Actin Cytoskeletal Organization in CDH23 Mutant Mice

By

Grant Baxter

April, 2014



Allegheny College

Department of Biology

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A Senior Comprehensive Project in Partial Fulfillment of the Requirements for a Bachelor of Science Degree from Allegheny College

I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code

and to maintain the integrity of both myself and the College community as a whole.

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Pledge

Approval by the Senior Thesis Committee:

_____

Dr. Donmoyer

_____

Dr. Nelson

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Dr. Donmoyer, big thanks for being such a good comp advisor. Thanks especially for always being patient with me, even when my comp was in limbo for several months while I fruitlessly tried to get mice. I can't imagine you felt very comfortable with the status of my comp at times, and I knew there were problems, so it was always a big relief when you never yelled at me about it. Who knows how I would've gotten it done without your patience and availability.

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Abstract

Usher syndrome is a genetic disorder resulting in deaf-blindness if the genes are defective or missing. CDH23 is one example of a gene responsible for Usher syndrome, thought to play a role in development of the ear's stereocilia and the eye's retina. This study examined a possible pathologic mechanism for Usher symptoms in mice with mutated CDH23: abnormalities in the actin cytoskeleton. The hypothesis was that defects would be noticeable in regions between cells using images with a fluorescent actin stain. Compared to age-matched wild-type mice, CDH23 mutants possessed thinner posterior eye tissue and cells in the retina were free-floating and did not connect to one another as cells in the wild-type retina did. Optic nerve cells were joined together in long filaments in wild-type eyes, while cells formed amorphous globules in mutant CDH23 eyes. This provides some evidence that actin cytoskeleton disorganization may be a possible mechanism for Usher symptoms.

Introduction

Usher Syndrome

Usher syndrome of the human auditory and visual systems describes ten genetic mutations that result in deaf-blindness. Usher syndrome is a rare condition, affecting only 1 in 23,000 in the United States, but it is nevertheless the leading cause of deaf-blindness, accounting for over 50% of deaf-blindness worldwide (Boughman *et al.* 1983, Vernon 1969). Usher syndrome is divided into three clinical subtypes, based on the rate of hearing loss progression; type 1 exhibits the fastest progression and type 3 the slowest. Usher syndrome-associated genes are distinguished by their importance in development and function of both the auditory and visual systems, resulting in the deaf-blind phenotype.

Visual function is lost progressively due to retinitis pigmentosa in all three Usher syndrome types. Patients with Usher syndrome 1 are born deaf, while types 2 and 3 retain hearing to varying degrees. Patients with type 2 are typically hard-of-hearing but not deaf. Patients with type 3 exhibit progressive hearing loss over many years. Mutations in six different genes have been associated with Usher syndrome 1, three mutations with type 2, and one with type 3 (Millan et al 2011; refer to Table 1). Cases of Usher syndrome are further described with a letter after the type number, denoting the exact gene responsible (e.g. Usher syndrome type 1 arising from a defect in the MYO7A gene is called type 1B). Usher genes encode for a variety of proteins with different roles. Because these proteins are so different, the exact mechanism by which each genetic defect produces

Usher symptoms is not known for every Usher gene (Cosgrove and Zallocchi 2014). Usher proteins, however, are often crucial in development of both the ear and the eye (Adato et al 2002, Cosgrove and Zallocchi 2014).

