Shroom3 Localization and Apical Constriction during the Development of the Crystalline Lens in Mouse Embryos

THESIS

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By

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Abstract

Purpose: The purpose of this research was to analyze the localization of the protein Shroom3, and determine its role in regulating lens fiber cell shape during the organization and development of the embryonic lens. It has previously been shown in the lens placode that Shroom3 is a crucial component in the pathway that leads to apical constriction. To determine if this is also the case in the lens fiber cells within the lens bow of developing lenses, the localization of Shroom3 expression was first evaluated, followed by an analysis of the degree of apical constriction.

Methods: Control and Shroom3-deficient mouse embryos were dissected, cryosectioned through the developing lens, and imaged. The images were analyzed following histological labeling using the x-gal assay, and immunofluorescent labeling of specific proteins including Shroom3, beta-catenin, non-muscle myosin IIb and F-actin.

Results: Shroom3 mRNA expression is localized to the cells within the lens epithelium, and the lens fiber cells in contact with the lens fulcrum, but not in older, more centrally located lens fibers. Antibody staining showed Shroom3 protein localization within the membranes of these same cells. This stands in contrast to the more specific apical localization observed in other epithelial tissues.

As the cells were differentiating, they changed from a columnar-shaped cell to a conical-shaped cell. F-actin and non-muscle myosin expression was diminished apically and increased basally in the Shroom3 null mice embryos. Differentiating lens fiber cells
at the lens fulcrum apically constricted less in the Shroom3 null mouse embryos than the controls.

*Conclusion:* Shroom3 directs apical constriction of lens cells as they differentiate from lens epithelial cells to lens fibers in part by modulating the distribution of F-actin and non-muscle myosin.
Dedication

This document is dedicated to my fiancé, Jon.

Thank you for your unconditional love even throughout all of my ramblings of mice embryos and Shroom staining.

You constantly inspire me and support me in any task I pursue.
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Fields of Study

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Chapter 1: Introduction

The crystalline lens is an important optical system within the eye that is mostly comprised of tightly packed and highly ordered cells called lens fibers. Lens fiber cells are elongated, narrow epithelial cells that make up the bulk of the lens mass. The incredible organization of the fibers aids in providing a specific refractive index and maintaining transparency of the lens as a whole. This in turn decreases the amount of unintended light scatter, and focuses incident light in the back of the eye on the retina. Many factors contribute to the transparency and refractive properties of the lens, including the morphogenesis of the crystalline lens fiber cells and the resulting structure of the fully developed lens.

Lens Anatomy

The crystalline lens is a unique structure in the body in that it is made up of only two cell types, the lens epithelial cells and lens fiber cells. These cells are encased by a thick basement membrane called the lens capsule. The outer layer of the anterior lens consists of lens epithelial cells. These epithelial cells migrate and differentiate into lens fiber cells. Lens fiber cells make up a majority of the lens and are conceptually grouped into different layers. The innermost grouping of lens fiber cells is the embryonic nucleus, followed by the fetal nucleus, the adult nucleus, and finally the cortex (Figure 1). Each of these layers surrounds the one before it. The cells making up the central embryonic
nucleus are not associated with the lens capsule and instead form apical-apical and basal-basal connections forming the first seams, or sutures, within the lens. There are multiple areas of the lens where the lens fibers come together and interdigitate during development. These sutures are normal and visible in healthy lenses. There are distinctive shapes to the sutures and these can differ among species (Al-Ghoul et al., 2003).

The lens has the highest protein content in the entire body, and relatively low water content, which provide a high refractive index. A large majority of the proteins within the lens are water-soluble proteins known as crystallins (Hoehenwarter et al., 2006). It has been hypothesized that as the lens ages, α-crystallins bind to β and γ-crystallins, and prevent the formation of high-molecular weight bundles. These aggregations can cause an opacification of the lens resulting in a cataract (Andley, 2007). An absence of organelles is also important for maintaining transparency in the lens and preventing additional light scatter. An extensive cytoskeleton aids in maintaining the lens fiber cells’ specific hexagonal shape as well as their uniform packing structure (Bloemendal et al., 2004). Research has shown the unique structure of the lens fiber cells in normal developing lenses, but it is not known how altering the pathway for cell shape change will affect this unique organization and transparency.

