Mechanotransduction activity facilitates hair cell toxicity caused by the heavy metal cadmium

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Abstract

Hair cells are sensitive to many insults including environmental toxins such as heavy metals. We show here that cadmium can consistently kill hair cells of the zebrafish lateral line. Disrupting hair cell mechanotransduction genetically or pharmacologically significantly reduces the amount of hair cell death seen in response to cadmium, suggesting a role for mechanotransduction in this cell death process, possibly as a means for cadmium uptake into the cells. Likewise, when looking at multiple cilia-associated gene mutants that have previously been shown to be resistant to aminoglycoside-induced hair cell death, resistance to cadmium-induced hair cell death is only seen in those with mechanotransduction defects. In contrast to what was seen with mechanotransduction, significant protection was not consistently seen from other ions previously shown to compete for cadmium uptake into cells or tissue

24 including zinc and copper. These results show that functional mechanotransduction
25 activity is playing a significant role in cadmium-induced hair cell death.
26

27 **Introduction**

28 Hearing loss is one of the most common sensory disorders affecting upwards of
29 20% of Americans over the age of 12 (Goman and Lin, 2016; Lin et al., 2011). One
30 common cause of hearing loss is the death of sensory hair cells. This is also a cause of
31 vestibular dysfunction. Hair cells are sensitive to numerous insults including loud noises,
32 certain therapeutic drugs, and aging (Cheng et al., 2005; Kurabi et al., 2017; Schacht et
33 al., 2012; Yamasoba et al., 2013). There is also evidence that environmental toxins
34 such as heavy metals can lead to hearing loss (Choi and Kim, 2014; Schaal et al.,
35 2017), though this form of hearing loss has not been as well-established or studied as
36 other forms.

37 Heavy metal toxicity, in general, is a growing environmental concern. Heavy
38 metals are naturally occurring and while some are required in the body at trace levels,
39 higher levels are often associated with toxicity. Other heavy metals, such as cadmium,
40 have no known function or benefit to animals. Cadmium is used in batteries and some
41 pigments and is produced as a by-product of zinc mining. It can be released into the
42 environment through industrial run-off from a variety of different sources including mine
43 drainage water, sewage treatment plans, and hazardous waste sites (IARC, 2012).
44 Working in certain industries comes with a risk of occupational exposure to cadmium,
45 and nonoccupational exposures can occur through diet or smoking (Faroon et al.,
46 2012). Elevated levels of blood or urinary cadmium correlate with a number of health

problems including dysfunction of the kidneys, liver, cardiovascular system, osteoporosis, and cancer (Ferraro et al., 2010; Gallagher et al., 2008; García-Esquinas et al., 2014; Hyder et al., 2013; Tellez-Plaza et al., 2012).

Studies looking at the link between cadmium exposure and hearing loss have had conflicting results. Some studies have shown increased hearing thresholds or balance impairments in individuals with higher blood cadmium levels (Choi et al., 2012; Choi and Park, 2017; Min et al., 2012). However, other studies have failed to show a significant association between elevated urinary or blood cadmium levels and hearing loss (Kang et al., 2018; Liu et al., 2018; Shiue, 2013). Some of these conflicting results may come from the different methods of quantifying cadmium levels and hearing loss used by the different groups. Studies in rodents have likewise drawn varying conclusions over whether or not cadmium exposure can cause hearing loss. Hair cell death in organ of Corti cultures and changes in auditory brain response (ABR) and distortion product otoacoustic emissions (DPOAEs) have been seen in mice and rats by some groups (Kim et al., 2008; Liu et al., 2014; Ozcaglar et al., 2001). However, other groups have failed to see vestibular dysfunction or hearing loss following exposure to cadmium alone (Carlson et al., 2018; Klimpel et al., 2017; Whitworth et al., 1999). Again different groups have used different cadmium treatment paradigms which may be responsible for the conflicting results. Defects in auditory and lateral line systems have been more consistently seen in fish following cadmium exposure (Baker and Montgomery, 2001; Faucher et al., 2006, 2008; Low and Higgs, 2015; Montalbano et al., 2018; Sonnack et al., 2015), making fish a useful model to study the mechanisms by which cadmium may cause hair cell death. The lateral line is a superficial sensory

70 structure in aquatic animals that is used to detect water movements and contains hair
71 cells similar to those used for hearing and balance in the mammalian inner ear
72 (Larsson, 2012). It has previously been shown that hair cells of the zebrafish lateral line
73 system are sensitive to many of the same insults as mammalian hair cells (Harris et al.,
74 2003; Ou et al., 2007; Ton and Parng, 2005), and the presence of lateral line hair cells
75 on the surface of the animal facilitates both observation of hair cells and the access of
76 potential ototoxic drugs.