Type	Gene	Protein
USH1B	*MYO7A*	Myosin VIIA
USH1C	*USH1C*	Harmonin
USH1D	*CDH23*	Cadherin 23
USH1E	?	?
USH1F	*PCDH15*	Protocadherin 15
USH1G	*USH1G*	SANS
USH2A	*USH2A*	Usherin
USH2C	*GPR98*	VLGR1b
USH2D	*DFNB31*	Whirlin
USH3A	*CLRN1*	Clarin-1

Table 1. An overview of the genes responsible for different Usher syndrome types. www.wikipedia.org

Cadherin 23

One Usher gene is CDH23, which encodes the protein cadherin 23. CDH23 is associated with Usher type 1D (Bork et al 2001; also see Table 1). Three distinct isoforms of cadherin 23 have been identified, splice variants of different lengths (Reiners 2003). Like other proteins in the cadherin family, cadherin 23 is involved in cell-cell adhesion, *i.e.* holding separate cells together. Cadherin 23 has been found to localize to the photoreceptors (Siemens et al 2002) in the retina as well as the area around the ear's stereocilia, small hairs that aid in detection of sound (Cosgrove and Zallocchi 2014). Cadherin 23's function in the ear is better understood than its function in the retina (Cosgrove and Zallocchi 2014).

This study examined whether actin cytoskeletal disorganization in the retina is a cause of Usher symptoms in type 1D, for the following reasons: cadherins are known to associate with actin, and other Usher proteins like Clarin-1 (associated with Usher type 3A) are also known to cause actin

abnormalities in the stereocilia and in the retina (Adato et al 2002).

Actin

Actin is an abundant protein present in all eukaryotes. Actin forms microfilaments, one of the three major components of the cytoskeleton. The cytoskeleton helps a cell to maintain its shape and structure against external physical pressure. The cytoskeleton serves a wide variety of other roles, such as aiding cell division and providing "rails" for organelle movement (Doherty et al 2008). It is found in two forms: F-actin, which forms the long filaments of muscle and the cytoskeleton, and G-actin, which forms free globules in the cell. F-actin is simply made up of many G-actin monomers linked together. Cadherins anchor themselves to the actin filaments using several intermediate proteins such as catenin, α-actinin, and the vinculin family (see Figure 1). These intermediate proteins anchor F-actin to the proteins in the cell membrane.

If cadherin 23 is missing or defective, it is likely that actin will not be able to anchor to it properly, resulting in poor cytoskeletal organization. This is the basis for this study.

