

IDENTIFICATION OF THE MOLECULAR PATHWAYS
AFFECTED IN USHER SYNDROME TYPE 1 USING
ZEBRAFISH AS AN ANIMAL MODEL

by

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Using Zebrafish as an Animal Model

Approved: _____

Monte Westerfield

Usher Syndrome (USH) is the leading cause of hereditary deafblindness. It is characterized by hearing and vestibular impairment as well as progressive retinal degeneration. This syndrome has been classified into three types based on the severity of the symptoms. Patients affected with USH1 present the most severe symptoms. *CADHERIN 23 (CDH23)*, the focus of this research, is one of the 6 genes causing USH1 when mutated. Previous work in zebrafish has determined that the encoded protein is present in an USH1 complex that preassembles at the endoplasmic reticulum (ER), a subcellular compartment. When the *cdh23* gene is mutated, the USH1 complex assembly and trafficking are disrupted, which leads to a cellular response called ER stress. It was also shown that inner ear hair cell and retinal cell death increase in the *cdh23* mutants. As a first step to understand if ER stress is the cause of increased cell death in the *cdh23* zebrafish mutants, I characterized the molecular pathways activated by ER stress and tested if FDA approved drugs targeting specific components of the activated pathways could lower ER stress and decrease cell death. I found that the Ire1 pathway is potentially activated in the inner ear hair cells and that the Perk pathway is activated in both the inner ear hair cells and the retinal cells of *cdh23* mutants. Furthermore, these mutants showed decreased levels of ER stress when treated with FDA approved drugs. The preliminary data presented in this thesis expand the current understanding of the molecular mechanism leading to USH1 and point toward directions for future research.

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1. Introduction

1.1 Usher syndrome

Usher syndrome (USH) is the leading cause of deafblindness in the United States and affects approximately 1/6000 individuals (Kimberling et al., 2010). Patients present with hearing and vestibular impairment, as well as vision loss through progressive retinal degeneration, known as retinitis pigmentosa (RP) (Yan et al., 2010).

There are three clinical types of USH based on the severity of the symptoms. USH Type 1 (USH1) is the most severe form of the disease. It is characterized by congenital, profound deafness, balance problems, and progressive RP that has an onset within the first decade of life (Mathur et al., 2015). USH2 is the most common clinical type (USHbases). Patients with USH2 have moderate to severe congenital hearing loss, normal vestibular function, and an onset of RP in the second decade of life (Mathur et al., 2015). USH3 is more rare. Loss of hearing and onset of RP are progressive, and vestibular dysfunction is variable (Mathur et al., 2015).

USH type	Gene name	Protein name
USH1	<i>MYO7A</i>	MYOSIN VIIA
	<i>USH1C</i>	HARMONIN
	<i>CDH23</i>	CADHERIN 23
	<i>PCDH15</i>	PROTOCOLADHERIN 15
	<i>USH1G</i>	SANS
	<i>CIB2</i>	CIB2
USH2	<i>USH2A</i>	USHERIN
	<i>GPR98</i>	VLGR1
	<i>DFNB31</i>	WHIRLIN
USH3	<i>USH3A</i>	CLRN1
	<i>USH3B</i>	HARS-1

Table 1: USH genes and proteins.

So far, researchers have been able to identify 11 genes that cause the disorder. Six genes are linked to USH1, three to USH2, and two to USH3 (Table 1) (Mathur et al., 2015). More genes related to USH remain to be discovered.

The focus of this thesis will be on USH1, and specifically on one of the causative genes, *CDH23*, and its effects when mutated.

To date there is no cure for USH1 patients. Using zebrafish as an animal model for USH1, this thesis aimed first to characterize the molecular mechanisms leading to USH1 and then to select and test FDA approved drugs that could potentially be used to treat symptoms in human USH1 patients.

1.2 Molecular basis for deafness and blindness in USH1

USH1 severely affects both inner ear and retinal function. To gain understanding about what exactly is happening in USH1, researchers have explored the effects of USH1 mutations in model organisms. A substantial amount of progress has been made with a particular focus on the inner ear.

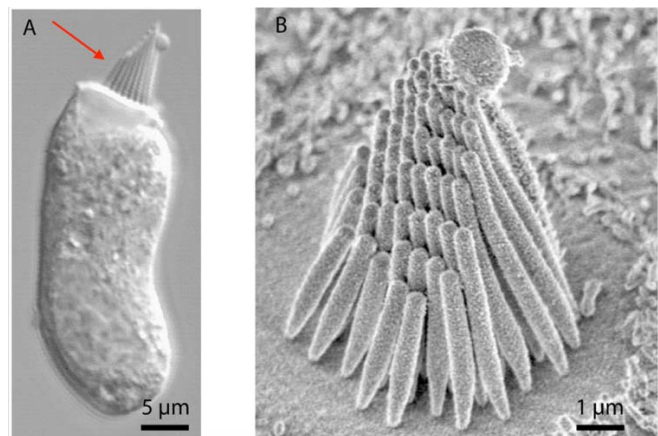


Figure 1: Hair cell of the bullfrog inner ear (A). Mechanoreceptor of the hair cell (B). Source: Prof. Jim Hudspeth, Rockefeller University and Prof. Peter Gillespie, OHSU. <http://www.labex-celtisphybio.fr/active-mechanosensitivity-by-hair-cells-in-the-inner-ear/>

The hair cells are the sensory cells of the inner ear (Figure 1A). They can sense

motion and sound through a structure called the mechanoreceptor (Arrow in Figure 1A and Figure 1B), and transmit this information to the brain.

The mechanoreceptor is made up of a single kinocilium composed of microtubules and multiple actin-based stereocilia that are bound together in a highly-organized fashion (Figure 1B). The latter form the hair bundle (Mathur et al., 2015). In response to sound, movement or gravity, the mechanoreceptor moves, which induces the opening of ion channels at the tip of the stereocilia (Mathur et al., 2015). A subsequent influx of ions leads to changes in membrane potential and release of neurotransmitters at synapses. The conversion of the mechanical stimulus into an electrical response is a process known as mechanotransduction.

The integrity of the hair bundle is essential. Any change in its morphology will disrupt hair cell function, which can lead to deafness. The USH1 proteins are responsible for maintaining the correct morphology of the hair bundle by forming links between the stereocilia (Figure 5A). Therefore, if one USH1 protein is missing or the USH1 complex is unable to form, it can compromise the integrity of the hair bundle and disrupt hair cell function (Mathur et al., 2015).

Many studies have been done to understand the role of USH1 proteins in inner ear hair cell function, but less is known about their role in visual function. RP is caused by a degeneration of the photoreceptor cells of the retina. Photoreceptors are the sensory cells of the eye (Figure 2) and are responsible for converting light to sensory

information, a process called phototransduction. In macaque monkeys and mice, researchers have found that USH1 proteins localize at the inner and outer segments of the photoreceptors and in the photoreceptor synapses, respectively (Ahmed et al., 2008; Reiners et al., 2005; Sahly et al., 2012). Despite these results, the exact function of the USH1 proteins in the photoreceptor cells is still not clear.

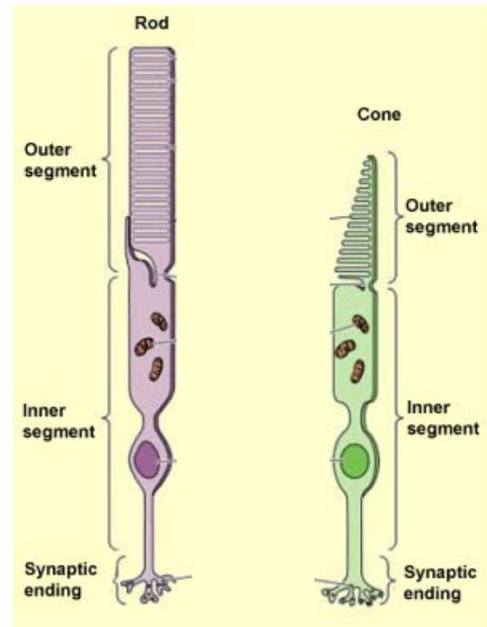


Figure 2: Photoreceptor cells from the retina.
Adapted from <http://thebrain.mcgill.ca/>

However, it has been described that mutations in some *Ush1* genes can lead to photoreceptor death and vision impairment in mice at several months of age (Lentz et al., 2010). Additionally, one *USH1* gene, *MYO7A*, has been shown to have a variety of functions within the mouse retina (Williams and Lopes, 2011). Specifically, the absence of *MYO7A* in photoreceptors leads to abnormally high levels and accumulation of opsin, which are light sensitive proteins found in the retina. This suggested that *MYO7A* may play a role in opsin transport and thus light sensitivity in mice (Sakai et al., 2011).

1.3 Zebrafish model

Different animal models have been developed to characterize the molecular mechanisms underlying USH but zebrafish is now emerging as an excellent organism to study this syndrome. First, fertilization is external, allowing researchers to follow early stages of development. Second, many eggs are laid at once, giving an abundance of

offspring to work with. Third, embryos are transparent, allowing researchers to visually track their overall development or the development of a particular organ. Fourth, zebrafish develop very fast. By 5 days post-fertilization (dpf), the visual and vestibular systems of the zebrafish larvae are already functional (Figure 3). Fifth, as in humans, zebrafish are diurnal animals. They are awake during the day and sleep at night. Finally, zebrafish mutants for *ush1* genes present with balance and visual defects that are comparable to symptoms in human USH patients.

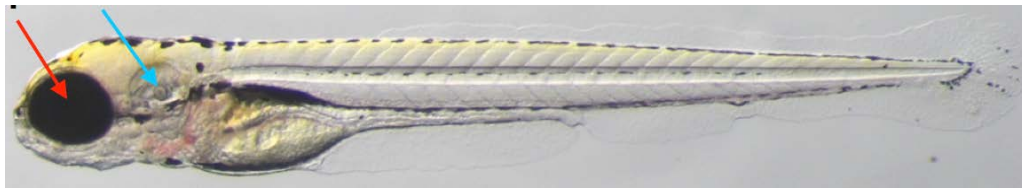


Figure 3: Zebrafish larvae at 5 dpf. Red arrow points to the eye. Blue arrow points to the inner ear. 5 dpf larvae are approximately 4 mm long. Source: Dr. Aurélie Clément.