Throughout differentiation, lens cells grow and mature from relatively short, squat columnar-shaped cells to long, hexagonally conically-shaped differentiated cells that reach from the anterior side of the lens to the posterior side. Apical-basal polarity of the lens fiber cells is constantly maintained in fiber growth. The apical membrane, or apical
face, of the epithelial cells abuts the apical ends of inversely oriented lens fiber cells while the basal ends point toward the lens capsule. The apical faces of the lens fiber cells abut one another, and even share junctions with neighboring cells. This aids in cell-to-cell communication, increases stiffness, and decreases the amount of light scatter that passes through the lens.


**Lens Development**

In humans, the development of the crystalline lens begins during the fourth week of gestation. The process of lens development in mice begins at embryonic day 9 (E9)
and, although the timing and rate differs between the human and mouse, the following description of embryonic development is comparable. Prior to the development of the lens, a portion of the brain begins to grow laterally outward and contacts the surface ectoderm (Fig. 2A). This tissue is called the optic vesicle and its contact with the overlying layer of surface ectoderm induces changes essential for further eye and lens development. Around E9 the surface ectoderm adjacent to the optic vesicle begins to thicken and forms the lens placode (Fig. 2B). In a process called invagination, the lens placode bends inward concomitantly with the underlying optic vesicle to form the lens pit and optic cup (Fig. 2C and 2D). By E11 the peripheral margins of the lens pit eventually come together, fuse, and pinch off from the rest of the original surface ectoderm to form a hollow sphere of cells suspended within the optic cup, called the lens vesicle (Fig. 2E). The remaining surface ectoderm will develop into the corneal epithelium (Cvekl and Ashery-Padan, 2014). The posterior cells of the lens vesicle are influenced by multiple growth factors circulating in the nearby vitreous humor. This stimulates the posterior cells to elongate and stretch toward the anterior cells, filling the cavity and becoming what are known as the primary lens fibers (Fig. 2F and 2G) (Griep, 2006). Proliferation and differentiation of the anterior epithelial cells produces the secondary lens fibers (Fig. 2H) (Cvekl and Ashery-Padan, 2014). The adult crystalline lens maintains the same basic anatomy as the developing lens and continues the same developmental processes, although at a slower rate.
The anterior portion of the lens is comprised of a single layer of epithelial cells conceptually divided into three concentric zones. The central zone is found at the anterior pole, and consists of cells similar to stem cells. These cells are usually stable and
do not undergo mitosis unless there is an insult, such as an infection, within the eye (Shi et al., 2014). This zone is peripherally neighbored by additional zones referred to as the pre-germinative zone, the germinative zone, and the equatorial zone. These zones demonstrate the transition from epithelium cells into lens fiber cells. The pre-germinative zone contains cells that occasionally undergo mitosis, and it is an area that contains cells that are in the process of migrating from the central zone to the germinative zone. The germinative zone contains cells that are actively undergoing mitosis and elongating to become new lens fiber cells.

The equatorial zone is the transition point between the lens epithelial cells and lens fibers cells. This zone is found at the lateral aspects of the crystalline lens and is the location where the new lens fiber cells undergo terminal differentiation. After passing through the equatorial zone, the lens fiber cells begin to migrate around the lens fulcrum. The lens fulcrum is the seam made from apical-apical apposition during lens fiber cell differentiation, and is depicted by the asterisk in Figure 3. At the lens fulcrum, the basal aspect of the lens fiber cells migrate along the lens capsule while the apical aspect turns in toward the center of the lens. The pathway around the lens fulcrum involves a narrowing of the apical portion of the cells. This research analyzed three different zones along the lens fiber cell differentiation pathway and subjectively defined these areas as the germinative zone, equatorial zone, and outer cortical fibers (Figure 3).

As the cells migrate around the lens fulcrum, they elongate to reach from the equatorial zone to the posterior portion of the lens (Cheng et al., 2013). As more and more lens fibers are produced and differentiate, they are continuously added on to the
outside of the existing differentiated lens fiber cells. Figure 1H shows the transition of lens epithelium into lens fiber cells near the lens fulcrum, the hexagonal shape of the lens fiber cells with interdigitations at the sutures, and the different layers of nuclei.