77 One open question regarding cadmium-induced hair cell death is how cadmium
78 enters hair cells. Cells do not have designated cadmium channels or transporters as
79 cadmium has no function in the cell. Instead, studies looking at other cell types have
80 found that cadmium enters through channels and transporters for other ions. Studies
81 have shown that cadmium can enter cells via zinc transporters ZIP8 and ZIP14, the iron
82 transporter DMT1, the organic cation channels OCT1 and OCT2, and the TRP channels
83 TRPV5, TRPV6, and TRPM7 (Bannon et al., 2003; Dalton et al., 2005; Fujishiro et al.,
84 2009; Girijashanker et al., 2008; Kovacs et al., 2011, 2013; Lévesque et al., 2008;
85 Martineau et al., 2010; Olivi et al., 2001; Soodvilai et al., 2011). It has also been shown
86 that some metals such as copper and zinc can compete with cadmium for uptake into
87 tissues, presumably due to the use of shared uptake routes (Barbier et al., 2004;
88 Komjarova and Bury, 2014). Another potential uptake route for cadmium into hair cells
89 is the hair cell mechanotransduction channel. Other hair cell toxicants including
90 aminoglycoside antibiotics and the chemotherapeutic cisplatin have been shown to
91 require functional mechanotransduction to enter hair cells (Alharazneh et al., 2011;
92 Marcotti et al., 2005; Thomas et al., 2013).

93 We have found that cadmium can consistently kill hair cells of the zebrafish
94 lateral line system in a dose-dependent manner. This hair cell death is reduced
95 following both genetic and pharmacological inhibition of hair cell mechanotransduction
96 suggesting that mechanotransduction plays a role in cadmium-induced hair cell death,
97 potentially as the route through which cadmium enters hair cells. In contrast to this, we
98 did not see consistent significant protection from cadmium-induced hair cell death when
99 cotreating fish with either zinc or copper, suggesting that these ions are not competing
100 with cadmium for hair cell entry.
101

102 **Materials and Methods**

103 **Animals**

104 All experiments used five-day post-fertilization (dpf) *Danio rerio* (zebrafish)
105 larvae. Experiments were carried out with either *AB wild type zebrafish, *cdh23*^{tj264}
106 (Nicolson et al., 1998; Söllner et al., 2004), *ift88*^{tz288} (Brand et al., 1996; Tsujikawa and
107 Malicki, 2004), or *cc2d2a*^{w38} (Owens et al., 2008) mutants. Mutant alleles were
108 maintained in the *AB background and experiments were carried out on offspring of
109 incrosses of heterozygous parents comparing homozygous mutants to both
110 homozygous and heterozygous wild-type siblings from the same clutch. Mutants were
111 separated based on secondary phenotypes; vestibular defects in the case of *cdh23*
112 mutants and body morphology defects in the case of *ift88* and *cc2d2a* mutants.
113 Larvae were raised in petri dishes containing embryo media (EM) consisting of 1
114 mM $MgSO_4$, 150 μM KH_2PO_4, 42 μM Na_2HPO_4, 1 mM $CaCl_2$, 500 μM KCl, 15 mM NaCl,
115 and 714 μM $NaHCO_3$. They were housed in an incubator maintained at 28.5°C. The

116 Lafayette College or University of Washington Institution Animal Care and Use
117 Committee approved all experiments.
118
119 **Drug Treatment**
120 Fish were treated with cadmium chloride hemipentahydrate (Fisher Scientific)
121 dissolved in EM for three hours at 28.5°C. Some fish were additionally treated with
122 benzamil hydrochloride hydrate (Sigma-Aldrich), zinc sulfate heptahydrate (Sigma-
123 Aldrich), or copper sulfate pentahydrate (Sigma-Aldrich). For all treatments, fish were
124 put into 6 well plates containing net well inserts and the net wells were moved to plates
125 containing the different solutions the fish were exposed to. Following cadmium
126 treatment, animals were washed three times in EM, euthanized with MS-222 and
127 immediately fixed for immunostaining.
128
129 **Immunostaining and Hair Cell Counts**
130 Fish used for immunohistochemistry were fixed for either two hours at room
131 temperature or overnight at 4°C in 4% paraformaldehyde. Antibody labeling was carried
132 out as previously described (Stawicki et al., 2014). Fish used for hair cell counts were
133 labeled with a rabbit anti-parvalbumin primary antibody (ThermoFisher, PA1-933)
134 diluted at 1:1,000 in antibody block (5% goat serum in PBS, 0.2% Triton, 1% DMSO,
135 and 0.2% BSA). Hair cells were counted in the OP1, M2, IO4, O2, MI2, and MI1
136 neuromasts (Raible and Kruse, 2000) and then an average number of hair
137 cells/neuromast was calculated. Statistics were calculated in GraphPad Prism 6.
138