1.3.1. *ush1* mutants

Mutations in four *ush1* genes, *ush1c*, *cdh23*, *myo7aa* and *pcdh15a*, have been studied and characterized in zebrafish. Mutants for each of these genes all display hearing and vestibular impairment. These impairments are caused by changes in the hair bundle morphology that result in a splayed and/or bent phenotype of the bundle (Figure 4) (Blanco Sánchez et al., 2014; Ernest et al., 2000; Phillips et al., 2011; Seiler et al., 2005; Söllner et al., 2004). Additionally, *ush1c*, *cdh23*, and *myo7aa* mutants have been found to have fewer hair bundles than their wild-type siblings (Blanco Sánchez et al., 2014; Phillips et al., 2011). Although more commonalities are seen with the *ush1* mutants in respect to the inner ear, past research has illuminated the differences between

these *ush1* mutants in terms of their visual systems. Retinal degeneration and lessened visual function have been shown in *ush1c* and *myo7aa* mutants through, respectively, elevated photoreceptor cell death and a reduced optokinetic response, which assays the ability of the fish to track a moving object visually (Wafsy et. al., 2014; Phillips et. al., 2011). Unlike the *ush1c* and *myo7aa* mutants, effects on vision have not been observed in *cdh23* or *pcdh15a* mutants. Immunohistochemistry experiments have not shown photoreceptor cell degeneration for either of these mutants and both maintain wild-type responses to the optokinetic response test (Glover et. al., 2012; Seiler et. al., 2005).

1.3.2. *cdh23* mutant

Analysis of the *cdh23* mutant via measurements of microphonic potentials has revealed reduced or even absent mechanotransduction from the hair cells of a neuromast, a sensory organ of fish within the lateral line system (Nicolson et. al., 1998). Researchers found that when stimulated, the *cdh23* mutant hair cells do not respond normally and cannot transduce the sensory signals (Nicolson et al., 1998). Behavioral analysis of *cdh23* mutants show a circling phenotype in response to a startle, which is a possible consequence of their reduced lack of mechanotransduction (Glover et al., 2012; Nicolson et. al., 1998; Söllner et al., 2004). This circling behavior is typical of mutants with vestibular dysfunction.

In mutants carrying nonsense or missense mutations in the *cdh23* gene, balance defects are due to a loss of structural integrity of the hair bundle and defective development of this organelle. Specifically, the stereocilia are splayed and/or bent and a decrease in the number of hair bundles formed is observed (Figure 4) (Blanco Sánchez et al., 2014; Nicolson et al., 1998; Söllner et al., 2004). An increase in hair cell death is also observed (Blanco Sánchez et al., 2014).

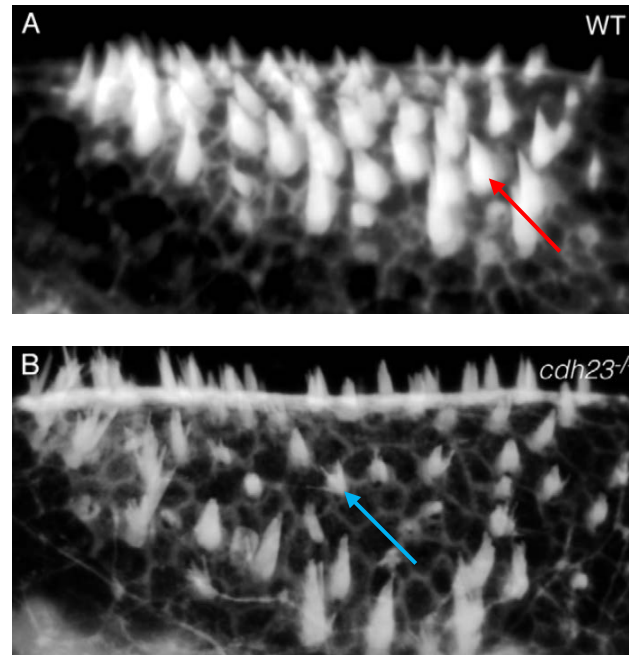


Figure 4: Phalloidin labeling of the anterior macula in 5dpf zebrafish inner ear. Red arrow points to highly organized wild-type hair bundles (A). Blue arrow points to a hair bundle with splayed stereocilia in *cdh23* mutants (B). Source: Blanco Sánchez et al., 2014.

In the zebrafish retina, *cdh23* is expressed in amacrine cells, interneurons of the retina, from 70 hpf and continuing throughout adulthood (Glover et al., 2012). However, no expression is found in the photoreceptors (Glover et al., 2012). Additionally, mutant larvae do not display retinal dysfunction or degeneration, and they respond normally to the optokinetic response test (Glover et al., 2012).

1.4. Molecular mechanisms leading to abnormal hair bundle development in *ush1* zebrafish mutants

A recent study shows that Ush1 proteins, Cdh23, Harmonin and Myo7aa, congregate together to form a complex at the endoplasmic reticulum (ER) (Blanco Sánchez et al., 2014). The ER is a subcellular compartment surrounding the nucleus where transmembrane proteins and proteins that are secreted from the cell are synthesized before they traffic to their final location. Once it is pre-assembled, the Ush1 complex traffics towards the mechanoreceptor, where it will accomplish its function. However, mutations in one or more of the genes encoding Ush1 proteins, prevent formation of the complex and consequently its transport from the ER. As a result, the proteins accumulate in the ER, creating ER stress (Figure 5) (Blanco Sánchez et al., 2014).

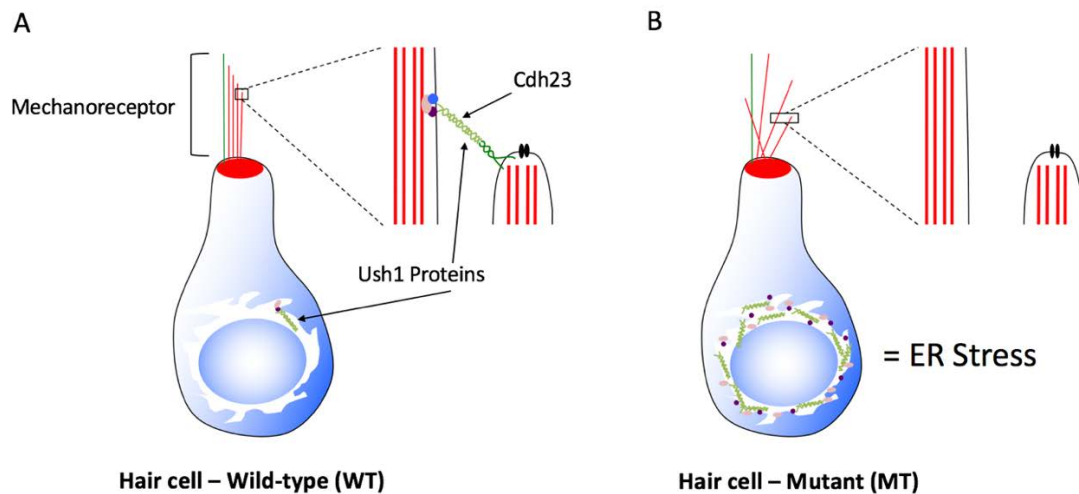


Figure 5: Schematic of Ush1 protein localization in the inner ear hair cells from wild-type (A) and *ush1* mutant zebrafish (B). Blue arrow indicates protein trafficking from the ER toward the mechanoreceptor.
Source: Dr. Aurélie Clément.

One possible consequence of ER stress is cell death (Hertz, 2012). This latter process is known to be responsible for deafness and blindness (Mathur et al., 2015). In zebrafish mutants for *ush1* genes, an increase of inner ear hair cell and photoreceptor cell apoptosis, a programmed cell death, has been observed (Blanco Sánchez et al., 2014; Phillips et al., 2011). At this point, a direct link between ER stress and cell death has not been established. Making a first link between these events is the purpose of my project.

1.5 Project Rationale

In zebrafish, accumulation of Ush1 proteins, including Cdh23, at the ER creates a stress on this subcellular compartment, called ER stress (Blanco Sánchez et al., 2014). There is always a basal level of ER stress. When the load of abnormal proteins is too high and these proteins accumulate, the stress exceeds its threshold. One response a cell has to this ER stress is the activation of the Unfolded Protein Response (UPR).

The UPR is made up of three pathways, Ire1, Perk, and Atf6, that can work individually or together, depending on the cause of activation (Figure 6). My work will focus on the Ire1 and Perk pathways.

For these two pathways, activation occurs through phosphorylation of the Ire1 and Perk proteins. Phosphorylation is a reversible modification of a protein that leads to a specific cascade of events. In the case of the UPR, those events lead to a decrease in translation and/or a degradation of proteins to decrease the load of proteins present at the ER. If the cell cannot resolve ER stress, it will enter into apoptosis.

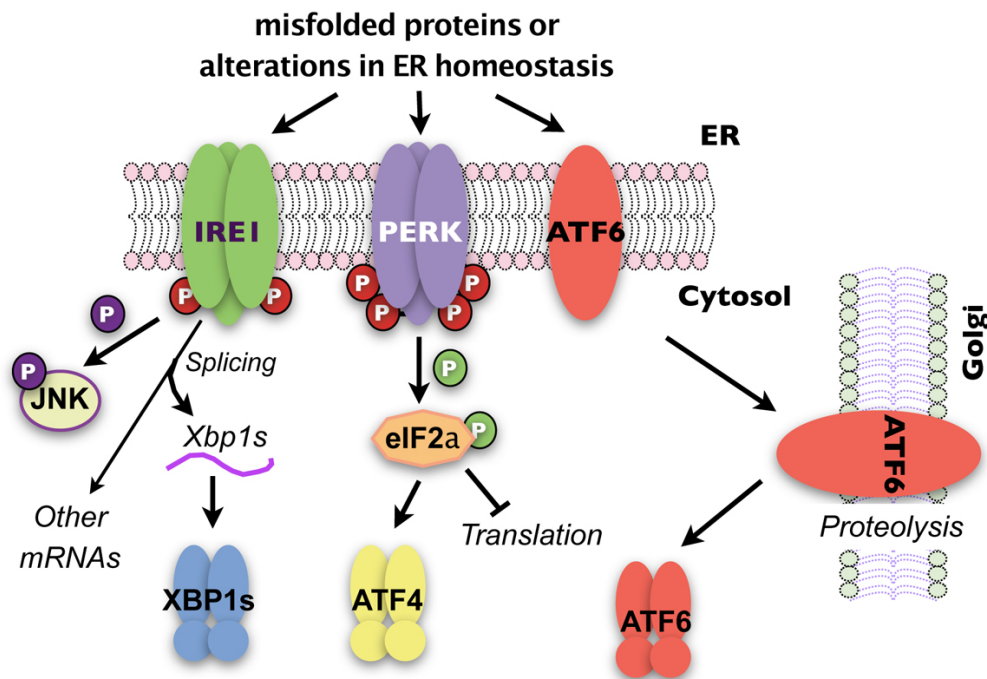


Figure 6: Schematic of the three UPR pathways. Source: <http://www.robertlfurler.com/2012/12/07/life-cycle-of-a-cell/>

Because any one or a combination of the three UPR pathways can be activated by ER stress, characterization of the exact molecular pathways implicated in the steps between ER stress activation and cell death would be essential to finding a specific drug treatment. Currently, FDA approved drugs are used to prevent ER stress in other diseases, such as Alzheimer's and Parkinson's. Similar drugs could be used in treatment of USH1. This is critical because, to date, there are no drugs available to treat USH1.