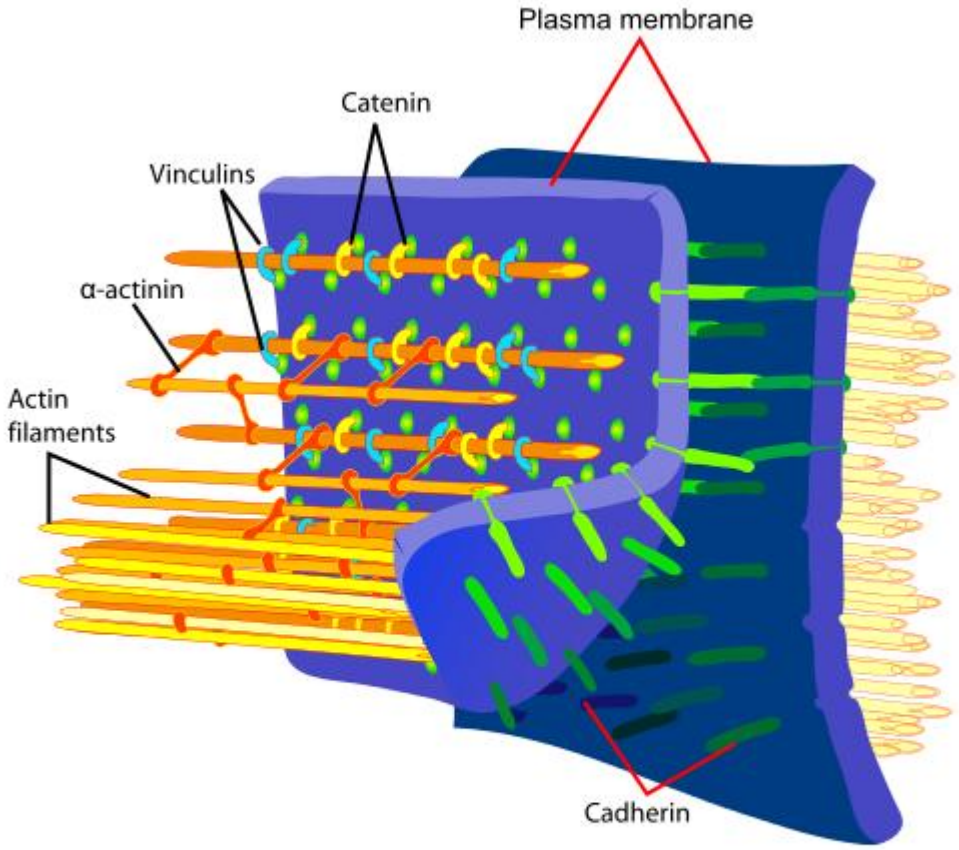


Figure 1. A drawing of actin-cadherin interactions near the edge of a cell. F-actin associates with cadherin via a number of intermediary proteins like vinculins and alpha-actinin. Cadherins on the cell membrane link up with other cadherins on the neighboring cells (Figure from public domain, Wikimedia Commons).

Actin cytoskeletal organization was assessed in a CDH23 mouse mutant called CDH23^{ahl} (ahl stands for 'age-related hearing loss'), which produces symptoms like those of Usher syndrome 1D (Bork 2001). Mice with this mutation display hearing and vision loss from birth. The mice strain used are DBA/2J, an inbred strain from Jackson Labs (Bar Harbor, ME) possessing this mutation (among others, see Appendix B). C57BL/6J mice were selected as the wild-type based on availability. F-actin was stained

with a fluorescent marker and visualized via fluorescence microscopy.

Hypothesis

Actin organization in CDH23 will show abnormalities in the regions between cells, due to being unable to properly link with the cadherin 23 mutant protein. These abnormalities should localize to the retinal photoreceptors, where cadherin 23 localizes (Reiners 2003).

Methods

Age-matched groups of C57BL/6J (wild-type) and DBA/2J mice were used, with a sample size of n=3 for both groups. C57BL/6J mice came from the animal colony at Allegheny College. Jackson Labs supplied the DBA/2J eyes already embedded in paraffin cassettes. DBA/2J mice were six weeks of age at enucleation, while C57BL/6J mice were 6-7 weeks of age. The age of six weeks was selected so that eyes would be full-sized; Usher syndrome 1 is not progressive, and older ages are not expected to produce different phenotypes.

Enucleation & Fixation

C57BL/6J mice were euthanized via 2 minutes of CO_2 inhalation followed by cervical dislocation as approved by the Animal Research Committee of Allegheny College. The eyes of the mice were then enucleated, *i.e.* the intact eye was removed from the socket with forceps

and placed immediately into fixative (Moshfeghi et al 2000).

Enucleated C57BL/6J eyes were fixed for 24 hours in 200 µl of Baker's Formalin Fixative (BFF) in individual microcentrifuge tubes. Stock solution of BFF was prepared with 1.0 g cadmium chloride, 1.0 g calcium chloride, and 10.0 ml of formalin in 100 ml of deionized water. DBA/2J eyes were fixed with 6% neutral buffered formalin (NBF); an example preparation of NBF includes 1.0 grams of sodium phosphate and 10 ml of 37% formaldehyde into 90 ml of distilled water.

Paraffin Embedding

The C57BL/6J eyes were dehydrated in four one-hour 100% ethanol baths, then left overnight in another 100% ethanol bath. After dehydration, eyes were embedded in paraffin blocks following the protocol from Sainte-Marie 1962. All embedded eyes were then sectioned with a Reichert-Jung 1130 Biocut rotary microtome into 3-5 µm slices. The slices were placed in a water bath at 60°C for about 1 minute to flatten them, and were then placed onto microscope slides.

