mutants are resistant to multiple hair cell toxicants

The *merovingian* mutant was identified in a genetic screen for mutations that conferred resistance to neomycin-induced hair cell death (Owens et al., 2008). *merovingian* mutants show a number of phenotypes in addition to resistance to neomycin-induced hair cell death, including a failure to inflate their swim bladders, an enlarged

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yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B). The average size of the posterior oolith in wild-type zebrafish larvae at 5 days post-yolk, and impaired oolith formation (Fig. 1A,B).
merovingian mutants contain a missense mutation in the transcription factor gcm2

To identify the gene mutated in merovingian mutants, we performed genetic mapping using zebrafish microsatellite markers (Knapik et al., 1998; Shimoda et al., 1999). The merovingian mutation co-segregated with a region on chromosome 24 containing 10 genes (Fig. 4A), which were sequenced to identify potential mutations. Only one gene, gcm2, contained a coding sequence mutation. This G-to-A nucleotide change causes a cysteine to tyrosine amino acid change (Fig. 4B). This cysteine is highly conserved among diverse species (Fig. 4C).

merovingian mutants show many of the phenotypes previously reported in fish injected with gcm2 antisense morpholino oligonucleotides (MO), including a failure to inflate their swim bladders, an enlarged yolk and impaired otolith formation (Fig. 1A) (Hogan et al., 2004). To test whether knockdown of gcm2 would cause similar hair cell defects as seen in merovingian mutants, we injected fish with a gcm2 MO. Like merovingian mutants, gcm2 morphants showed a reduction in initial lateral line hair cell number (8.1±1.9 hair cells/neuromast as compared with 10.7±1.4 in controls) and resistance to neomycin-induced hair cell death.

Fig. 1. merovingian mutants have inner ear defects. (A) merovingian (mero) mutants show multiple phenotypes including a failure to inflate their swim bladders, an enlarged yolk, and impaired otolith formation. Arrowhead points to otolith. (B) Quantification of the size of the posterior otolith in wild-type siblings and merovingian mutants. Otolith size is significantly reduced in merovingian mutants. Mutants were selected randomly and included eight fish lacking a posterior otolith and therefore having an otolith size of 0 (n=10 fish). (C) Hair cells expressing the bm3c:gfp transgene in the lateral crista of both wild-type siblings and merovingian mutants. (D) Quantification of the number of hair cells/crista in wild-type siblings and merovingian mutants. All three cristae were used for counting. There is a significant reduction in hair cell number in merovingian mutants (n=9 fish). ***P<0.0001 by Student’s t-test; error bars indicate s.d. Scale bars: 250 μm (A), 10 μm (C).

Fig. 2. merovingian mutants are resistant to toxicant-induced hair cell death. (A) Quantification of lateral line hair cell number in wild-type siblings and merovingian mutants; ***P<0.0001 by Student’s t-test (n=10 fish). (B) Hair cells labeled with parvalbumin in merovingian mutants and wild-type siblings without (left) and with (right) neomycin treatment. merovingian mutants show reduced initial hair cell numbers, but no hair cell loss in response to neomycin. (C) merovingian mutants show a significant resistance to neomycin-induced hair cell death; P<0.0001 by two-way ANOVA (n=10 fish). (D) merovingian mutants are partially resistant to cisplatin-induced hair cell death. Genotypes are significantly different; P<0.0001 by two-way ANOVA (n=6-10 fish). Error bars indicate s.d. Scale bar: 10 μm.
(5.2±1.6 hair cells/neuromast following 200 μM neomycin as compared with 1.2±0.4 in controls) (Fig. 4D).

gcm2 has previously been shown to be necessary for the production of H⁺-ATPase-rich ionocytes (Chang et al., 2009; Esaki et al., 2009). To confirm that gcm2 function was impaired in merovingian mutants, we labeled H⁺-ATPase-rich ionocytes in 3-dpf zebrafish larvae by staining with an anti-vH-ATPase antibody. Robust staining was present on the yolk of wild-type zebrafish larvae and was absent in merovingian mutants (Fig. 5A). We also observed an enrichment of H⁺-ATPase staining in the vicinity of lateral line hair cells (Fig. 5B). This is in agreement with previous reports showing that H⁺-ATPases are expressed in hair cells (Shiao et al., 2005; Stanković et al., 1997). This staining, although reduced in level, was still present in merovingian mutants (Fig. 5B).