In a recent study, Hu et al. (2016) investigated the molecular pathways activated by ER stress and leading to hair cell apoptosis in a *Cdh23* mutant mouse. Their results indicate that the Perk pathway is activated in this *USH1* mutant. The researchers used the ER stress inhibitor Salubrinal, to inhibit dephosphorylation of eIF2α selectively, an active component of the Perk pathway. They found that there is significantly less hair

cell death and better hearing in the *Cdh23* mutant mice that received the drug than in the mice that did not.

The results of this study relate very closely to my project. However, the Hu et al. (2016) research focused on the inner ear hair cells and only addressed the question of whether the PERK pathway was activated in the *Cdh23* mouse mutants. My project aimed to characterize in more depth the cascades of events triggered in the zebrafish *cdh23* mutants. Specifically, I not only examined both the Perk and Ire1 pathways, but I also studied them in different cell types, the inner ear hair cells and the retinal cells. Because hearing and vestibular impairment in conjunction with vision loss are characteristic of USH1, it was important to characterize the activated UPR pathways in cells of both the inner ear and retina in zebrafish.

As mentioned above, analyzing the Perk and Ire1 pathways in both the inner ear and the eye of the *cdh23* mutants was the first goal of my project. Using these findings, my second goal was to test FDA approved drugs affecting the activated UPR pathways and to assay their effects on behavior as well as hair bundle integrity and development.

2. Materials and Methods

2.1 Husbandry

Zebrafish strains used were *cdh23^{tj264}* (Söllner et al., 2004), *cdh23^{tj264};eif2 α ^{sa10155}* and *cdh23^{tj264};Tg[myo6b:Kdel-crimson]b1319*. Zebrafish were raised in a 14-hour light and 10-hour dark cycle and maintained at 28.5°C. Eggs were collected following fertilization and stored in embryo medium until 5 dpf. 0.003% 1-phenyl-2-thiourea was added to *cdh23^{tj264}* mutants before 25 hours post fertilization (hpf) to prevent pigmentation and allow study of the retina. All analyses were done at 5 dpf, when vestibular and visual systems are known to be functional. All experiments followed protocols approved by the University of Oregon Institutional Animal Care and Use Committee.

2.2 Immunohistochemistry

Larvae were anesthetized and fixed in BT fix solution (4% paraformaldehyde, 4% sucrose, 0.15mM CaCl₂ in phosphate buffer saline (PBS)) for 24-48 hours, then washed 4 times 5-min in 0.1% Tween-20 in PBS (PBT). Larvae were permeabilized in 2.5% Tween-20 at room temperature for 18 hours, followed by a 15-min acetone crack at -20°C. Following 3 times 5-min washes in PBT, samples were blocked overnight in PBS blocking solution (PBS, 5% goat serum, 5% bovine serum albumin) at 4°C and then incubated overnight at 4°C in primary antibody solution (PBS blocking solution + primary antibody) (Figure 7). Primary antibodies used were rabbit anti-Ire1 (Cell Signaling), rabbit anti-Phospho-Ire1 (Novus Biologicals), rabbit anti-Perk (Cell Signaling), rabbit anti-Phospho-Perk (Cell Signaling), mouse anti-eiF2 α (Santa Cruz),

rabbit anti-
Phospho-eiF2 α
(Cell Signaling).
Primary antibodies
were all added at a
1:400 dilution in
PBS blocking
solution. For double
immunolabeling,
both primary
antibodies were
added concurrently.

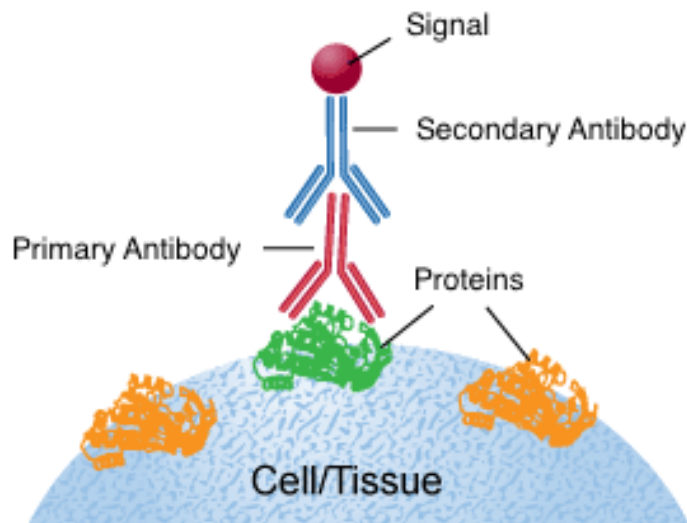


Figure 7: Schematic representation of immunohistochemistry. The primary antibody binds to a specific protein. Secondary antibodies specifically recognize and bind to the primary antibodies. The signal results from a catalytic reaction when a substrate is added. Adapted from: <http://www.leinco.com/immunohistochemistry>

Unbound primary antibodies were removed by 4 times 30-min washes in PBT. Following an overnight incubation at 4°C in PBS blocking solution, secondary antibodies, biotinylated horse anti-rabbit (Vector Laboratories; 1:500), were added overnight at 4°C (Figure 7). Unbound secondary antibodies were removed by 4 times 30-minute washes in PBT.

For the detection step, the Vectastain ABC Elite kit (Vectorlabs) was used to amplify the signal and allow for better visualization of proteins. This amplification system utilizes the ability of the Avidin and Biotin molecules to form complexes. Horse radish peroxidase (HRP) conjugated-Biotin and Avidin molecules were pre-incubated in PBS blocking solution (AB solution) at a 1:1:100 ratio for 20 minutes at room

temperature. The AB solution was then added to the samples for 25 minutes at room temperature. Following 2 PBT rinses and 4 times 5-min washes, the samples were incubated for 25-min with a fluorescent substrate, cyanine-3 (TSA kit, Vector Laboratories), diluted at 1:75 in amplification buffer (PBT, 0.006% H₂O₂). The catalytic reaction was stopped by 3 times 5-min washes in PBT.

In the case of single immunolabeling (only one protein detected), samples were stored in Vectashield (Vectorlabs) at 4°C. In the case of double immunolabeling (two proteins detected), the catalytic reaction was stopped by 4 times 5-min washes in PBT and the HRP enzyme was inactivated by incubation in 0.1 M hydrochloric acid for 10-min. Following 6 times 5-min PBT washes, larvae were post-fixed for 20-min in BT fix solution. After 4 times 5-min PBT washes, 1 drop of avidin solution was added for 1-hour and removed. 1 drop of HRP conjugated-Biotin was then added for 1-hour. Samples were washed twice in PBT for 5-min and incubated in PBS blocking solution overnight. Next, secondary antibodies, biotinylated horse anti-mouse (Vector Laboratories; 1:500), were added. Steps for detection were the same as described above, with fluorescein as a substrate.

2.3 Genotyping

To genotype the double mutants *cdh23*^{*ij264*};*ef2α*^{*sa10155*}, larvae were anesthetized in Mesab and cut in two. Heads were immersed in BT fix for 48 hrs at 4°C and then phalloidin-labeled (see below section). Tails were incubated 20-min in a DNA lysis buffer (NaOH 25mM, EDTA 0.2mM in H₂O) at 95°C to extract genomic DNA (gDNA). A polymerase chain reaction (PCR) was then run using the GoTaq system

(Promega) to obtain the amplicon that would be sequenced (Genewiz). Sequences of the primers and PCR conditions are described in Table 2, 3 and 4.

PCR Step	Temperature	Time
1	95 °C	5 min
2	95 °C	30 s
3	60 °C	20 s
4	72 °C	30 s
5	Go back to step 2, 39 times	
6	72 °C	5 min
7	END	

Table 2. PCR conditions for amplification of the DNA fragment for *cdh23*^{tj264}.

PCR Step	Temperature	Time
1	95 °C	2 min
2	95 °C	20 s
3	67.5 °C	30 s
4	72 °C	30 s
5	Go back to step 2, 34 times	
6	72 °C	5 min
7	END	

Table 3. PCR conditions for amplification of the DNA fragment for *eif2α*^{sa10155}.

tj64a-F2	5'-ACTCCAGTTGGGACGTCAGT-3'
tj64a-R2	5'-TTGTATTGCTTGATTGGTATGG-3'
eif2α.f2	5'-CACTGCAGTTCTTGTACCG-3'
eif2α.r2	5'-CAATGTGCACATTGCGATGTTGATGC-3'

Table 4. Primer sequences for *cdh23*^{tj264} and *eif2α*^{sa10155}.

2.5 Actin labeling

Steps were the same as for an immunolabeling, but phalloidin was used in place of a primary antibody and incubated overnight at 4°C (0.08 unit/ul phalloidin) (Thermofisher Scientific) in PBS blocking solution). Phalloidin solution was removed by 4 times 30-min washes in PBT and larvae were stored in Vectashield.

2.6 Behavior Assay

The behavior of treated and untreated mutants and siblings was assessed at 5 dpf using the Startle Response Test. This test was performed by tapping the side of the dish and observing the larvae's response.

2.7 FDA Approved Drugs

cdh23^{tj264};eif2α^{sa10155} and *cdh23^{tj264};Tg[myo6b:Kdel-crimson]b1319* larvae were incubated with dimethylsulfoxide (DMSO) or FDA approved drugs, SP600125 and Losmapimod, from 3 dpf for 48 hours or from 4 dpf for 24 hours. DMSO, SP600125 and Losmapimod were diluted in EM at a concentration of 10μM. Larvae were then fixed in BT fix at 5 dpf.