Phalloidin Staining

Each section was bathed in phosphate-buffered saline (PBS) with 0.1% Triton X-100 for 2-3 minutes to permeabilize the cells. Eye sections were then stained with phalloidin, an actin-binding protein from the mushroom *Amanita phalloides*, fluorescent-labeled with fluorescein isothiocyanate (FITC). Phalloidin binds to F-actin with high specificity. FITC-labeled phalloidin from Sigma Aldrich was stored as a powder in the dark at -20°C,

wrapped in a light-resistant cloth in a tin canister until the day of staining. One mg of the powder was reconstituted in 1.0 ml methanol, then diluted to 50 µM in PBS at room temperature. The eye sections were stained in this solution for 20 minutes. After staining, the sections were rinsed twice with PBS briefly (15 seconds) to wash away excess, unbound phalloidin.

After this preparation, actin cytoskeletal organization in the eye sections was assessed immediately via fluorescence microscopy. Images were visualized on a Nikon Eclipse E600 fluorescent microscope using the FITC setting (fluoresces at approximately 495 nm). Digital images were acquired with SPOT software and an RT slider camera, both from Diagnostic Instruments. Most images were taken at 20X magnification. Light microscopy images were taken on the same microscope. Eyes were visualized in the sagittal plane, from a nasal view (see Figure 2).

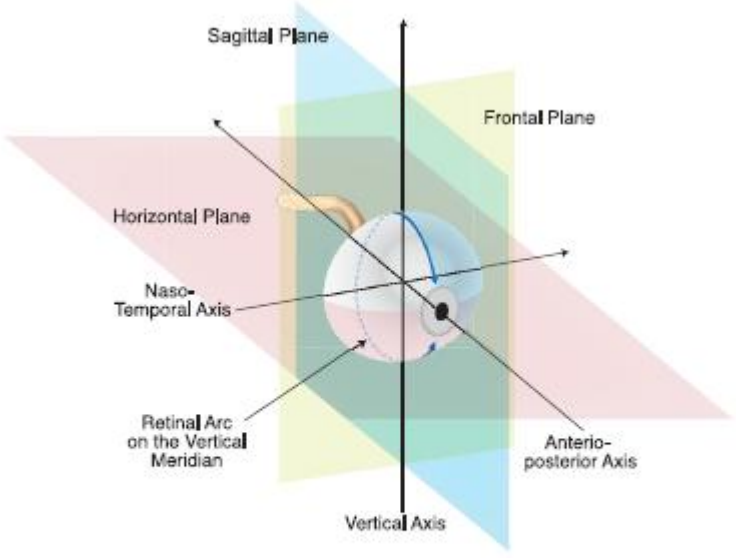


Figure 2. Diagram of planes and axes of the vertebrate eye. Eyes were imaged in the sagittal plane, from the nasal side, the left end of the naso-temporal axis in this diagram (Figure from Wisard et al 2011).

The regions between cells were of particular interest, specifically, if actin was correctly linked between cells. Defective cadherin 23 protein would be expected to interfere with proper actin linkage between cells.

Results

Mouse eyes were imaged on the fluorescent microscope, with particular attention to the tissue around the posterior of the eye (connected to the optic nerve) and the retina. Sections used a nasal view of the eye, *i.e.* looking from the nose side of the eye toward the outward side. Light microscopy images showed no noticeable differences between strains (Figure 3A and 3C). Actin staining images showed significant abnormalities. Posterior tissue in wild-type eyes adhered together into a solid ribbon structure, whereas tissue in $Cdh23^{ahl}$ eyes was thinner and lacked good cell-cell adhesion, forming disorganized, amorphous globules (Figure 3B and 3D). Figure 3 shows representative images, more examples can be found in Appendix A.