Results

Acute cadmium treatment can kill hair cells of larval zebrafish in a dose-dependent manner

To investigate potential mechanisms of cadmium uptake into hair cells we first worked to develop an acute cadmium treatment paradigm that would reliably kill hair cells. Cadmium has previously been shown to kill hair cells in both adult and larval zebrafish (Montalbano et al., 2018; Sonnack et al., 2015; Wang and Gallagher, 2013); however, none of these experiments quantified hair cell death in larval fish following acute treatment paradigms. We found that after a three-hour treatment with cadmium, we could see a dose-dependent decrease in lateral line hair cell numbers (Figure 1). This treatment paradigm was thus used in subsequent experiments looking for protection from cadmium-induced hair cell death.

Impaired mechanotransduction activity reduces hair cell death in response to cadmium

Multiple hair cell toxicants have been shown to enter hair cells in a mechanotransduction-dependent manner (Alharazneh et al., 2011; Marcotti et al., 2005; Thomas et al., 2013) and manipulations that decrease hair cell mechanotransduction activity therefore protect hair cells from these toxicants (Seiler and Nicolson, 1999; Stawicki et al., 2014; Thomas et al., 2013; Wang and Steyger, 2009). To test if impairing mechanotransduction would likewise protect hair cells from cadmium-induced hair cell death, we both genetically and pharmacologically manipulated hair cell mechanotransduction activity in zebrafish larvae. First, we tested *cadherin 23* mutants,

162 also known as *sputnik*. Cadherin 23 is one of the proteins that make up the tip links that
163 link neighboring stereocilia in hair cells. Mutants no longer have tip links and thus do not
164 have functional mechanotransduction activity (Nicolson et al., 1998; Söllner et al.,
165 2004). We found that there was significantly less cadmium-induced hair cell death in
166 these mutants at all doses tested though hair cell death was not eliminated at higher
167 cadmium doses (Figure 2A). We next pharmacologically inhibited mechanotransduction
168 activity by cotreating fish with cadmium and 200 μM benzamil, an analog of amiloride
169 that has been shown to block hair cell mechanotransduction activity (Hailey et al.,
170 2017). We again saw significant protection against cadmium-induced hair cell death at
171 all cadmium doses (Figure 2B).

172 We next tested whether genetic mutants identified through a screen looking for
173 mutants resistant to aminoglycoside-induced hair cell death (Owens et al., 2008) would
174 also be resistant to cadmium-induced hair cell death. We tested two different cilia-
175 associated gene mutants. One, *ift88,* is believed to be resistant to hair cell death in
176 response to the aminoglycoside neomycin due to reduced neomycin uptake as a result
177 of reduced mechanotransduction activity. The other, *cc2d2a,* appears to have normal
178 neomycin uptake and mechanotransduction activity (Stawicki et al., 2016). We found
179 reduced hair cell death in response to cadmium in *ift88* mutants (Figure 3A), further
180 suggesting that mechanotransduction plays a role in cadmium-induced hair cell death.
181 In contrast, we saw no reduction of hair cell death in *cc2d2a* mutants in response to
182 cadmium (Figure 3B).
183

184 **Zinc and copper cotreatment do not protect hair cells from cadmium-induced hair**
185 **cell death as effectively as impairing mechanotransduction does**