2.8 Imaging

Images of labeled larvae were obtained using a LSM510 Zeiss confocal microscope. Larvae were mounted laterally in Vectashield between slide and coverslip. Scans of the inner ear anterior macula and of the retina were done using a 63x objective. ImageJ (v. 1.51) was used to measure pixel intensity and count hair bundles and hair cells.

2.9 Statistical Methods

A student's t-test was performed to test if the difference between sets of data was significant. Each t-test gave a p-value, which is the calculated probability that two data sets are different due to random chance. If the p-value was less than 0.05 the data were said to be significant (* in graphs). If the p-value was less than 0.01 the data were said to be highly significant (** in graphs). If the p-value was more or equal to 0.05 the

data were said to be non significant (ns in graphs). All means for intensity were normalized.

3. Results

3.1 Identification of the UPR pathways activated in the *cdh23* mutant

The first goal of this research was to identify the UPR pathways activated in the *cdh23* zebrafish mutants. There are three UPR pathways named after the first protein activated for each of them, Ire1, Perk, and Atf6. I focused on the Ire1 and Perk pathways.

Both the Ire1 and Perk pathways are activated by phosphorylation. Therefore, by comparing the levels of phosphorylated Ire1 and Perk with their total amount utilizing immunohistochemistry, in both *cdh23* mutants and their siblings, I was able to assay activation.

3.1.1 Ire1 pathway

To determine the amount of phosphorylated Ire1 and total Ire1 in the inner ear hair cells, I measured the intensity of the signal in all the hair cells of one of the sensory patches, the anterior macula (Figure 8). In the eye, the levels were determined for 1) all retinal cell types and 2) the photoreceptors only (Figure 9). For each larva labeled, images were acquired from both the hair cells of the anterior macula and

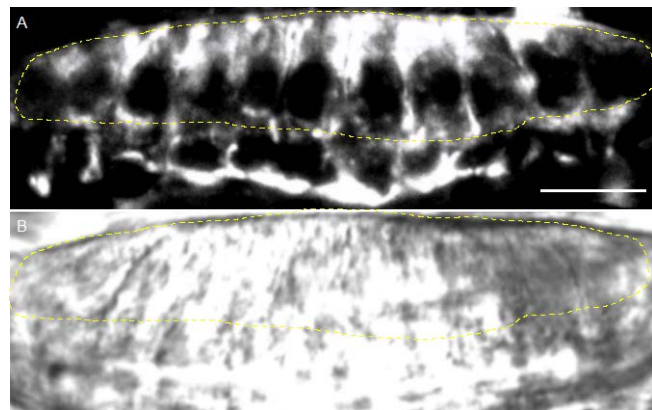


Figure 8: Optical section of the anterior macula of a *cdh23* wild-type sibling (A). Yellow dashed line encloses the hair cells. DIC image of the same section (B). Scale bar: 10 μ m.

the retinal cells. Labeling for Ire1 and phosphorylated Ire1 (PhosphoIre1) were repeated three times.

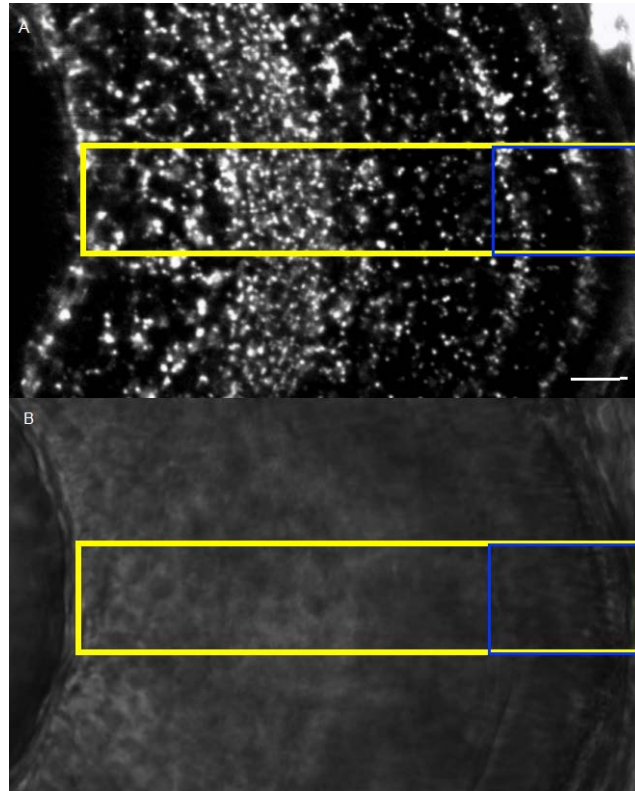


Figure 9: Optical section of the retina of *cdh23* wild-type sibling (Sib). (A) Perk labeling. (B) DIC image of the same section. Yellow box delimits the retinal cells for which intensity will be measured. Blue box delineates the photoreceptor cells. Scale bar: 10 μ m.

In the hair cells of the anterior macula, there was an apparent increase of the level of phosphorylated Ire1 in the mutants, but this increase was not significant (Figures 10 and 11). This suggests that, with the current data, the Ire1 pathway might be activated in the inner ear hair cells of *cdh23* mutants.

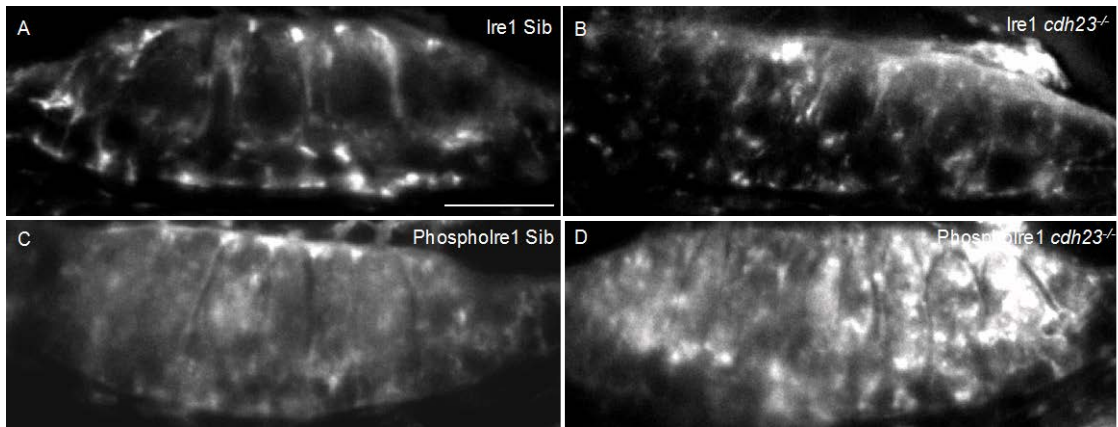


Figure 10: Levels of Ire1 in hair cells of the anterior macula. Ire1 labeling in sibling (A, n=11) and *cdh23* mutant (B, n= 12). PhosphoIre1 labeling in siblings (C, n=16) and *cdh23* mutant (D, n= 17). Scale bar: 10 μ m.

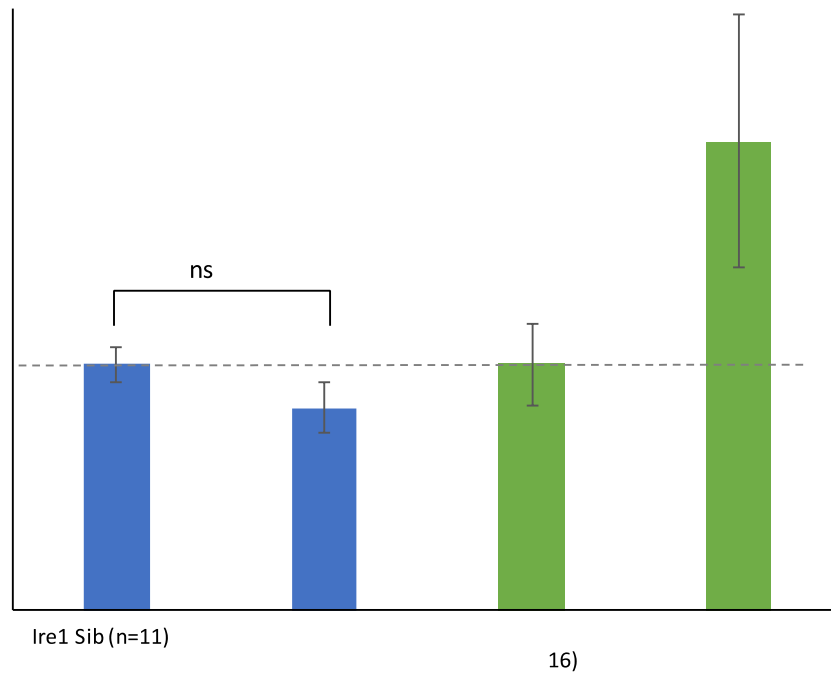


Figure 11: Quantification of Ire1 and PhosphoIre1 in the inner ear hair cells. Black lines represent $\pm$ S.E.M. ns = not significant.

Analysis in the whole retina showed no significant difference between the levels of phosphorylated Ire1, but did show a significant decrease of the total amount of Ire1

between *cdh23* and their siblings (Figure 12 and Figure 13A). Similarly, no significant change was observed within the photoreceptors (Figure 13B). This likely indicates that the Ire1 pathway is not activated in the retina of the *cdh23* zebrafish mutant.