Figure 3. (A) Lens bow region (anterior lens is at the top and posterior lens is at the bottom) of an E15.5 mouse lens. Designated zones for cell shape analysis. GZ: germinative zone, EZ: equatorial zone, and OCF: outer cortical fibers. Asterisk (*): tip of lens fulcrum.
As these anterior epithelial cells migrate toward the lens fulcrum there is also an inhibition of cell proliferation (Griep, 2006). The newly divided cells elongate and lose their organelles, undergo cell differentiation, and remodel into lens fiber cells. These cells then begin the gradual formation of “ball-and-socket” junctions with one another. These interdigitations refer to a ball or outpouching of one cell that is firmly inserted into a notch of an adjacent cell (Cheng et al., 2016). These connections increase the strength and uniformity of the refractive index of the lens (Cvekl and Ashery-Padan, 2014), as well as aid in maintaining homeostasis of fluids and ions between the cells (Cheng et al., 2016).

The differentiating cells are osmotically driven to increase in volume, and accumulate large quantities of crystallin proteins (Parmelee and Beebe, 1988). The crystallin proteins help maintain the structural integrity of the lens as well as being a significant inhibitor of lens fiber cell apoptosis (Cvekl and Ashery-Padan, 2014). Expression of the crystallin proteins is regulated by pathways involving extracellular proteins like fibroblast growth factor (FGF) as well as transcription factors such as c-Maf, and Pax6. Expression of FGF is required during embryonic development of the crystalline lens for modulation of numerous processes such as cell proliferation, cell survival, cell migration, and cell differentiation. The developing retina secretes FGF and the lens capsule regulates how much FGF enters (Xie et al., 2016). An FGF gradient regulates the process of differentiation of lens epithelial cells into lens fiber cells (Audette et al., 2016). Studies have shown that a high FGF concentration activates differentiation of lens fiber cells, a moderate FGF concentration regulates cell migration,
and a low FGF concentration regulates cell survival (McAvoy and Chamberlain, 1989; Xie et al., 2016). When FGF signaling is disrupted, a fulcrum does not develop and lens fiber cells do not elongate (Zhao et al., 2008).

The processes that form lens fibers embryonically are preserved throughout life and continue with age. Lens fiber cells are not degraded and shed, but rather become more and more condensed. These cells are permanent and are maintained for life. The embryonic nucleus and fetal nucleus are the same cells as those formed initially during development. As the secondary lens fibers differentiate and become compacted into concentric spheres with different lens fiber cell densities, regions with distinct refractive indices are produced.

Apical Constriction and Shroom3

Many different developmental processes throughout the embryo require a mechanism called apical constriction. Apical constriction is an active shrinking of cellular apices which causes epithelial cells to change from a columnar shape to a more wedge-like shape. Figure 4 demonstrates the change in a tissue’s shape due to apical constriction (Sawyer et al., 2010). A small change in each cell can cause a significant change in the whole tissue.

Apical constriction is induced by activation and contraction of apically positioned non-muscle myosin proteins with filamentous actin (F-actin), creating actomyosin filaments networks at the apical end of the cell. Contraction of these networks causes a decrease in the surface area of the apical membrane and junctional circumference,
leading to the change in cell shape. Apical constriction is important for epithelial morphogenesis occurring in several developing organs that enables tissues to bend and form different shapes necessary for proper development. This cell shape change, and tissue folding, can be used to create different morphogenetic results, depending on the organism and/or tissue. Epithelial invagination [ex lens placode (Plageman et al., 2010)], organ asymmetry [gut tube bending (Plageman et al., 2011a)], and early tube formation [ex neurulation (Haigo et al., 2003)] are all known to be dependent on apical constriction (Sawyer et al., 2010).