**merovingian mutants show a whole body acidification, including in the extracellular environment of hair cells**

gcm2 expression in zebrafish is believed to be restricted to the pharyngeal arches and ionocytes and is not expressed in hair cells or support cells (Chang et al., 2009; Hanaoka et al., 2004; Hogan et al., 2004; Shono et al., 2011). This suggests that gcm2 acts globally to influence hair cells. gcm2 morphants have been shown to have impaired whole body proton excretion (Chang et al., 2009). We hypothesized that this impaired proton excretion would lead to internal acidification of the animal and, in turn, influence hair cell function. To test for acidification, we used the ratiometric pH-sensitive fluorescent protein pHluorin2 (Mahon, 2011). Ratiometric pHluorin contains two excitation peaks, one at 395 nm and one at 475 nm. The fluorescence intensity for the excitation peak at 395 nm decreases with decreasing pH, whereas that for the excitation peak at 475 nm increases with decreasing pH (Miesenböck et al., 1998).

For our experiments, we used 405-nm and 488-nm excitation lasers to excite the two peaks of pHluorin2. Given the known properties of pHluorin2, the ratio of 405-nm/488-nm fluorescence intensities should decrease with decreasing pH, whereas that for the excitation peak at 475 nm increases with decreasing pH (Miesenböck et al., 1998).

For our experiments, we used 405-nm and 488-nm excitation lasers to excite the two peaks of pHluorin2. Given the known properties of pHluorin2, the ratio of 405-nm/488-nm fluorescence intensities should decrease with decreasing pH, whereas that for the excitation peak at 475 nm increases with decreasing pH (Miesenböck et al., 1998).
To test whether the extracellular environment of hair cells was similarly acidified, we expressed cytoplasmic and GPI-linked pHluorin under the control of the hair cell-specific myosin6b promoter (Obholzer et al., 2008) (Fig. 6C,E). We found that the GPI-link pHluorin construct showed a decreased 405/488 fluorescence ratio in merovingian mutants in both lateral line and inner ear hair cells, which is indicative of an acidified extracellular environment of these cells (Fig. 6D,F). Inner ear hair cells, similar to muscle cells, also showed a reduction in the 405/488 fluorescence ratio of cytoplasmic pHluorin (Fig. 6D). By contrast, lateral line hair cells showed the same cytoplasmic pHluorin 405/488 fluorescence ratio in both wild-type siblings and merovingian mutants (Fig. 6F). Thus, although the extracellular environment of these cells is acidified in merovingian mutants, they are able to maintain a normal intracellular pH.

Fig. 4. merovingian is a mutation in gcm2. (A) merovingian was mapped to a ~170,000 bp region on chromosome 24 (arrows) containing 10 genes. Neighboring microsatellite markers used for mapping are shown as well as the number of recombinant animals found for each marker. (B) gcm2 cDNA sequencing results from pooled groups of merovingian wild-type siblings and mutants. Mutants contain a G-to-A mutation resulting in a cysteine to tyrosine amino acid change. (C) The cysteine residue mutated in merovingian is conserved across numerous species. (D) Injection of a gcm2 morpholino (MO) reduced hair cell number in control fish and causes neomycin resistance; ***P<0.001 by two-way ANOVA and Bonferroni post-hoc test (n=14 fish).

Fig. 5. merovingian mutants lack H^+-ATPase-rich ionocytes, but not hair cell H^+-ATPases. (A) vH-ATPase labeling on the yolk of 3-dpf zebrafish. merovingian mutants lack H^+-ATPase-rich ionocytes present in wild-type siblings. (B) vH-ATPase labeling in neuromasts of 5-dpf zebrafish. Although reduced in level, staining is still present in merovingian mutants. Scale bars: 100 μm (A), 10 μm (B).
Exposure to certain therapeutic drugs, particularly aminoglycoside antibiotics and chemotherapeutics, can damage hair cells and cause subsequent hearing loss. However, there is a large amount of variation seen in the degree of hearing loss that patients suffer when taking these drugs (Mulheran et al., 2001; Rybak et al., 2009; Skinner et al., 1990; Xie et al., 2011). This variability is due in part to genetic differences between patients. Although some genes have been identified that alter the susceptibility to drug-induced hearing loss (Hema Bindu and Reddy, 2008; Guan, 2011; Mukherjea and Danks, 2008) and therefore is not necessarily involved in human parathyroid rather than merovingian mutants, we hypothesized that there would be a global acidification of the animal’s internal environment. To confirm that merovingian mutants show increased acidification, we used the fluorescent pH indicator pHluorin2 (Miesenböck et al., 1998). These results show that the extracellular environment of muscle cells as well as inner ear and lateral line hair cells is acidified in merovingian mutants, as measured by a decreased 405/488 ratio in the ratiometric fluorescent pH indicator pHluorin2 (n=15-19 fish). (E) Images of both GPI-linked and cytoplasmic pHluorin2 expressed under the control of the myosin6b promoter in hair cells of the lateral line. (F) The extracellular, but not intracellular, environment of lateral line hair cells is acidified in merovingian mutants, as measured by a decreased 405/488 ratio in the ratiometric fluorescent pH indicator pHluorin2 (n=13-16 fish). Values were normalized to the 405/488 ratio of wild-type siblings for each construct. *P<0.05, ***P<0.001 by ANOVA and Bonferroni post-hoc test. Error bars indicate s.d. Scale bars: 10 μm.