186 As we still saw some hair cell death in *cadherin23* mutants, which completely
187 lack mechanotransduction, we wanted to test if there were alternative means by which
188 cadmium could enter hair cells. Previous experiments have shown that cadmium enters
189 other cell types through zinc transporters (Dalton et al., 2005; Fujishiro et al., 2009), and
190 that zinc can block cadmium uptake (Barbier et al., 2004; Girijashanker et al., 2008).
191 Zinc has also previously been shown to protect against impaired behavioral responses
192 to odorants caused by cadmium in larval zebrafish (Heffern et al., 2018) and to protect
193 against impaired auditory responses caused by cadmium in rats (Agirdir et al., 2002).
194 To test whether zinc could likewise protect against cadmium-induced hair cell death in
195 zebrafish, we co-treated fish with 30 μM of cadmium and varying doses of zinc, based
196 on doses that had previously been shown to protect against cadmium-induced defects
197 in olfactory behavior in larval zebrafish (Heffern et al., 2018), with and without a one-
198 hour zinc pretreatment. We found no significant reduction in hair cell death in response
199 to cotreatment alone (Figure 4A), and only a slight reduction at the highest zinc dose,
200 88 μg/L, in response to the one-hour pretreatment combined with cotreatment (Figure
201 4B). When treating fish with 88 μg/L zinc an hour before and in combination with a
202 range of cadmium doses, we failed to see significant protection (Figure 4C). Zinc has
203 also been shown to kill hair cells on its own (Montalbano et al., 2018), or to exacerbate
204 hair cell death in response to other toxicants (Nakagawa et al., 1997); however, we saw
205 no evidence of this at the doses we used (Figure 4).

While cadmium is not believed to travel through copper transporter family gene products, it has been shown that copper cotreatment can impair cadmium uptake in fish (Komjarova and Bury, 2014). Copper has also been shown to protect against hair cell death in response to the platinum-based chemotherapeutic cisplatin and the heavy metal lead in mammals (Liu et al., 2011; More et al., 2010), though it was unable to protect against cisplatin in fish (Thomas et al., 2013). To determine whether copper might protect hair cells in zebrafish from cadmium toxicity, we first tested various doses of copper to see if they could protect against 30 μM of cadmium when fish were treated with copper for an hour before and while treated with cadmium. Copper has previously been shown to be toxic to zebrafish hair cells on its own (Hernández et al., 2006; Linbo et al., 2006; Mackenzie et al., 2012; Olivari et al., 2008) and we likewise found all but the smallest dose of copper tested, 0.25 μM, caused significant hair cell death on its own while offering no protection against cadmium (Figure 5A). We subsequently tested whether pre- and cotreatment with 0.25 μM copper would protect against a range of cadmium doses but again failed to see any significant protection (Figure 5B).

Discussion

Here we show that cadmium can consistently cause hair cell death in the lateral line system of zebrafish larvae. This is consistent with prior work that has shown cadmium can cause hair cell death in fish (Faucher et al., 2006; Montalbano et al., 2018; Sonnack et al., 2015). Studies looking at hair cell death and/or hearing loss in response to cadmium exposure in mammals and humans, on the other hand, have had much less consistent results, with some studies showing that cadmium is damaging

229 (Choi et al., 2012; Choi and Park, 2017; Kim et al., 2008; Liu et al., 2014; Min et al.,
230 2012; Ozcaglar et al., 2001) and others showing no effect (Carlson et al., 2018; Kang et
231 al., 2018; Klimpel et al., 2017; Liu et al., 2018; Shiue, 2013; Whitworth et al., 1999). This
232 disparity might come from the fact that lateral line hair cells in fish are on the surface of
233 the animal and therefore easily accessible to toxins. In contrast to this, for cadmium to
234 reach hair cells in mammals it must first travel through the bloodstream where it is
235 actively removed by the liver and kidneys (Satarug, 2018; Swiergosz-Kowalewska,
236 2001; Yang and Shu, 2015), and then must pass through the blood-labyrinth barrier.
237 This means presumably only a small percentage of the cadmium an individual is
238 exposed to will ever make it to the hair cells. The fact that some researchers did see
239 either hair cell damage or hearing impairment in mammals following cadmium exposure
240 suggests that it can cause damage in these systems as long as sufficient amounts are
241 present. Therefore, understanding how this damage occurs is an important question to
242 research further.

243 In this study, we attempted to elucidate how cadmium enters hair cells. We did
244 this by blocking potential uptake mechanisms and looking for a decrease in cadmium-
245 induced hair cell toxicity. We showed that blocking mechanotransduction activity either
246 via a genetic mutation in *cdh23* mutants or pharmacologically via benzamil lead to a
247 significant reduction in cadmium-induced hair cell death. While *cdh23* mutants still
248 showed significant cadmium-induced hair cell death at higher doses of cadmium,
249 benzamil appeared to show an almost complete elimination of hair cell death across the
250 dose range tested. This is different from what was previously shown for copper, where
251 amiloride could protect against only lower doses of copper (Olivari et al., 2008). The