Figure 4. The effect of apical constriction on a tissue (Sawyer et al., 2010). (A) A flat tissue before apical constriction. (B) A curved tissue following apical constriction. (A₁) An individual cell before apical constriction. (B₁) An individual cell after apical constriction.
Shroom3 is a cytoskeletal protein found in cellular membranes that is a major contributor of tissue morphogenesis by controlling the assembly of apically positioned contractile actomyosin networks and, thus, apical constriction (Chauhan et al., 2015). Previous research has shown that Shroom3 expression is enriched in cells that are actively going through the process of apical constriction (Hildebrand and Soriano, 1999). Shroom3 can initiate apical constriction by recruiting, organizing, and activating the contraction of sarcomeric-like structures. These structures are comprised of non-muscle myosin and F-actin and associated with the apical junctions of epithelial cells. To accomplish this, Shroom3 directly binds to and recruits Rho-kinase (Rock) to the zonula adherens of epithelial cells. Shroom3-induced apical constriction is dependent on Rock activity, which leads to the phosphorylation and activation of non-muscle myosin (Zalewski et al., 2016). When non-muscle myosin is activated, F-actin and non-muscle myosin fibers (actomyosin) become anchored to the apical junctions (Roh-Johnson et al., 2012). Shroom3-dependent apical constriction requires an association with actomyosin filaments and the apical junctional complex via the cadherin associated protein, p120-catenin. When p120-catenin is absent from lens placodes, localization of Shroom3 and non-muscle myosin are both reduced at the apical junctions of cells (Lang et al., 2014).

Apical constriction is also necessary in other developing organ systems including closure of the neural tube (Brodland et al., 2010; Das et al., 2014), as well as gut looping asymmetry (Plageman et al., 2011a), lungs (Kim et al., 2013), and thyroid (Loebel et al., 2016). Shroom3 has been shown to regulate the tissue apical constriction necessary for many of these formation changes. The absence of Shroom3 has caused systemic
malformations such as body wall herniations and incomplete neural tube closure resulting in spina bifida (Hildebrand and Soriano, 1999). Figure 5 shows Shroom3 mutant mice embryos compared to wild type mice embryos at different ages.

Figure 5. Systemic appearance of Shroom3 null embryos compared to wild type embryos (Hildebrand and Soriano, 1999). (A-D) show wild type embryos on the left and Shroom3 null on the right at different embryonic ages. (D) The Shroom3 mutant exhibits neural malformations and herniation of the liver and intestines. (E) Wild type neural tube compared to (F) Shroom3 mutant malformed neural tube.

In the lens placode, Shroom3 is expressed and localized to the apex of the epithelial cells (Plageman et al., 2010). In mutant mouse embryos lacking the Shroom3 gene, the lens pits at E10.5 were smaller and irregularly shaped compared to the lens pits of wild type mouse embryos. Instead of lens placodes with a smooth, rounded curvature
in the controls, the lens pit developed into a V-like shape comprised of two straight epithelia flanking a kinked region (Plageman et al., 2010). Normal curvature of the lens placode during invagination was disrupted by an inhibition of apical constriction. When compared to the control embryos, the epithelial cells of the mutants showed a decrease in apical constriction and possessed larger apical areas (Plageman et al., 2010). A decreased occurrence of F-actin and myosin at the apical aspect of the epithelium in the lens placode was also observed, demonstrating that Shroom3 is also important for the apical recruitment of the actomyosin network (Plageman et al., 2010). Phalloidin is a stain that binds to f-actin and can be used to visualize the location of the F-actin in the cell membranes.

Further studies have demonstrated the importance of other pathway components that contribute to apical constriction during placode invagination. Appendix A shows one hypothesized pathway outlining apical constriction that includes intrinsic and extrinsic forces that are involved. Shroom3 is transcriptionally regulated in the lens placode by the critical transcription factor Pax6. In the absence of Pax6, Shroom3 mRNA and protein expression are diminished (Plageman et al., 2010) suggesting Pax6 may initiate Shroom3-dependent morphogenesis. The activity of the GTPase protein, RhoA, is necessary for Shroom3 apical localization and apical constriction in the invaginating lens (Plageman et al., 2011b). It was also found that a guanine nucleotide exchange factor called Trio, which was initially found to be required for skeletal muscle and neuronal development, acts upstream of RhoA during Shroom3-dependent apical constriction.
Although previous studies have shown that the Shroom3 pathway is necessary for apical constriction of the lens placode, it is not currently known if Shroom3 has the same influence on apical constriction later in developmental of lens fiber cells that also undergo narrowing of apices. The mechanisms that regulate lens fiber cell morphogenesis are not well known and it is plausible that Shroom3 has a similar effect in the lens fiber cells as the younger lens epithelial cells. It was hypothesized that Shroom3 is localized to lens fiber cell and aids in facilitating morphogenesis by modifying lens fiber cell shape, and eliminating Shroom3 will result in decreased apical constriction of lens fiber cells in the developing lens of mice embryos. To test this hypothesis, lenses of wild type and Shroom3-deficient mouse embryos were sectioned and examined histologically. Apical and basal areas of the epithelial cells at and near the lens fulcrum were measured in mice with and without the Shroom3 gene and compared to one another.
Chapter 2: Materials and Methods