In fish, acid excretion occurs primarily at the gills rather than the kidneys (Claiborne et al., 2002). H+-ATPase-rich ionocytes have been shown to be important for acid secretion in larval zebrafish (Lin et al., 2006). Because these cells are absent in merovingian mutants, we hypothesized that there would be a global acidification of the animal’s internal environment. To confirm that merovingian mutants have an acidified internal environment, we used the genetically encoded pH indicator pHluorin2 (Mahon, 2011; Miesenböck et al., 1998). These results show that the extracellular environment of muscle cells as well as inner ear and lateral line hair cells in merovingian mutants is acidified, consistent with a whole body acidification. Additionally, the intracellular environment of both muscle and inner ear hair cells are also acidified in merovingian mutants, although to a lesser degree than the extracellular environment. By contrast, lateral line hair cells only show an extracellular acidification. As lateral line hair cells are on the surface of the animal it makes sense that they would have additional mechanisms to control their intracellular pH. Indeed, we found an enrichment of H+-ATPase staining around the hair cells of the lateral line and this staining was still present in merovingian mutants. These
data support our hypothesis that gcm2 functions globally to control whole body pH instead of locally at the hair cells. Additionally, it suggests that the defects we are seeing in merovingian mutants are due to changes in extracellular rather than intracellular pH.

Cellular pH regulation has previously been shown to regulate cell death processes, although this regulation is complex (Matsuyama and Reed, 2000). Extracellular acidification influences the response of cancer cells to cisplatin, making cells more susceptible (Atema et al., 1993; Groos et al., 1986; Laurencot et al., 1995; Murakami et al., 2001). However, aberrant cellular pH regulation is also a hallmark of many cancers (Hargunin et al., 2005), which makes it difficult to extend these conclusions to other cell types. Transient application of an acidic solution to the round window potentiated cisplatin ototoxicity in mammals (Tanaka et al., 2003; Tanaka et al., 2004), in contrast to our findings that suggest an acidic environment can partially protect lateral line hair cells from cisplatin. Several differences might account for these different findings. Tanaka and colleagues used transient application of an acidic solution, whereas our mutants are presumably chronically exposed to an acidified environment. Alternatively, mammalian hair cells might use alternative mechanisms of cisplatin uptake that are less sensitive to pH or perturbations in mechanotransduction. It has been previously shown that, unlike zebrafish, mammalian copper transporters Oct2 and Ctr1 appear to play a role in cisplatin ototoxicity (Ciaramboli et al., 2010; Ding et al., 2011; More et al., 2010; Thomas et al., 2013). It is therefore possible that acidification of the mammalian hair cell environment would not have the same protective effects.

There are multiple possible mechanisms by which acidification of the hair cell environment could lead to defects in hair cell function. Mutations in the H+-ATPase subunit Atp6v0a4 as well as pharmacological manipulations of pH regulatory measures cause dramatic decreases in endocochlear potential (EP) (Ikeda et al., 1987; Kuijpers and Bonting, 1970; Lorente-Cánovas et al., 2013; Nortegg et al., 2012; Sierkens et al., 1984; Wagemann et al., 2004). The Na+-K+-ATPase has been shown to have impaired function at acidic pH (Kuijpers and Bonting, 1969), leading to the hypothesis that inhibition of this pump leads to the decrease in EP seen in an acidified environment (Kuijpers and Bonting, 1970). The cupula of Xenopus has been shown to have an elevated endocochlear potential and increased K+ concentration (Russell and Sellick, 1976), therefore a similar mechanism of action could occur in the lateral line.