252 reason for the differences in resistance seen in *cdh23* mutants versus benzamil
253 treatment may be that unlike *cdh23,* benzamil is likely not blocking
254 mechanotransduction selectively. Amiloride and benzamil are both considered
255 nonselective epithelial sodium channel blockers and have been shown to be capable of
256 blocking a number of channel types including some Ca^{2+}, TRP, and K^{+} channels
257 (Bielefeld et al., 1986; Castañeda et al., 2019; Dai et al., 2007; Tang et al., 1988).
258 Therefore, while the mechanotransduction channel may serve as a major entry route for
259 cadmium, cadmium could also be entering at a lower level through other channel types
260 blocked by benzamil, thus allowing for the low level of hair cell death seen in cdh23
261 mutants. The small remaining level of hair cell death seen in *cdh23* mutants in the
262 absence of mechanotransduction activity is in contrast to other toxicants entering via the
263 mechanotransduction channel, such as cisplatin and aminoglycosides, where toxicity is
264 completely blocked (Thomas et al., 2013; Wang and Steyger, 2009).
265 To test other potential uptake routes for cadmium into hair cells, we next
266 investigated whether zinc or copper could protect hair cells from cadmium-induced hair
267 cell death potentially be competing for entry. We failed to see consistent protection from
268 either of these ions; however, these experiments were complicated by the fact that both
269 copper and zinc can kill hair cells on their own (Hernández et al., 2006; Linbo et al.,
270 2006; Montalbano et al., 2018), requiring the use of lower concentrations of these ions.
271 Copper, in particular, kills hair cells at doses considerably lower than cadmium. The zinc
272 doses we used were based on previous work showing that 22 μg/L zinc could protect
273 against cadmium-induced olfactory damage whereas lower and higher doses could not
274 (Heffern et al., 2018). We failed to see protection at 22 μg/L of zinc. We did see

275 protection at the higher dose 88 μg/L zinc; however, that protection was inconsistent.
276 Also, while benzamil was able to protect following cotreatment with cadmium, zinc
277 required a one-hour pretreatment to show any effect.

278 While we failed to show consistent protection against cadmium-induced hair cell
279 death from zinc, it has previously been shown that zinc can protect against cadmium-
280 induced hearing loss in rats (Agirdir et al., 2002). Zinc can also protect against
281 cadmium-induced nephrotoxicity (Liu et al., 1992; Tang et al., 1998). These experiments
282 were looking over a longer period of cadmium treatment than we did. Potential
283 mechanisms for this protection include the upregulation of metallothionein, changes in
284 gene expression changes caused by cadmium that normally lead to toxicity, and
285 prevention of cadmium from displacing zinc off metal-binding enzymes (Andrews, 2000;
286 Pan et al., 2017; Pinter and Stillman, 2015). It is possible that these mechanisms do not
287 have time to activate in the three-hour treatment window we are using. Also, we are
288 exposing the fish to cadmium by adding it to the water they are swimming in. This is in
289 contrast to mammalian studies that use more systemic treatments where cadmium is
290 injected or given through drinking water. Presumably, given our short treatment times,
291 cadmium is primarily exposed to the apical part of the hair cells where they are most
292 accessible to the surrounding water. This may be the cause of the dominant role of
293 mechanotransduction as a means of protecting against cadmium-induced hair cell
294 death, as fewer other channels may be present on the apical area of the cell.

295 We also investigated whether two cilia-associated gene mutants that are
296 resistant to aminoglycoside-induced hair cell death were similarly resistant to cadmium-
297 induced hair cell death. Mutations in one of those genes, *ift88,* did lead to resistance in

298 cadmium-induced hair cell death similar to what had previously been seen with
299 aminoglycosides. These mutants have previously been shown to have a decrease in
300 FM1-43 uptake (Stawicki et al., 2016) and a decreased response to water jet stimulation
301 (Kindt et al., 2012), suggesting impaired mechanotransduction activity though
302 mechanotransduction is not eliminated as in *cdh23* mutants where no response is seen
303 (Nicolson et al., 1998). Thus, *ift88* mutant's resistance to cadmium-induced hair cell
304 death further supports the role of mechanotransduction in this process. They are not as
305 resistant as *cdh23* mutants, which fits the fact that mechanotransduction is not as
306 impaired. We failed to see significant resistance to cadmium-induced hair cell death in
307 mutants of the other gene tested, *cc2d2a.* Unlike *ift88* mutants, these mutants do not
308 show any impairment in FM1-43 uptake, suggesting normal mechanotransduction
309 activity (Owens et al., 2008; Stawicki et al., 2016). They also do not show any
310 impairments in aminoglycoside loading into hair cells (Owens et al., 2008; Stawicki et
311 al., 2016), suggesting *cc2d2a* plays an intracellular role in the aminoglycoside-induced
312 hair cell death process. The fact that these mutants are not resistant to cadmium-
313 induced hair cell death suggests that these two toxins are behaving differently once
314 entering the cell.
315
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318
319 **Funding**