Animal Maintenance and Use

All procedures for this research were approved by The Ohio State University Institutional Animal Care and Use Committee and were done under the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were obtained from a breeding colony maintained at OSU from mice originally acquired from Jackson Laboratory (Bar Harbor, ME). A description of Shroom3$^{Gt/Gt}$ mice [in full, Shroom3Gt(ROSA)53Sor] has previously been published (Hildebrand and Soriano, 1999). The mice were maintained under proper Ohio State University policies in a pathogen-free habitat. A gestational age of 0.5 days was described as the day when a vaginal plug was discovered [embryonic day (E) 0.5]. At specific gestational ages, the pregnant female mice were anesthetized with isoflurane, and a hysterectomy was performed to remove the fetuses. Eleven total embryos were used producing over 120 total sections that were analyzed.

Genotyping protocol

Yolk sacs were separated from mouse embryos and the DNA was isolated. This was done using 50 mL of a 10X ear digest buffer. This solution was made by combining 25mL KCl (500mM), 5mL Tris-HCl pH 8.3 (100mM), 750uL MgCl2 (15mM), 2.25mL
NP40 (4.50%), 2.25mL Tween 20 (4.50%), and 14.75mL double distilled water. The solution was stored at 4C.

Ear punch tissues were used for genotyping the mice embryos. A 1X ear digest buffer solution was made with 1mL of the 10X ear digest buffer mixed with 1mL proteinase K (1mL=20mg, Invitrogen 255530-049) and 8mL of double distilled water. This solution was stored at -20C. Eighty microliters of 1X ear digest buffer was used to digest an ear clip overnight at 65C. The next day the mixture was boiled in the thermocycler for 30 minutes at 99.99°C. 1.5uL of isolated DNA was used in the PCR reaction.

Polymerase Chain Reaction

The PCR reaction mixture was made by combining the following, making 25uL total mixture per reaction: 2.5uL/reaction 10X buffer, 2uL/reaction dNTPs, 0.25uL/reaction Primer 1 (50pmol/uL), 0.25uL/reaction Primer 2 (50pmol/uL), 1uL DNA template, 0.2uL/reaction Taq (Clontech’s titanium Taq polymerase), 3 uL/reaction of loading dye, and 75.2 uL water. Ten microliter ladder was added to each row in 1% agarose gel. The agarose gel was prepared by mixing 100mL TAE with 1g Agarose. This was microwaved for 1-2 minutes and 2.5uL Ethidium Bromide is added per 50mL of gel. One liter of 50X TAE solution was made by mixing Tris base (BP152-1, Fisher Sci) into water with 57.1mL acetic acid and 100mL of 500mM EDTA with pH 8.0 (O2793-500, Fisher Sci). This 50X TAE solution was diluted down by mixing 200mL with 9.8L of distilled water. One microliter DNA Ladder was combined with 1uL 6X loading dye and 4uL deionized water, and mixed gently.
The mouse genotyping protocol performed in this research utilized PCR with the following primers to identify the mutant allele: 5'-atcctctgcatggtcaggtc and 5'-cgtgccctgtcattcc (315 base pairs) and the wild-type Shroom3 allele: 5'-ggtgactgaggagtagagtcc (1000 base pairs)5'-gcaaccacatggtgggagacaagc. Thirty PCR cycles were performed using the following procedure: 94 degrees for 30 seconds, 60 degrees for 30 seconds, and 72 degrees for 30 seconds.