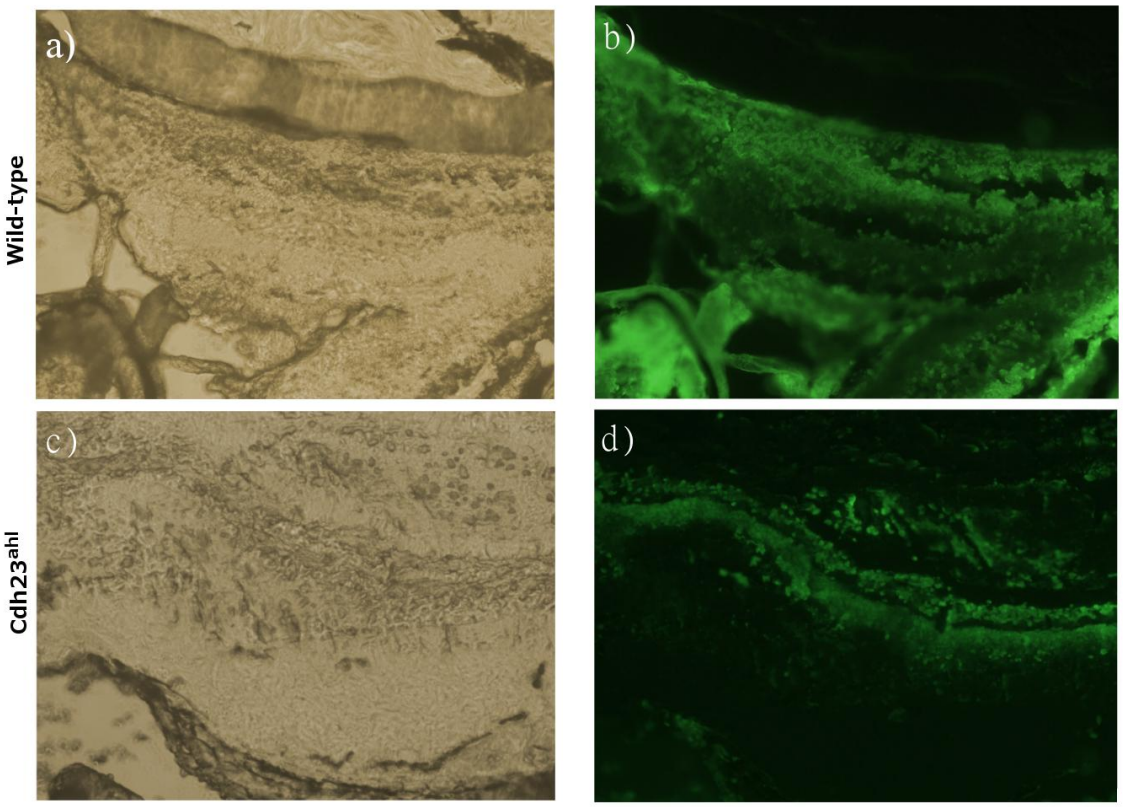


Figure 3. FITC-phalloidin-stained mouse retinas and posterior eye tissue. Images are at 20X magnification, nasal view with eyes oriented with the anterior pole facing upwards in the plane of the page. A) Light microscopy of a wild-type mouse retina. B) FITC-phalloidin stain image of the same region in the wild-type eye. C) Light microscopy of a DBA/2J mouse retina. D) FITC-phalloidin stain of the same region in the DBA/2J eye.

Additionally, significant abnormalities were observed in the optic nerves of Cdh23^{ahl} mice. Actin at the optic nerve showed long, filamentous stretches, while Cdh23^{ahl} nerves showed scattered bunches of cells that did not adhere together (Figure 4).