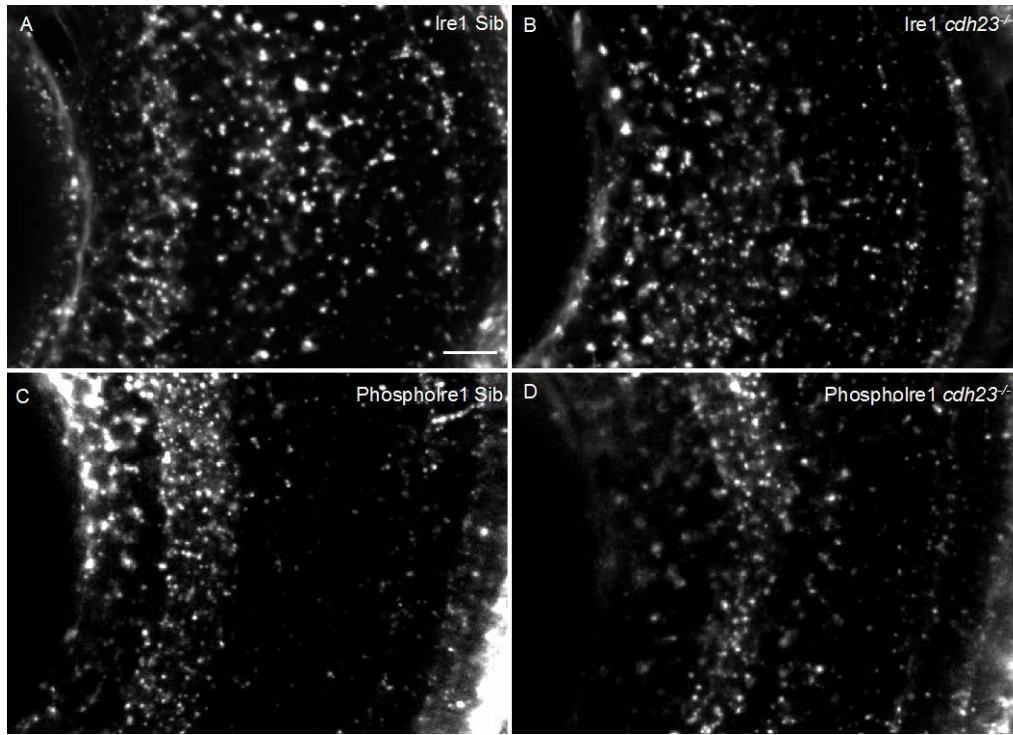


Figure 12: Levels of Ire1 in the retina. Ire1 labeling in sibling (Sib) (A, n=11) and *cdh23* mutant (B, n= 15). PhosphoIre1 staining in siblings (C, n=15) and *cdh23* mutant (D, n= 16). Scale bar: 10 μ m.

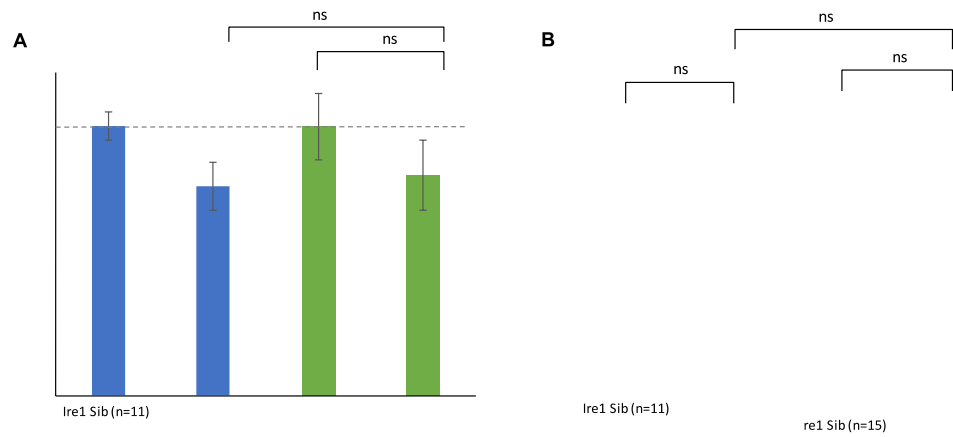


Figure 13: Quantification of Ire1 and PhosphoIre1 in the retina (A) and photoreceptors (B). Black lines represent $\pm$ S.E.M. * $p < 0.05$, ns = not significant.

3.1.2 Perk Pathway

The activation of this pathway was investigated via the activation of two proteins, Perk and a downstream target of this kinase, Eif2 α . The amounts of phosphorylated Perk and total Perk and phosphorylated Eif2 α and total Eif2 α in the hair cells and retinal cells were determined as explained above. Labelings for Perk, phosphorylated Perk (PhosphoPerk), Eif2 α , and phosphorylated Eif2 α (PhosphoEif2 α) were repeated twice.

Analysis of the anterior macula showed significant changes between the levels of phosphorylated Perk and total Perk in the *cdh23* mutants compared to siblings (Figure 14 and Figure 15). This result suggests that in *cdh23* mutants the Perk pathway is activated in the hair cells.

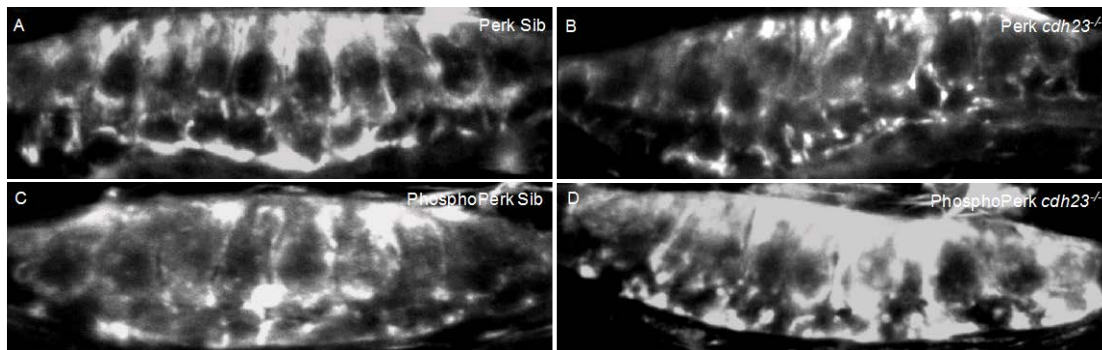


Figure 14: Levels of Perk in hair cells of the anterior macula. Perk labeling in sibling (Sib) (A, n=8) and *cdh23* mutant (B, n= 8). PhosphoPerk labeling in siblings (C, n=8) and *cdh23* mutant (D, n= 11). Scale bar: 10 μ m.

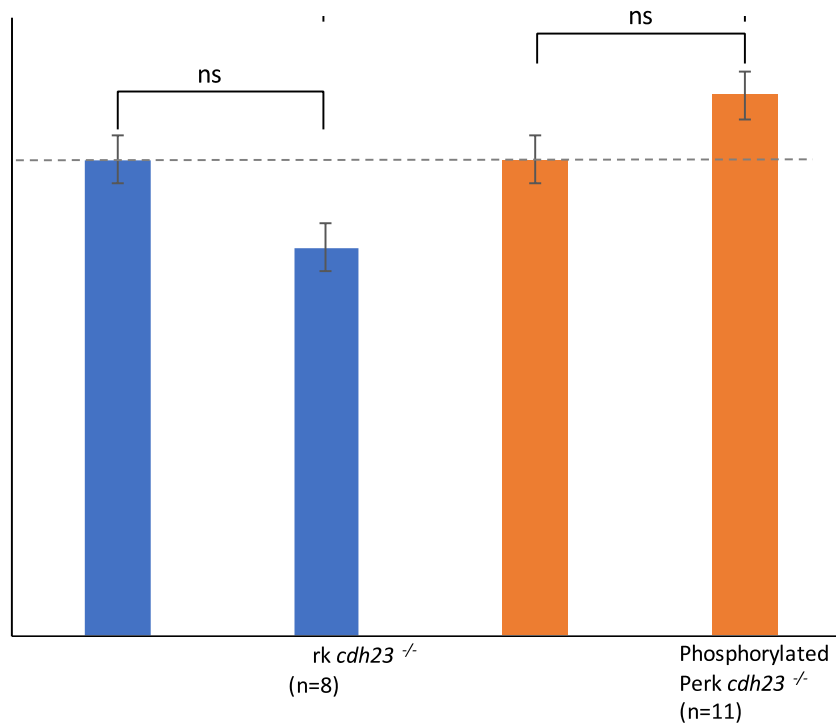


Figure 15: Quantification of Perk and PhosphoPerk in hair cells. Black lines represent $\pm$ S.E.M. * $p < 0.05$, ns = not significant.

Analysis in the retina showed a highly significant difference between the levels of phosphorylated Perk or in the total amount of Perk proteins (Figure 16 and Figure 17A). However, no significant difference between the levels of phosphorylated or total Perk was found in the photoreceptors (Figure 17B). These results indicate that, with the current data, there is activation of the Perk pathway in the eye, but not in the photoreceptors of *cdh23* mutant zebrafish.

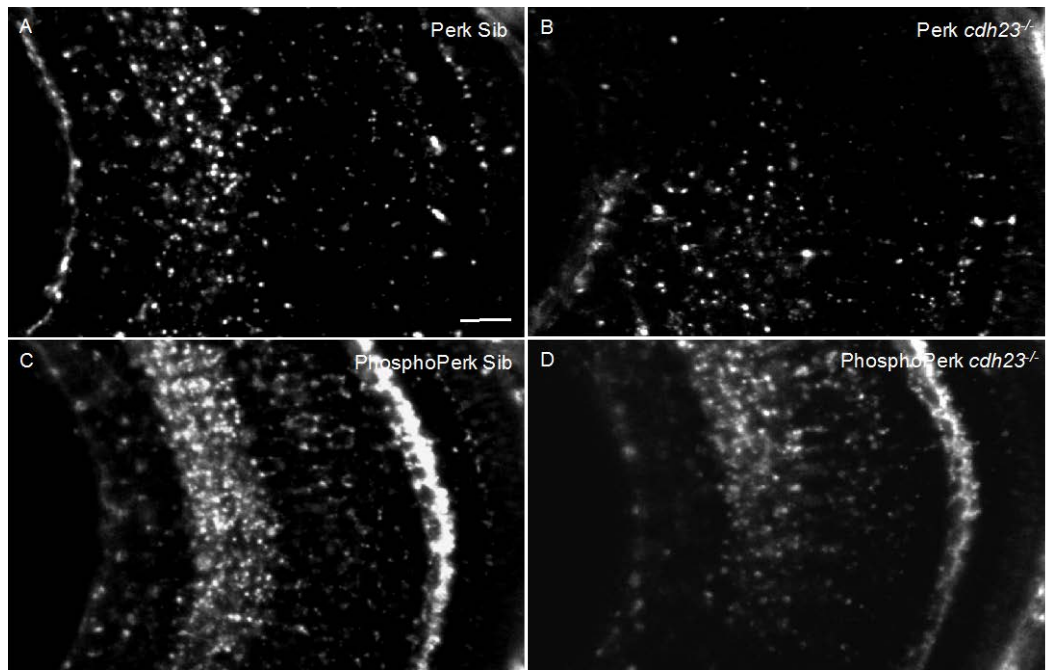


Figure 16: Levels of Perk in the retina. Perk labeling in sibling (Sib) (A, n=8) and *cdh23* mutant (B, n= 8). PhosphoPerk staining in siblings (C, n=10) and *cdh23* mutant (D, n= 11). Scale bar: 10 μ m.