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FIGURES

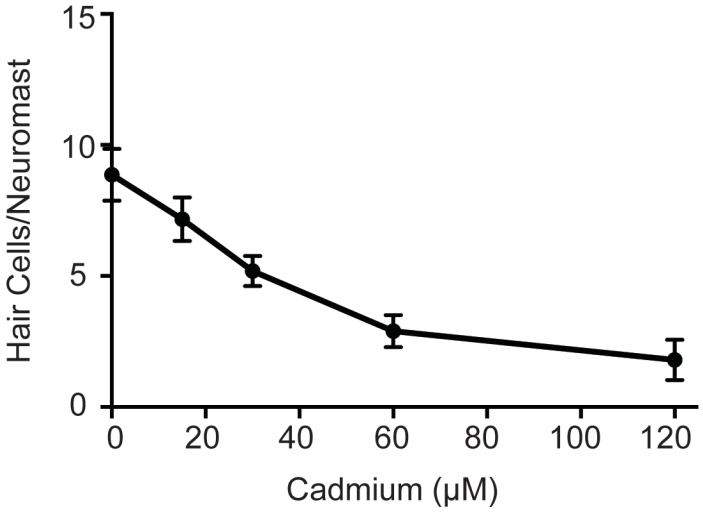

Figure 1: Cadmium kills hair cells in zebrafish larvae in a dose-dependent manner. Treating 5-day post-fertilization (dpf) zebrafish with cadmium doses ranging from 0 - 120 μM for three hours showed an increasing amount of hair cell death with increasing cadmium dose. Data are displayed as mean ± standard deviation. Fish were fixed and stained with parvalbumin and hair cells from six neuromasts were counted (OP1, M2, IO4, O2, MI2, and MI1).

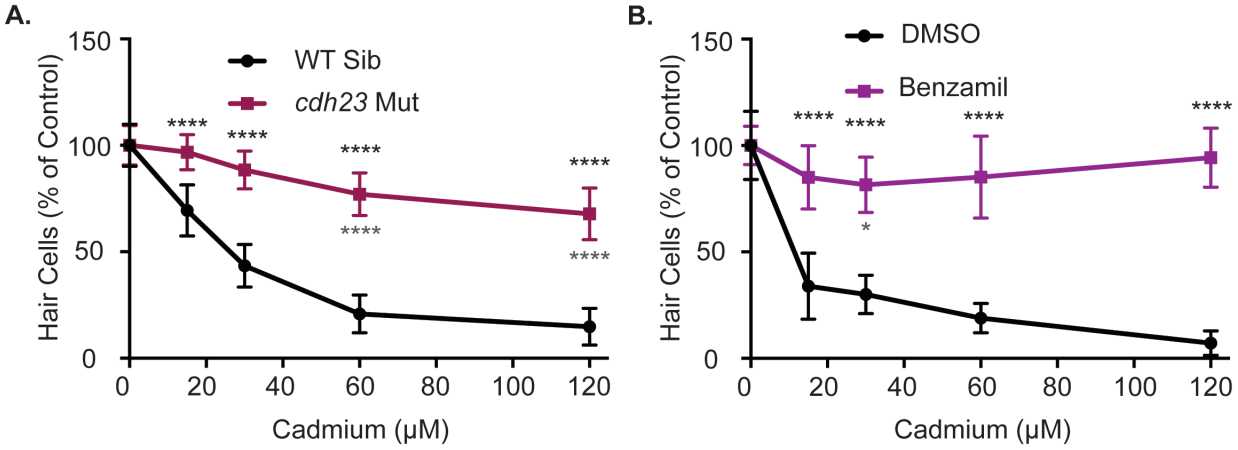

Figure 2: Cadmium toxicity is dependent on functional mechanotransduction activity. (A) *cdh23* mutants, which lack functional mechanotransduction activity, are resistant to cadmium-induced hair cell death. (B) Benzamil, a mechanotransduction blocker, significantly blocks cadmium-induced hair cell death. Data is normalized to the 0 cadmium control for each treatment group. Data are displayed as mean ± standard deviation. * = $p<0.05$, **** = $p<0.0001$ by Two-Way ANOVA and Šídák multiple comparisons test. Black stars above the error bars denote significant differences in *cdh23* mutants or benzamil treated fish as compared to controls treated with the same dose of cadmium. Gray stars below the error bars denote significant differences comparing different cadmium doses in *cdh23* mutants or benzamil treated fish to the 0 cadmium control group.