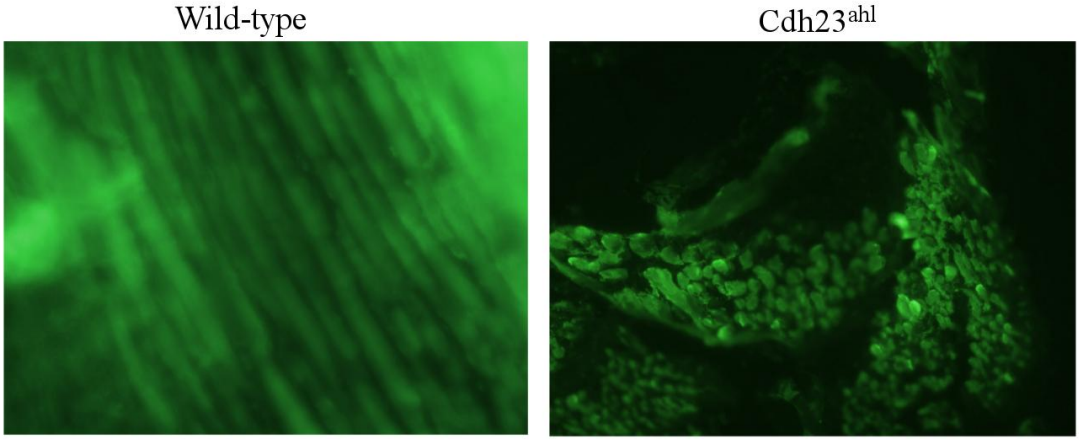


Figure 4. FITC-phalloidin stains of optic nerves. Images are at 40X and 20X magnification, respectively, nasal view with eyes oriented with anterior pole facing upwards in the plane of the page. A) FITC-phalloidin stain of the wild-type optic nerve. B) FITC-phalloidin stain of a DBA/2J optic nerve.

Discussion

This study examined whether symptoms of Usher syndrome type 1D are due to poor actin cytoskeleton organization. Other types of Usher syndrome also involve actin-related proteins, like protocadherin 15 (type 1F) and Clarin-1 (type 3A). Research on Usher proteins in the eye is somewhat lacking, and most research has focused on hearing loss. In the retina, little has been confirmed except the presence of many Usher-1 proteins in the mouse photoreceptor (Reiners 2003). Harmonin, cadherin 23, and myosin VIIA have all been found to localize to the photoreceptor (Reiners et al 2003).

A finding of actin disorganization and poor actin linkage between cells supports the hypothesis that this Usher syndrome mutation causes

actin abnormalities in the eye that may lead to vision loss. This is consistent with other research on the hearing loss aspect of Usher syndrome, where defects in actin stereocilia in the cochlea are observed (Cosgrove and Zallocchi 2014). In past research these stereocilia defects have been ascribed to the problems in the mechanotransduction apparatus in the ear, rather than the actin organization of the stereocilia itself. Proponents of this idea acknowledge that this does not explain the visual symptoms (Cosgrove and Zallocchi 2014). The defects in actin cytoskeletal organization observed in this study may provide another avenue of research into the pathologic mechanism of Usher proteins. Further investigation could include examination of actin organization in retina specimens of other Usher syndrome types.

Limitations

The DBA/2J mice used for this study are an inbred strain with a number of gene mutations besides the $CDH23^{ahl}$ mutation of interest. While none of these other mutations are known to be linked with cell-cell adhesion besides CDH23, they are nonetheless confounding factors that may have contributed to the actin phenotype in these mice. Thus, it is difficult to say with certainty that the observed differences from the wild-type retinas were due to the $CDH23^{ahl}$ mutation and not one or a combination of the others. In particular, DBA/2J mice possess a mutant form of the Fscn2 gene, known to be involved in actin bundling. In future research, a dedicated CDH23 knockout would be advantageous.

A more in-depth investigation might also include mutants or knockouts of other Usher syndrome type 1 genes such as USH1C and PCDH15, with which cadherin 23 is known to interact (Kazmierczak *et al.* 2007 and Siemens *et al.* 2002). The functions of USH1C and PCDH15 are intact in the DBA/2J mice used in this study. Employing some method of statistical rigor to the images acquired would also yield more powerful results.