Mouse embryo embedding and cryosectioning

Following dissection of the eye with surrounding tissues, the tissues were immediately placed in 15% sucrose solution and rocked until they sunk in the vial. Tissues were then transferred to 30% sucrose and rocked until they sunk in the vial. Tissues were embedded in OCT and placed in -80C chamber for at least 24 hours. Cryosections of frozen embryos were taken in 10 micron increments, and placed on slides. The slides were hydrated in PBS prior to the addition of antibodies. The cryosections were stained according to the antibody procedures described below.

Immunofluorescence

Six fetuses between the ages of E14.5 and E16.5 were used for beta-catenin staining, three being Shroom3 mutant embryos and three being control embryos. The same three control embryos were also co-immunolabeled with Shroom3. The primary antibodies used were anti-beta-catenin (1:500, Santa Cruz, sc-7199) and Shroom3 (1:1000) specific rabbit polyclonal antibody. Jeff Hildebrand generously donated the
Shroom3 (1:1000) specific rabbit polyclonal antibody used for staining cryosectioned embryos (n=3) (Hildebrand, 2005).

One Shroom3 mutant embryo at age E15.5 and one control embryo at age E15.5 were used for F-actin and myosin staining. Phalloidin (Invitrogen, A12381, A12379) staining was at 1:1000 and was used to visualize F-actin. Additionally, Hoechst 33342 was used as a counterstain to visualize the cell nuclei (Sigma, B-2261).

In order to co-immunolabel cryosections with Shroom3 and beta-catenin antibodies, slides with cryosections were placed on a warmer (37°C) to dry for a minimum of 1 hour. The slides were washed with PBS-Tween 0.1% (PBS-T) 3 times for 5-10 minutes each to wash off the OCT material. The slides were placed on a warmer (37°C) to dry again for 15 minutes. Once dry, the slides were placed into Tris (pH=9) and then put into a pressure cooker for 15 minutes. The slides were again washed with PBS-T 3 times for 5-10 minutes each. A pap pen was used to trace around the tissues on the slides and allowed to dry for 1-2 minutes. Next the primary antibody solution was prepared in a 4% blocking buffer. 40mg non-fat dry milk was mixed into 1000 ul PBS-T. The primary antibodies were added as follows: Mouse beta-catenin: dilution of 1:200, Rabbit Shroom3 (N-term): dilution of 1:500, and Rabbit Shroom3 (UPT131): dilution of 1:1000. The primary antibody solution was applied over the tissues in the pap pen wells, assuring that there was no spillover. If a spillover did occur, the excess was removed with a pipette, the slide was dried carefully with a Kimwipe, the pap pen markings were replaced, and the primary antibody solution was reapplied. The slides were incubated with the primary antibody solution in a humid chamber at 4°C for 12-48 hours.
On the second day, the antibody solution was rinsed off of the slides with PBS-T, and the slides were washed 3 times for 5-10 minutes each. The secondary antibody solution was prepared in a 4% blocking buffer. 40mg non-fat dry milk was mixed into 1000 ul PBS-T. The secondary antibodies were added at a dilution of 1:1000, matching the species of the primary antibody to the secondary antibody as follows: for primary Mouse beta-catenin primary, the anti-mouse secondary antibody was used; for primary Rabbit Shroom3 [N-term and UPT131 (Hildebrand, 2005)], a goat anti-rabbit secondary antibody was used; and the Hoechst counterstain was used at a dilution of 1:1000. The secondary antibody solution was applied over the tissues in the original pap pen wells, again assuring that there was no spillover. The slides were incubated with the secondary antibody solution in the dark at room temperature for 1-2 hours. The slides could be incubated for up to a week as long as they were kept in a dark humid chamber at 4C.

After incubation, the antibody solution was rinsed off of the slides with PBS-T, and the slides were washed 3 times for 5-10 minutes each. The slides were soaked in PBS-T and stored at 4C until imaging. Once the slides were ready to be imaged, Fluorogel was applied followed by a coverslip. The slides were then imaged using a Zeiss Axiovision inverted fluorescent microscope.