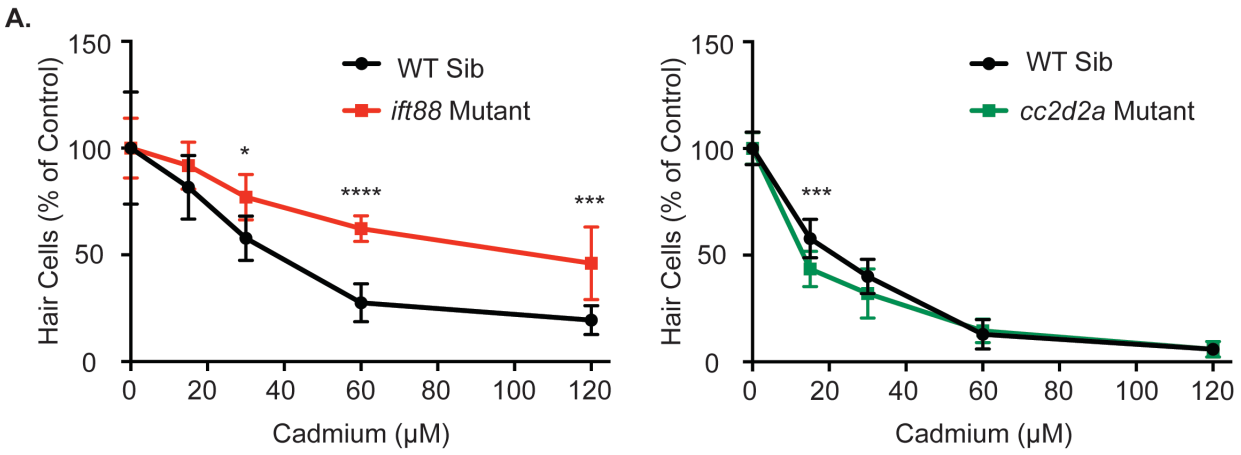

Figure 3: Genetic mutants resistant to neomycin-induced hair cell due to impaired mechanotransduction activity are also resistant to cadmium-induced hair cell death. (A) *ift88* mutants which have previously been shown to have reduced mechanotransduction activity are partially resistant to cadmium-induced hair cell death. (B) *cc2d2a* mutants, which appear to have normal mechanotransduction activity, are not resistant to cadmium-induced hair cell death. Data is normalized to the 0-cadmium control for each treatment group. Data are displayed as mean ± standard deviation. * = $p<0.05$, *** = $p<0.001$, **** = $p<0.0001$ by Two-Way ANOVA and Šídák multiple comparisons test.

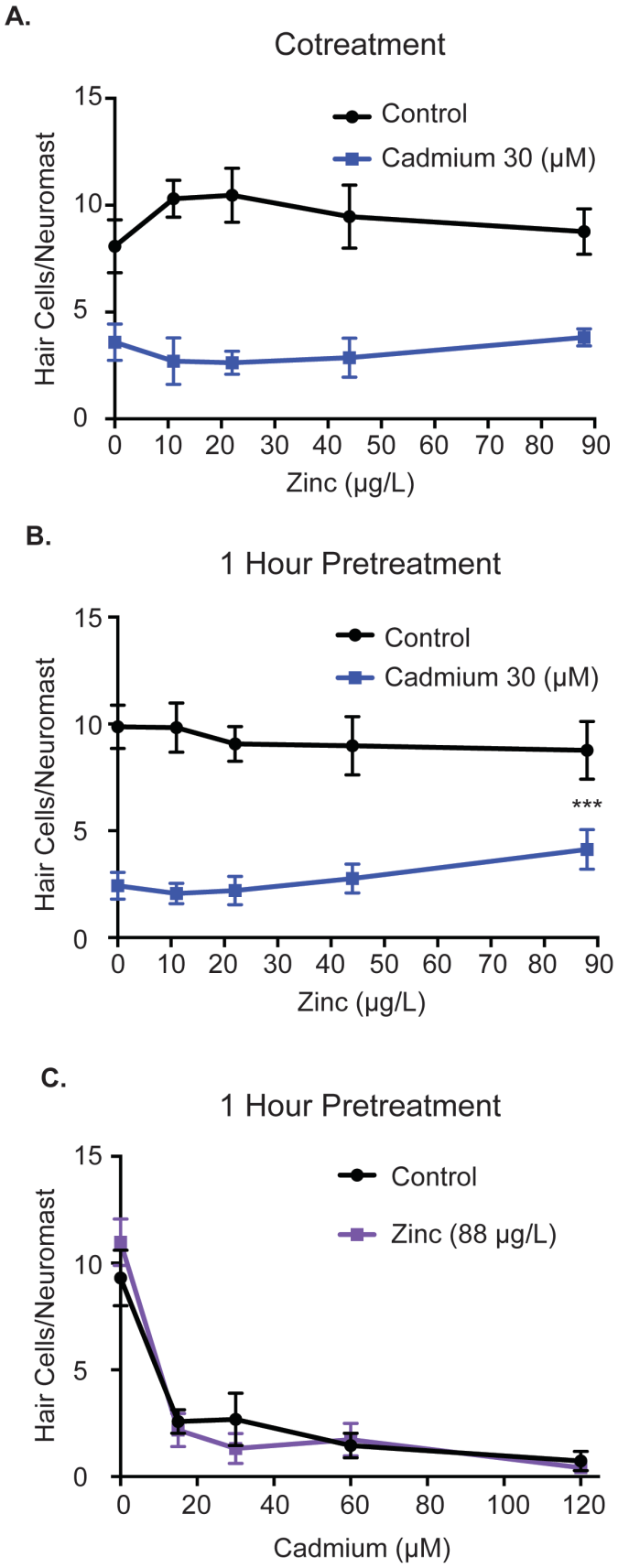
A.
Cotreatment
Control
Cadmium 30 (μM)
Hair Cells/Neuromast
Zinc (μg/L)
B.
1 Hour Pretreatment
Control
Cadmium 30 (μM)
***
Hair Cells/Neuromast
Zinc (μg/L)
C.
1 Hour Pretreatment
Control
Zinc (88 μg/L)
Hair Cells/Neuromast
Cadmium (μM)