Alternatively, altered pH homeostasis might be affecting hair cell function by influencing Ca2+ regulation. Fish raised in an acidic environment or with knocked down H+-ATPase function show decreased whole body Ca2+ levels (Horn et al., 2007; Horn et al., 2009). Mutations in Ca2+-modulating proteins are associated with defects in otolith and otoconia formation (Cruz et al., 2009; Hughes et al., 2007; Kozel et al., 1998; Lundberg et al., 2006). Because CaCO3 is a major otolith component, a decrease in Ca2+ levels could be responsible for the otolith formation defects in gcm2 mutants. Acidification of the endolymph has also been associated with an increase in endolymphatic Ca2+ in the Tribolodon hakonensis (Trichobodan hakonensis) vh-ATPase, similar to the antibody described in Xiao et al., 2005; this transgene is referred to here as hmp3:gap3-GFP. Larvae were raised in embryo media (EM) consisting of 1 mM MgSO4, 150 μM KH2PO4, 42 μM Na2HPO4, 1 mM CaCl2, 500 μM KCl, 15 mM NaCl and 714 μM NaHCO3 at pH 7.2. pH was adjusted with NaOH and HCl. Given the 15 mM NaCl present in EM, changes in counterion concentrations during pH adjustments were negligible. The University of Washington Institution Animal Care and Use Committee approved all experiments.

**Otolith measurements**

For quantification of otolith size, fish were anesthetized using MS222 and immobilized in 1% low-melting-point agarose on a microscope slide. Fish were imaged on a Zeiss Axioplan 2 microscope using a Spot camera and Spot Advanced Imaging software (version 4.0.6). The posterior otolith was used for size measurements, and area quantification was carried out using ImageJ software (version 1.45s).

**Immunohistochemistry**

Zebrafish larvae were fixed in 4% paraformaldehyde in PBS for either 2 hours at room temperature or overnight at 4°C. For parvalbumin staining, fish were washed three times with PBS containing 0.1% Tween 20 (PBST), then incubated for 30 minutes in distilled water, at least 1 hour in antibody block (5% heat-inactivated goat serum in 1× PBS, 0.2% Triton, 1% DMSO, 0.02% sodium azide and 0.2% BSA), and overnight at 4°C in mouse anti-parvalbumin antibody (Millipore, MAB1572) diluted 1:500 in antibody block. Fish were then washed three times in PBST and incubated with fluorescently conjugated secondary antibody (Life Technologies) diluted 1:1000 in antibody block for 4 hours at room temperature, washed three times in PBST and stored in a 50:50 mixture of PBS and glycerol before use. For vh-ATPase staining, fish were fixed as before, washed three times with PBST, once with 50% MeOH in PBST, once with 100% MeOH, and then stored overnight at −20°C in fresh 100% MeOH. Fish were then washed once with 50% MeOH in PBST, once with PBST, and incubated with antibody block and antibody for the same durations as parvalbumin antibody staining. A rat antibody against the H+-ATPase B subunit of fish (Tribolodon hakonensis) vh-ATPase, similar to the antibody described in Hirata et al. (Hirata et al., 2003), was used at 1:500 dilution. The vh-ATPase antibody was a gift from Shigehisa Hirose (Department of Biological Sciences, Tokyo Institute of Technology).

**Drug treatment**

Animals were exposed to neomycin (Sigma-Aldrich) at the indicated concentrations for 30 minutes in standard EM, washed three times in EM and given 1 hour to recover in EM before being euthanized and fixed.
Animals were exposed to cisplatin (Teva, supplied by University of Washington Pharmacy) at the indicated concentrations for 24 hours in standard EM, washed four times in EM and immediately euthanized and fixed. The OP1, M2, I04, O2, M12 and M11 neuromasts (Raible and Kruse, 2000) were counted for all lateral line hair cell number quantifications.

**Neomycin-Texas Red**

Neomycin was conjugated to Texas Red-X-succinimidyl ester (Lefevre et al., 1996) in a modified version of the protocols for gentamicin labeling previously described (Sandoval et al., 1998; Stegner et al., 2003). Neomycin sulfate hydrate (Sigma-Aldrich) was used at 115.6 mg/ml final concentration. Neomycin sulfate hydrate solid was resuspended in deionized water up to 50% of the final solution volume, then 0.5 M K2CO3 at pH 9.0 was added at 17.6% final volume. Texas Red-X-succinimidyl ester (Life Technologies) was dissolved in dimethylformamide at 2.5 mM and was added at 12% final volume. The volume of the mixture was brought to 100% with deionized water and the solution incubated overnight at 4°C to allow the conjugation reaction to go to completion.