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Appendix A: Additional FITC-Phalloidin Images

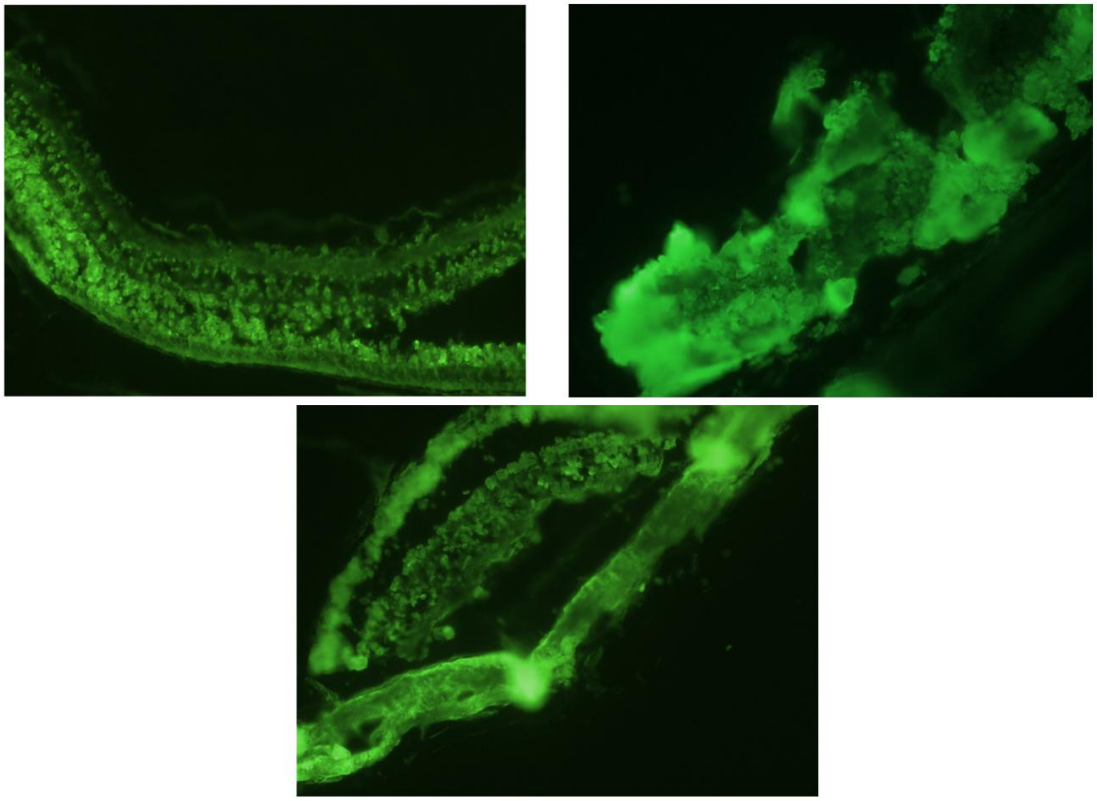


Figure 5. Additional FITC-phalloidin images of wild-type eyes. All images are at 20X magnification, nasal view. Eyes are oriented with the anterior pole facing upwards in the plane of the page.