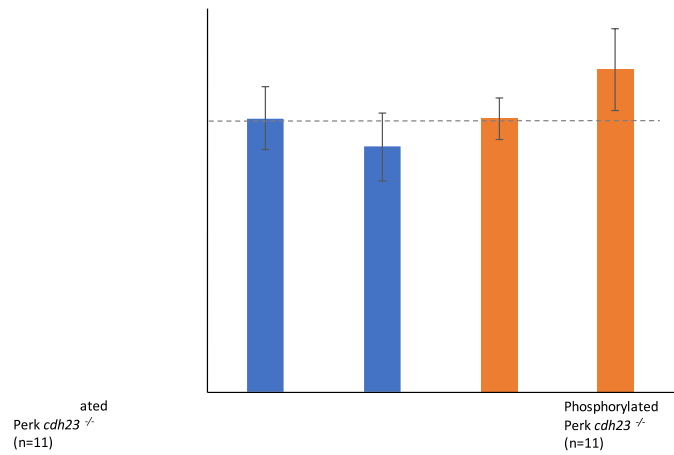


Figure 17: Quantification of Perk and PhosphoPerk in the retina (A) and photoreceptors (B). Black lines represent $\pm$ S.E.M. ** $p < 0.01$, ns = not significant

Labeling for Eif2 α and PhosphoEif2 α was conducted in the same larvae. In the hair cells of the anterior macula, I found a highly significant increase in the levels of phosphorylated proteins in the mutants compared to the total amount of Eif2 α (Figure 18 and figure 19). This is not a surprising result given the Perk pathway is activated in these cells in the *cdh23* mutants.

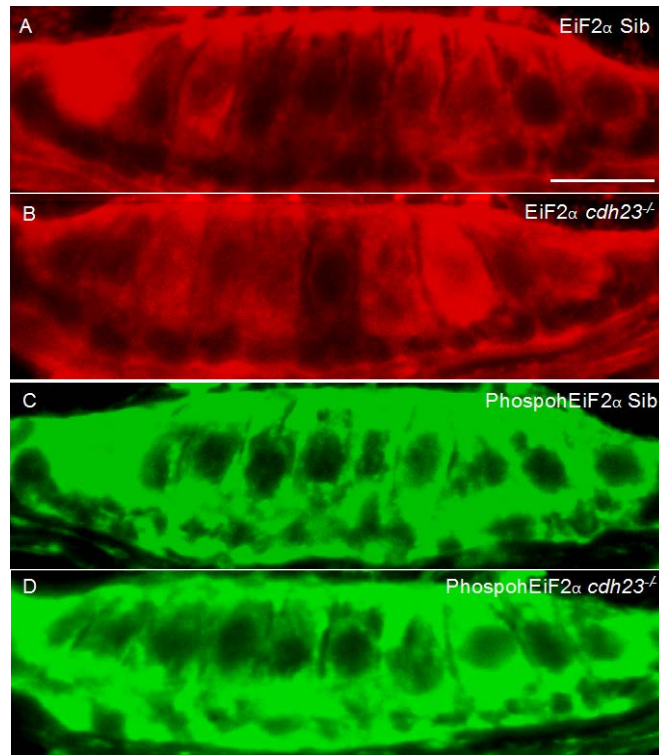


Figure 18: Levels of Eif2 α in hair cells of the anterior macula. Eif2 α labeling (red) in siblings (Sib) (A, n=9) and *cdh23* mutants (B, n= 11). PhosphoEif2 α labeling (green) in siblings (C, n=9) and *cdh23* mutants (D, n= 11). Scale bar: 10 μ m.

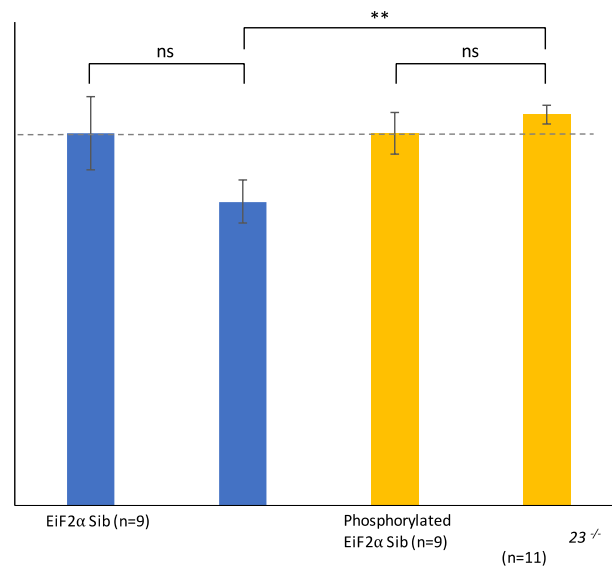


Figure 19: Quantification of Eif2 α and PhosphoEif2 α in hair cells. Black lines represent $\pm$ S.E.M. ** p < 0.01, ns = not significant.

In the various cells of the retina, including the photoreceptors, my experiments showed a significant increase in the levels of phosphorylated Eif2 α in *cdh23* mutants (Figure 20 and Figure 21). This is not surprising given that the Perk pathway is activated in this tissue in the *cdh23* mutants.

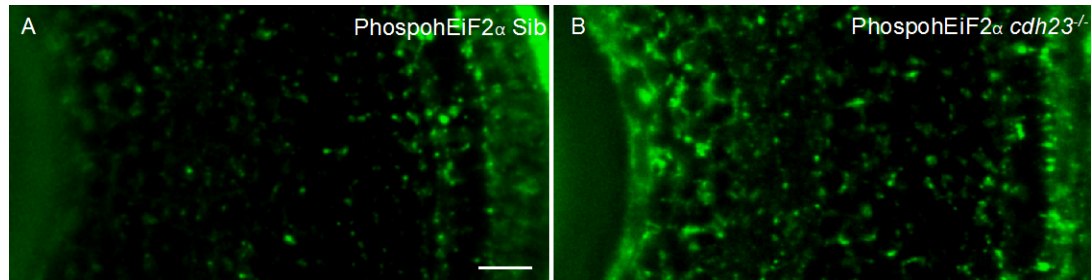


Figure 20: Levels of Eif2 α in the retina. Eif2 α labeled (red) in siblings (Sib) (A, n=9) and *cdh23* mutant (B, n= 11). PhosphoEif2 α (green) in siblings (C, n=9) and *cdh23* mutant (D, n= 11). Scale bar: 10 μ m.

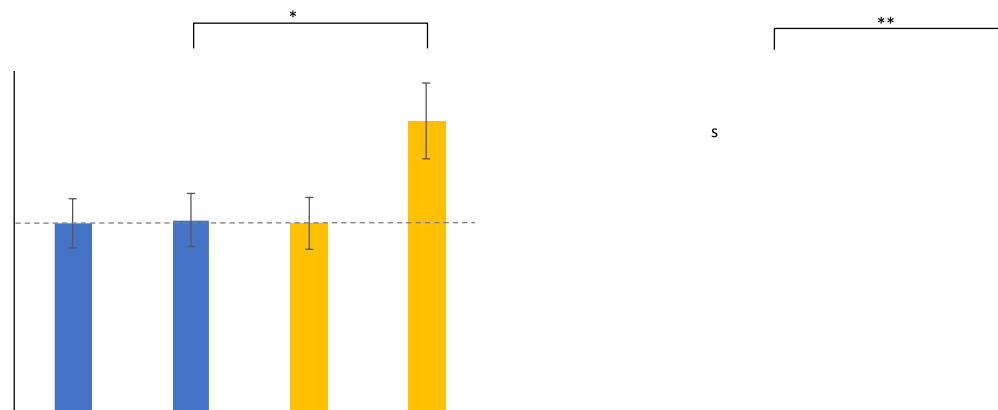


Figure 21: Quantification of Eif2 α and PhosphoEif2 α in the retina (A) and photoreceptors (B). Black lines represent $\pm$ S.E.M. * p<0.05, ** p<0.01, ns = not significant.

Overall, these results indicate that, in zebrafish, at this stage of development, the Ire1 pathway might be activated in the inner ear hair cells of *cdh23* mutants and the Perk pathway is activated in the inner ear hair cells and retinal cells.

*3.2 Effect of FDA approved drugs blocking different branches of the Ire1 pathway in *cdh23* mutants*

From the immunohistochemistry studies, I found not only that the Perk pathway is activated in the inner ear hair cells of the *cdh23* mutants, but that the Ire1 pathway may also be activated in this tissue. These preliminary results allowed me to select among FDA approved drugs that can specifically inhibit a UPR pathway or part of a UPR pathway.

3.2.1 Effects of FDA approved drugs on ER stress

To assay the efficiency of the FDA approved compounds chosen, I measured the amount of ER stress in treated and untreated *cdh23* mutants and siblings using the *cdh23^{tj264};Tg[myo6b:Kdel-crimson]b1319* mutant line. This line contains a transgene that allows expression of a ER stress marker specifically in the hair cells. The fluorescence of this marker allows quantification of ER stress.

For this assay, I treated mutant *cdh23^{tj264}* and their siblings with the FDA approved drugs Losmapimod and SP600125. Both drugs target the Ire1 pathway, but at two different levels. Losmapimod targets the mitogen-activated protein kinase (MAPK) branch of the pathway, whereas SP600125 targets the c-Jun N-terminal kinase (JNK) branch. Two separate experiments were carried out to assess the both drug's effects over a different amount of time.

In the first experiment, *cdh23* mutants and siblings were exposed to Losmapimod or SP600135 from 4 dpf for 24 hrs (Figure 22A-F). No significant difference in the level of ER stress was observed between untreated and Losmapimod

treated larvae (Figure 22C,D,G). However, *cdh23* mutants treated with SP600125 showed a highly significant decrease in the level of ER stress when compared to untreated mutants (Figure 22E-G). Interestingly, the level of ER stress was comparable to this of siblings treated or not with SP600125. These results support the hypothesis that the Ire1 pathway is activated in the inner ear hair cells.