**Uptake experiments**

For uptake experiments, fish were labeled with 2.25 μM FM1-43FX (Life Technologies) for 1 minute, 50 μM neomycin-TR for 15 minutes, or 25 μM Rhodamine-Universal Labeling System (Rho-Pt, Kreatech Diagnostics; Thomas et al., 2013) for 20 minutes. Fish were exposed to the indicated compound, washed three times and then imaged. To image drug uptake, fish were anesthetized in MS222 and transferred to a Nunc Lab-Tek Chambered Coverglass (Fisher Scientific) where they were immobilized under a nylon mesh and two stainless-steel slice hold-downs (Warner Instruments). One neuromast per fish was imaged, and each neuromast was imaged as a stack of 30 1-μm sections. Image stacks were obtained and analyzed using SlideBook software (version 5.5) running a Marinas spinning disk confocal system (Intelligent Imaging Innovations). Maximum projection images were generated of the entire neuromast stack (for FM1-43 and neomycin-TR labeling), or from nine planes (for Rho-Pt labeling). A mask was drawn around the neuromast based on the brn3c-gfp labeling, and the average intensity was calculated. An identical mask was drawn away from the region of the neuromast to calculate the background intensity. Data is shown as neuromast/background intensity.

**Genetic mapping**

Heterozygous carriers of the merovingian mutation in the *AB* strain background were crossed to the Tübingen strain. Hybrid *AB/Tübingen carriers of the merovingian mutation were identified by phenotype and intercrossed to generate progeny for linkage marker analysis. Mutant and wild-type fish were selected based on otolith and vestibular phenotypes as well as resistance to 200 μM neomycin. For bulk segregant analysis, two pools of 20 mutants and two pools of 20 wild-type fish were used. Microsatellite markers for each chromosome (Knapik et al., 1998; Shimoda et al., 1999) were amplified by PCR and evaluated for co-segregation with mutant phenotypes. Markers co-segregating with the merovingian allele were further evaluated with individual DNA from 294 mutant fish and 32 wild-type fish. Initial mapping localized the mutation between Z-markers Z23011 and Z24856. To narrow the region further, candidate SSR marker primer pairs for this work were generated using the Zebrafish Genome SSR search website (Massachusetts General Hospital, Charlestown, MA 02129; http://damio.mgh.harvard.edu/chrMarkers/zfssr.html). To sequence candidate genes, RNA was isolated from pools of 20 wild-type sibling or mutant embryos using TRIzol Reagent (Life Technologies). cDNA was prepared using SuperScript III Reverse Transcriptase (Life Technologies). Genes were amplified by PCR from CDNA and then sent to Eurofins MWG Operon for sequencing.

**gc2 morpholino oligonucleotide**

For knock-down experiments, we used a previously described gc2 antisense morpholino oligonucleotide (Hanaoka et al., 2004) with the sequence 5′-AAACCTGATCTGGATTGATTTGACAGT-3′ (Gene Tools, LLC). The MO (in 0.1% Phenol Red) was injected into the yolk of 1-cell stage embryos at 10 ng/embryo using previously described techniques (Nasevicius and Ekker, 2000). For a mock injection negative control, 0.1% Phenol Red was injected at comparable volumes as the MO injections.

**pHluorin2**

pHluorin2 DNA was obtained from Matthew Mahon (Massachusetts General Hospital, Harvard Medical School). Constructs were generated to express pHluorin2 (Mahon, 2011) under the control of the β-actin and myosin6b (myo6b) promoter in a Tol2 transposon backbone (Kwan et al., 2007) using standard Gateway cloning mechanisms (Walhout et al., 2000). The GPI targeting sequence of folate receptor alpha (Lacey et al., 1989) was fused to pHluorin2 to generate GPI-pHluorin2. DNA constructs were injected into single-cell embryos at 200 pg along with 40 ng of transposase mRNA. Transiently injected fish expressing pHluorin2 under the control of the β-actin promoter were used for quantification of muscle cells. For hair cell experiments, injected fish were grown to adulthood and screened for germline incorporation of the transgene. Two stable lines were generated, Tg(myo6b:pHluorin2) and Tg(myo6b:pHluorin2gfp), and used in all hair cell pHluorin experiments. Microscope and immobilization techniques used for uptake experiments (see above) were used for pHluorin imaging. For muscle cells, a 20-section stack of 1-μm sections was collected containing trunk muscle cells. For hair cells, a 30-section stack of 1-μm sections was collected from either the anterior crista or anterior lateral line. pHluorin2 fluorescence was acquired using both the 405 and 488 excitation lasers and a 535/30 emission filter. Single planes were used for image analysis. For hair cell data, the cell body was used for measurements. Background correction was carried out in SlideBook software. Fluorescence elicited by the 405 and 488 excitations was measured and then ratioed. One cell was analyzed per animal.

**Statistical analyses**

All statistics were calculated using the GraphPad Prism software (GraphPad, version 4.0). All data are represented as means and standard deviations. P-values are based on ANOVA and Bonferroni post-hoc tests or the Student’s t-test.

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Competing interests

The authors declare no competing financial interests.

Author contributions


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Supplementary material

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