Figure 4: Zinc shows limited protection against cadmium-induced hair cell death. Zinc failed to reduce hair cell death when administered (A) alongside cadmium and only showed only a slight reduction at the highest dose tested when administered (B) one hour before and during cadmium treatment. This protection was not seen when tested against a range of cadmium doses (C). Data are displayed as mean ± standard deviation. *** = $p<0.001$ as compared to the 0 zinc control by Two-Way ANOVA and Šídák multiple comparisons test.

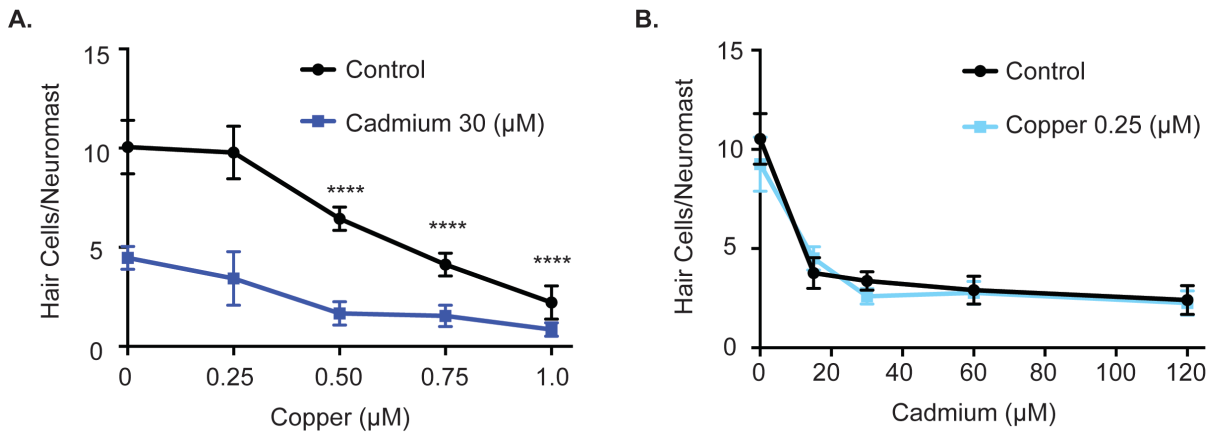

Figure 5: Copper fails to protect against cadmium-induced hair cell death. (A) At doses of 0.5 μM and higher copper caused significant hair cell death on its own. **** = $p<0.0001$ by Two-Way ANOVA and Šídák multiple comparisons test as compared to the 0-copper control. No dose of copper was able to protect against hair cell death in response to 30 μM of cadmium. (B) 0.25 μM of copper failed to show a significant reduction in hair cell death at all cadmium doses tested. Data are displayed as mean ± standard deviation.

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