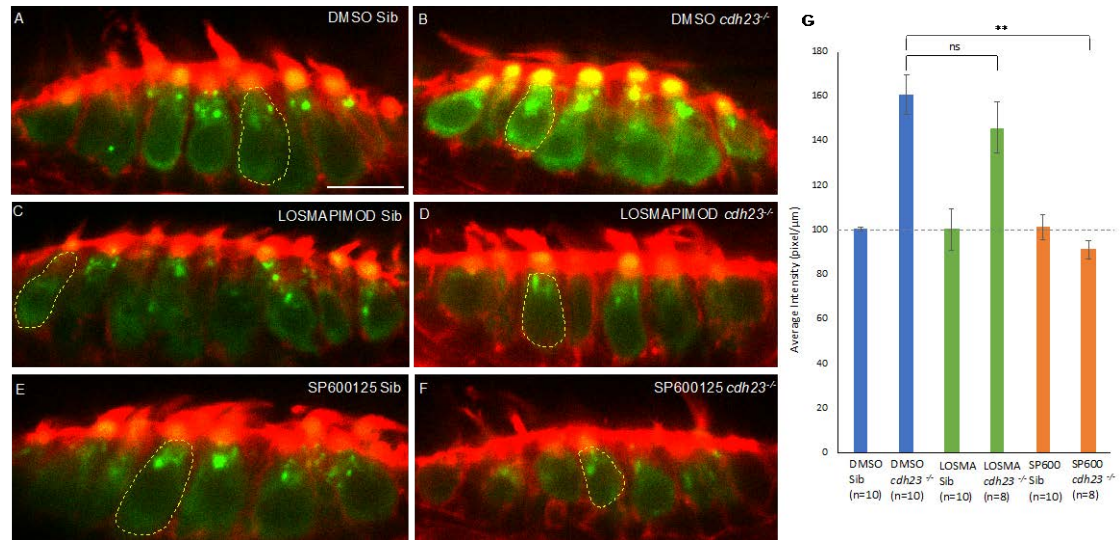


Figure 22: ER Stress level in the hair cells of the anterior macula. DMSO (control) treated siblings (A) and *cdh23* mutants (B). Losmapimod treated siblings (C) and *cdh23* mutants (D). SP600125 treated siblings (E) and *cdh23* mutant (F). (A-F) Actin in red, ER Stress marker in green. Yellow dashed lines enclose single hair cells. (G) Average intensity (pixels/μm) per hair cell for each drug condition. Black lines represent $\pm$ S.E.M. ** $p < 0.01$; ns = not significant. Scale bar: 10 μm.

Because it is possible that longer exposure, or exposure at an earlier stage in development leads to a stronger effect on the outcome of ER stress, I ran a second experiment where 3 dpf *cdh23* mutants and siblings were exposed for 48 hrs to Losmapimod or SP600125. Both sibling and mutant larvae that received SP600125 were necrotic and/or dead at 5 dpf. I was therefore unable to obtain data for this drug at this time point. However, I was able to analyze the level of ER stress in the larvae treated with Losmapimod (Figure 23). I did not find a significant decrease in the level

of ER stress in *cdh23* mutants that received Losmapimod compared to mutants that did not receive the drug (Figure 23E).

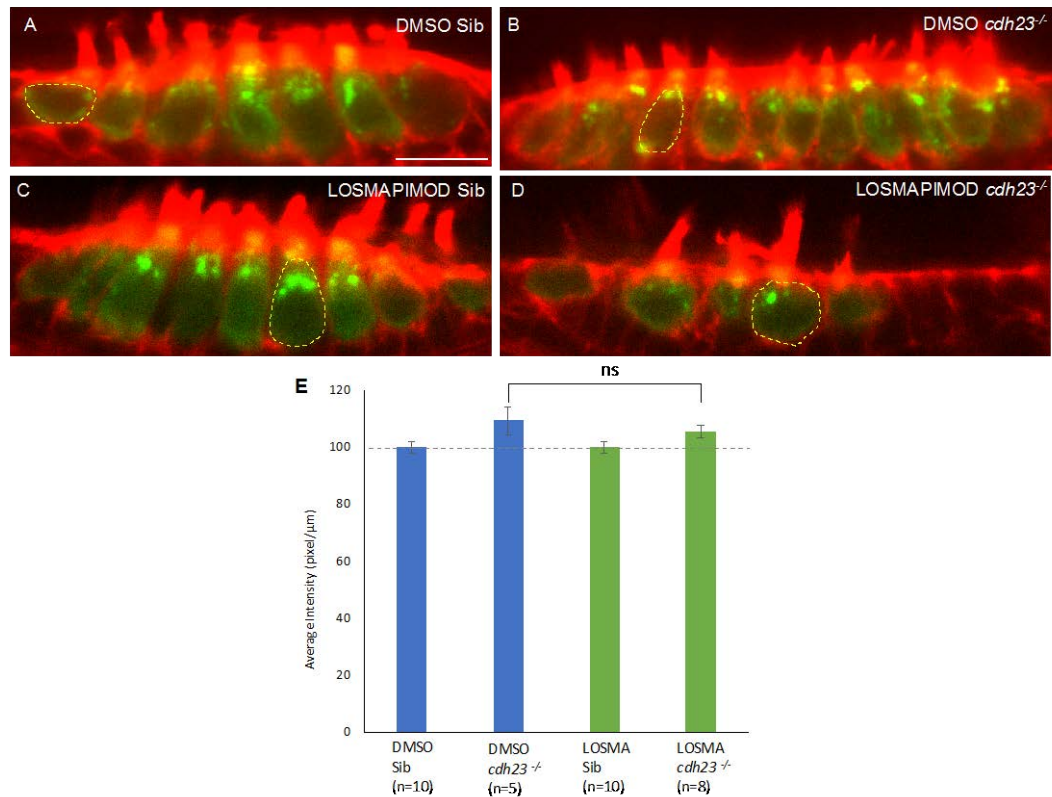


Figure 23: ER Stress level in the hair cells of the anterior macula. DMSO (control) treated sibling (A) and *cdh23* mutant (B). Losmapimod treated sibling (C) and *cdh23* mutant (D). (A-D) actin in red, ER Stress marker in green. Yellow dashed lines enclose single hair cells. (E) Average intensities (pixels/μm) per hair cell for each drug condition. (H). Black lines represent $\pm$ S.E.M. ns = not significant. Scale bar: 10 μm.

3.2.2 Effects of FDA approved drugs on mechanoreceptor integrity and behavior

cdh23 mutants present with disrupted hair bundle morphologies (Blanco Sánchez et al., 2014; Nicolson et al., 1998; Söllner et al., 2004) and circle or do not move as a response to the startle response test. Therefore, I also analyzed the behavior

and the hair bundle integrity of *cdh23* mutants treated with Losmapimod at 3 dpf for 48 hrs and 4 dpf for 24 hours or with SP600125 at 4 dpf for 24 hours.

Behavior was observed prior to genotyping and was evaluated with the startle response test, which assays vestibular function and evaluates the ability of the larvae to swim away from sound or vibration (Kimmel et al., 1974; Zeddies and Fay, 2005).

After genotyping, I found that larvae that responded to the startle by swimming away were wild-type or

heterozygous, and that larvae unresponsive to the startle or would occasionally circle were homozygous mutants (Table 5 and 6). Importantly, I did not observe a rescue of the mutant

	Wild-type like behavior	USH1 like behavior
DMSO siblings	18	0
DMSO <i>cdh23</i> mutants	0	6
Losmapimod siblings	34	1
Losmapimod <i>cdh23</i> mutants	0	8

Table 5. Behavior analysis of *cdh23* siblings and mutants treated with Losmapimod at 3 dpf for 48 hrs. Wild-type like behavior refers to responding to a startle or poke by swimming away. USH1 like behavior means circling or no response after startle or poke.

behavior in mutants treated with Losmapimod or SP600125 at either time point.

Following the behavior analysis, siblings and *cdh23* mutants treated with Losmapimod or not from 3 dpf were labeled with phalloidin to allow examination of the hair bundles. For this analysis, the

	Wild-type like behavior	USH1 like behavior
DMSO siblings	11	0
DMSO <i>cdh23</i> mutants	0	7
Losmapimod siblings	11	0
Losmapimod <i>cdh23</i> mutants	0	11
SP600125 siblings	10	1
SP600125 <i>cdh23</i> mutants	0	10

Table 6. Behavior analysis of *cdh23* siblings and mutants treated with Losmapimod or SP600125 at 4 dpf for 24 hrs. Wild-type like behavior refers to responding to a startle or poke by swimming away. USH1 like behavior means circling or no response to startle or poke.

larvae were mounted laterally but slightly tilted to better visualize the whole anterior macula.

Both *cdh23* siblings and mutants display a range of hair bundle phenotypes (Figure 24). These phenotypes were examined in detail and recorded to allow assessment of the differences between treated and untreated *cdh23* mutants and siblings.

I counted the total numbers of hair bundles and hair cells in the anterior macula and extensively examined the morphology of the hair bundles within a predetermined section of the anterior macula (Figure 25). Despite a small increase in the amount of hair bundles and hair cells in

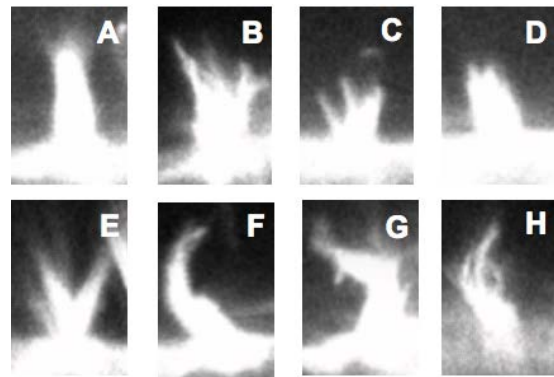


Figure 24: Range of hair bundle phenotypes. WT hair bundle phenotype (A). *cdh23* mutant hair bundle phenotypes: extreme splay (B), splay (C), slight splay (D), two-prong (E), hook (F), extreme hook (G), combination of morphologies (hook and splay) (H).

Losmapimod treated *cdh23* mutants versus untreated mutants, the differences were not significant (Figure 26A,B,D,E and Figure 27A,B). Similarly, although *cdh23* mutants treated with Losmapimod had more wild-type like hair bundles and fewer splayed hair bundles compared to untreated mutants, there was no significant rescue of the hair bundle morphology (Figure 27C).