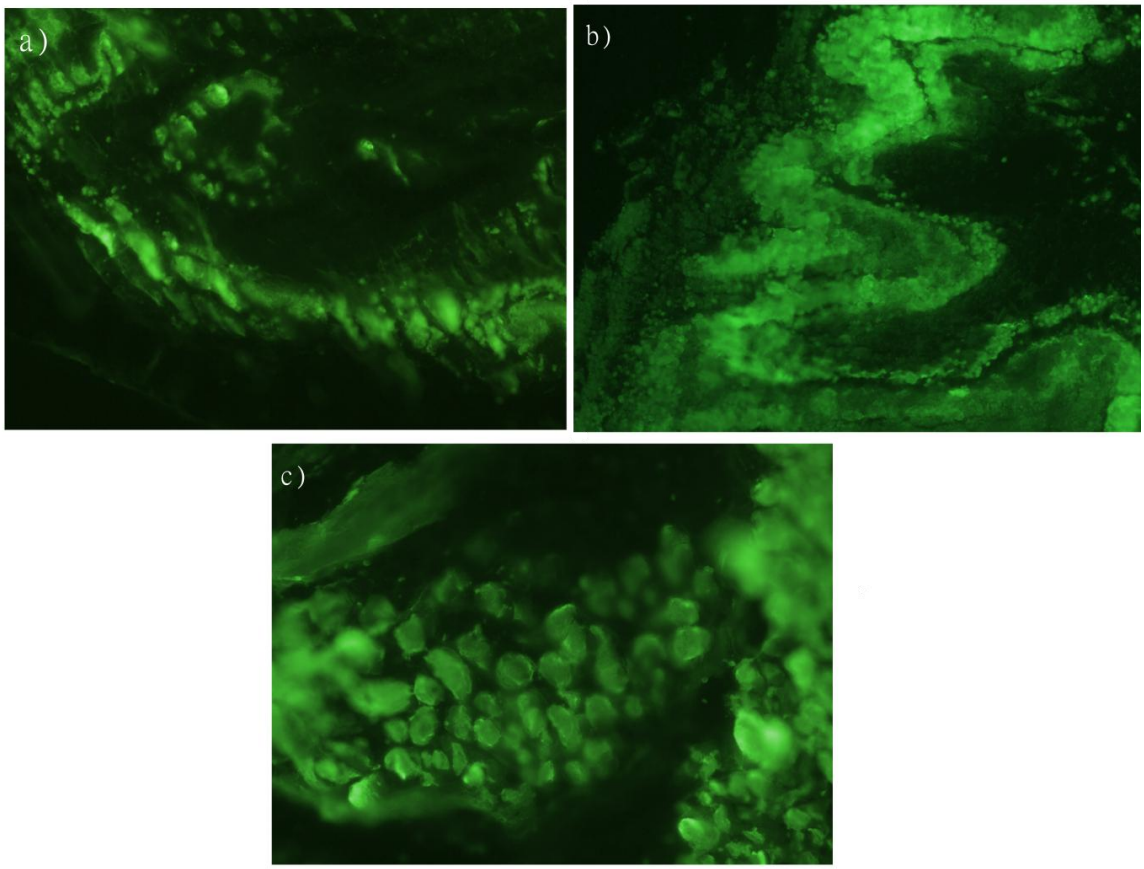


Figure 6. Additional FITC-phalloidin images of CDH23 mutant eyes. Images are at 20X magnification, nasal view. A & C) Eyes oriented with the anterior pole facing upwards in the plane of the page. B) Eye oriented with anterior pole facing to the right in the plane of the page.

Appendix B: List of DBA/2J Mutations
(Information adapted from Jackson Labs info sheet)

a – nonagouti

Ahr^{d} – aryl hydrocarbon receptor
- Mice with Ahr^{d} exhibit decreased inflammatory response to dimethylbenzanthracene applied to the skin, and have lower susceptibility to some subcutaneous and lung sarcomas.

asp2 – audiogenic seizure prone 2

$Cdh23^{ahl}$ – age related hearing loss 1

Fbrwt1 & Fbrwt2 – forebrain weight 1 & 2

$Fscn2^{ahl8}$ – age related hearing loss 18

$Gpnmb^{R150X}$- iris pigment dispersion
- DBA/2J mice develop glaucoma associated with cell loss from iris stromal atrophy.

Hc^{0} - hemolytic complement
- Associated with lung susceptibility to airborne allergens.

Klrd1 – killer cell lectin-like receptor

Myo5A – myosin VA

$P2rx7^{P451L}$ – purinergic receptor P2X
- Encodes for a ligand-gated ion channel

$Tyrp1^{isa}$ - iris stromal atrophy
– See above; DBA/2J mice develop glaucoma associated with cell loss from iris stromal atrophy