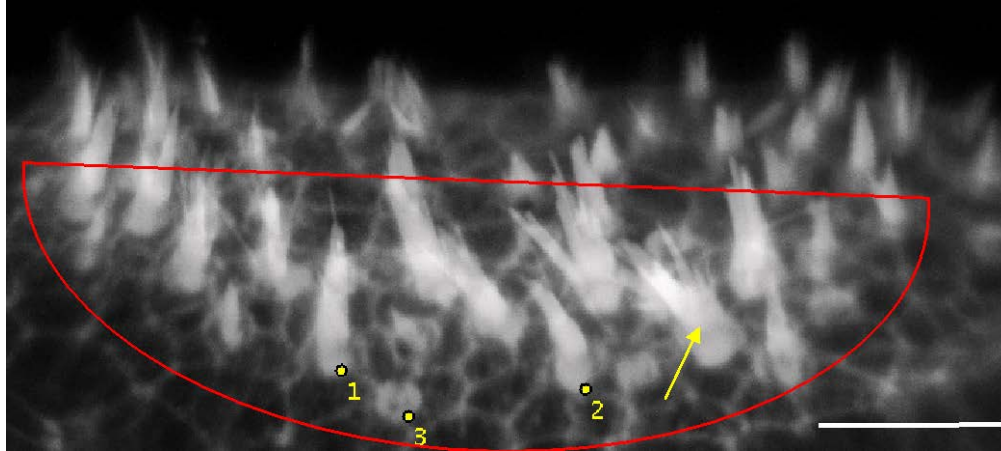


Figure 25: Anterior macula of a *cdh23* mutant. Red half circle refers to the section of the macula analyzed for hair bundle morphology. Numbers refer to how counting was done: 1 and 2 are bundles, 3 is a hair cell without a bundle. Yellow arrow points to a splayed hair bundle. Scale bar: 10 μ m.

3.3 Effect of inhibition of the Perk pathway on behavior and hair bundle integrity in *cdh23* mutants

To further investigate the Perk pathway, I took advantage of the genetics and utilized a mutant line carrying not only a mutation in *cdh23* but also in *eif2 α* , a gene encoding a downstream component of the Perk pathway, directly activated by Perk. The mutation in *eif2 α* should prevent its activation and therefore the triggering of the downstream events, thus blocking the Perk pathway. With this tool, I specifically wanted to see if inhibition of the Perk pathway in the *cdh23* mutants would lessen or alleviate the hair bundle morphology phenotype. The strategies used to quantify hair bundles and hair cells and analyze hair bundle morphology were the same as described above.

No significant difference was observed in the number of hair bundles and hair cells between *cdh23* mutants and *cdh23*;*eif2 α* double mutants (Figure 27A,B). Although the number of wild-type like hair bundles is higher in double mutants

cdh23;eiF2α compared to *cdh23* mutants, the difference was not significant (Figure 27). To summarize, I did not observe a significant rescue of the *cdh23* mutant phenotypes from blocking the Perk pathway.

3.4 Effect of combined inhibited Ire1 and Perk pathways on hair bundle integrity in *cdh23* mutants

As a final point of analysis, I wanted to see the effects of inhibiting both the Perk and Ire1 pathways in *cdh23* mutants by treating the *cdh23;eiF2α* double mutants and siblings with Losmapimod at 3 dpf for 48 hrs (Figure 26).

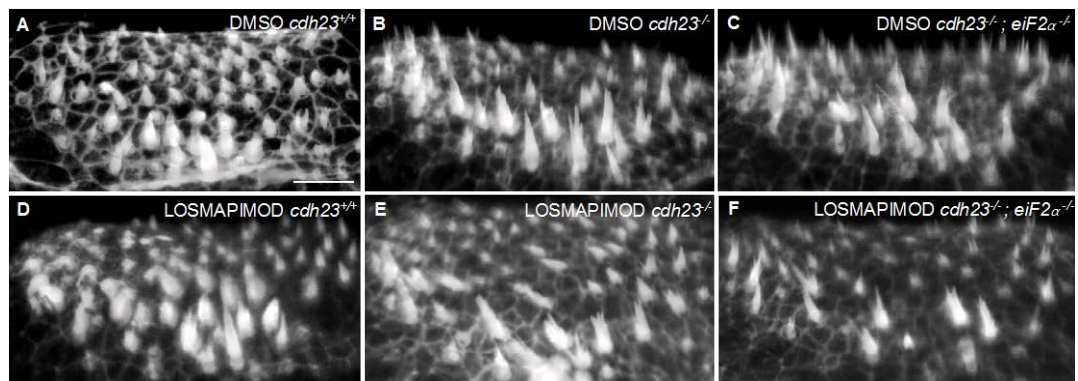


Figure 26: Actin labeling of the anterior macula. DMSO treated siblings (A), *cdh23* mutants (B) and *cdh23; eiF2α* double mutants (C). Losmapimod treated siblings (D), *cdh23* mutants (E) and *cdh23; eiF2α* double mutants (F). Scale bar: 10 μm.

Using the same strategies as above, I counted the total numbers of hair bundles and hair cells and analyzed the hair bundle phenotypes (Figure 27). I did not find a significant change in the numbers of hair bundles and hair cells in treated *cdh23;eiF2α* double mutants and siblings (Figure 27A,B) nor did I find a rescue of hair bundle morphology (Figure 26 and 27C). It is interesting to note that in larvae treated with Losmapimod, the amount of wild-type like hair bundles is decreased to the level of the

untreated *cdh23* mutant, suggesting that with the current conditions, the Losmapimod might affect hair bundle morphology.

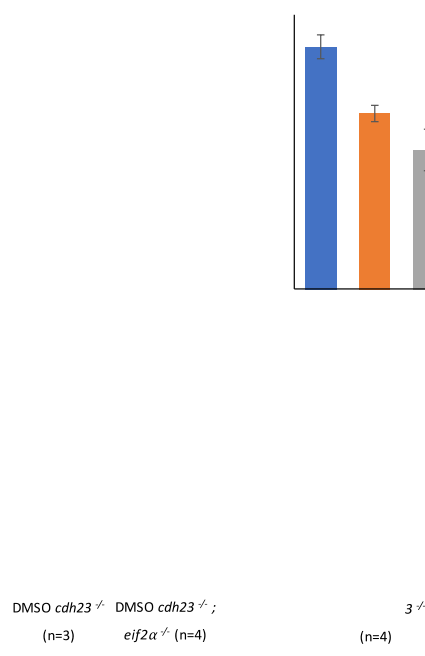


Figure 27: Quantification of anterior macula phenotypes. Graphs representing the numbers of hair bundles (A), hair cells (B). Bold numbers are average numbers of hair bundles (A) and hair cells (B) per larvae. Hair bundle phenotypes in DMSO and Losmapimod (LOSMA) treated *cdh23* mutants, *cdh23*;*eiF2α* double mutant and siblings larvae (C). ns = not significant.

4. Discussion

Past research on USH1 using zebrafish as a model system has provided essential information to comprehend the mechanisms leading to the disease. The discovery that ER stress might be the cause of some of the USH1 symptoms has allowed me to explore new possibilities of treatments.

This thesis looked specifically at one USH1 zebrafish gene, *cdh23*, and aimed first, to investigate the ER stress related pathways activated due to this mutation, and second, to test FDA approved drugs that would target these pathways and assay for rescue of the observed *cdh23* mutant phenotypes.

4.1 Activation of the UPR pathways in inner ear hair cells and retinal cells of cdh23 mutants

Here I have presented preliminary data that indicate that the Perk signaling pathway is activated in the inner ear hair cells of 5 dpf *cdh23* mutants. Our data support the finding in *Cdh23* mutant mice (Hu et al., 2016). Our results also show that the Ire1 pathway is likely activated in these cells. It is important to point out that my experiments were run at only one time point. It is possible that with development and continued ER stress, the Ire1 pathway becomes activated leading to an increase in levels of phosphorylated Ire1. To test this, we would need to look at the activation of the Ire1 pathway at later stage of development.

In the retina, I found that the Perk pathway is activated in *cdh23* mutants. The significant decrease in the total amount of Ire1 protein in the *cdh23* mutants could also

reflect the activity of the Perk pathway, as it is known to attenuate translation (Kim et al., 2008). However, I cannot rule out that the Ire1 pathway is not activated at later stages of development. Past research has shown that photoreceptor death within the retina is increased at 8 dpf in *ush1c* zebrafish (Phillips et al., 2011). Therefore, more research and adjustment of experimental conditions may be necessary.

Another reason why the Ire1 pathway does not seem activated could be the high variability I observe between the larvae. Despite running the experiments in duplicate or triplicate, the sample size is still small. By increasing the number of mutants and siblings for each condition, I would have a more representative measurement of what is occurring in the *cdh23* population.

4.2 Effect of FDA approved drugs on inner ear hair cells in cdh23 mutants

I found that the increase in ER stress observed in the *cdh23* mutants can be diminished by addition of SP600125, a FDA approved drug inhibiting the JNK branch of the Ire1 pathway. These results support the hypothesis that the Ire1 pathway might be activated in the inner ear hair cells. Addition of Losmapimod which inhibits another branch (MAPK) of the Ire1 pathway, did not have such an effect, suggesting that, at this stage of development, this part of the Ire1 pathway is not activated. Applying the drugs at variable stages of development and/or adjusting their concentration may have a greater effect on hair bundle morphology or even behavior.

With the current data, we do not know if the Ire1 and Perk pathways act together or independently. The third UPR pathway, Atf6, could also be affecting the phenotypes of *cdh23* mutants. Because we are unsure at this point of how these three pathways interact, it is possible that affecting one pathway could lead to changes in another.

The findings from my research have important implications for both the future study and potential treatment for USH1. Additional research building from these results will give an even better understanding of the molecular mechanisms leading to USH1 and the potential treatment of this syndrome.

5. Future Directions

Overall, the results from this study have increased our understanding of the mechanisms underlying USH1, but it has also brought forth many new questions and points of future research regarding *CDH23* and other USH1 genes.

The third UPR pathway, Atf6, has not been investigated in *ush1* zebrafish mutants. To build on our understanding of the effects of these pathways on USH1 phenotypes, we could investigate the effects of this pathway as well as its interaction with Ire1 and Perk.

It would also be interesting to look at other FDA approved drugs that affect other parts of the UPR pathways and see if it results in a rescue of the *ush1* mutant cell death level, phenotypes, and behavior. For example, the Ire1 pathway breaks into three separate pathways (Kim et al., 2008). I investigated two of them here, the MAPK and the JNK pathways. The third pathway is an RNase splicing pathway, which can be affected by other drugs (Kim et al., 2008). Toyocamycin has been shown to inhibit ER stress by blocking this latter pathway in human epithelial cells with multiple myeloma *in vitro* (Ri et al., 2012). Using drugs like Toyocamycin that target other parts of the UPR pathways may shed more light on the exact mechanism that leads to phenotypes of USH1 in zebrafish and in human patients. The work on SP600125 is definitely worth pursuing.

Finally, it would be valuable to investigate if the results obtained here are consistent across the various USH1 zebrafish mutants (*ush1c*, *cdh23*, *myo7aa* and *pcdh15a*).

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