.Modified procedure for staining with anti-Shroom3 and anti-beta-catenin antibodies

A few modifications were made to the Day 1 procedures to ensure that the cryosections were not washed off of the slides throughout the PBS-T washing and pressure cooker procedures. When the slides were placed on the warmer (37C) to dry a
second time, it was changed to 30 minutes instead of 15 minutes. Additionally, the Tris solution was adjusted to a pH = 9 before inserting the slides in the pressure cooker.

**X-gal staining protocol**

Three wild type embryos at age E17.5 were used for the x-gal staining. The Shroom3 knock-out embryos were created by an alteration of the mouse Shroom3 gene at the Soriano lab (Hildebrand and Soriano, 1999). The coding sequence for Shroom3 was replaced with a lacZ coding sequence. It was determined which cells normally express Shroom3 by using an X-gal assay. The dissected E17.5 mouse embryo tissues were placed into cold PBS. The tissues were washed 2 times for 15 minutes each in 1xPBS/0.02% NP-40 at 4C. An x-gal fixative was prepared by combining the following into 1xPBS: 1% formaldehyde, 0.2% glutaraldehyde, 2mM MgCl₂, 5mM EGTA, and 0.01% NP-40. The tissues were fixed for 45 minutes with x-gal fixative at 4C while gently rocking. Next the tissues were washed 2 times for 15 minutes each in 1xPBS/0.02% NP-40 at 4C. An x-gal solution was prepared by combining the following into 1xPBS: 5mM K₃Fe(CN)₆, 5mM K₄Fe(CN)₆, 1M MgCl₂, 0.01% NP-40, and 1 mg/mL X-gal. The tissues were stained in freshly made X-gal solution and stored at 37C overnight.

The tissues were washed 2 times for 15 minutes each in 1xPBS/0.02% NP-40 at 4C and then were post-fixed in 4% paraformaldehyde for 1 hour at 4C. They were washed again 2 times for 15 minutes each in 1xPBS/0.02% NP-40. The tissues were then
either stored in PBS or, if ready to proceed to the cryosection procedure, were embedded into the OCT medium.

*Shape/area quantification*

Images and measurements were obtained from beta-catenin and Shroom3-stained 10 micron thick cryosections. Measurements were taken on 52 wild type cryosections and 44 mutant cryosections. These cryosections were taken from 7 wild types and 4 mutants, for a total of 11 embryos. Each lens bow area was subjectively divided into three zones: the germinative zone, the equatorial zone, and the outer cortical fibers. The germinative zone was distinguished by cells that had not begun apically constricting, elongating, or migrating around the lens bow. The equatorial zone was distinguished by cells that were located at the tip of the lens fulcrum. The outer cortical fibers were distinguished by cells that had elongated and migrated past the lens bow and were the closest to becoming mature lens fiber cells. The measurements were only taken within 25 microns of the tip of the lens fulcrum. Three to five width measurements were obtained along the apical and basal axis of lens fiber cells in each zone. Measurements were calculated by drawing a line along the apical and basal membranes of stained 10 micron cryosections using Zen computer software. The widths of all the apical and basal membranes were each averaged. The D’Agostino-Pearson test was applied to each data set as a way to determine whether or not they were normally distributed. Because data sets were not normally distributed, the Wilcoxon-Mann-Whitney, non-parametric test
was applied between the wild-type and control data sets to evaluate statistically significant differences (Marx et al., 2016).
Chapter 3: Results

The purpose of this research was to analyze the localization of the protein Shroom3, and compare the degree of apically constriction of lens fiber cells in control and Shroom3-deficient mice embryos during the organization and development of the embryo lenses.

*Shroom3 Localization with X-gal Staining*

The Shroom3 knock-out allele was created by altering the mouse Shroom3 gene such that the coding sequence was replaced with the lacZ reporter gene. The lacZ gene encodes for beta-galactosidase protein, which is only produced in cells that express Shroom3. To determine whether Shroom3 mRNA is expressed during later lens development when the cells appear to undergo apical constriction, an X-gal assay was performed on cryosections of heterozygous E17.5 mouse embryos (*Shroom3*^Gt/+^) and analyzed for a blue signal that indicated beta-galactosidase activity.

Both lens epithelial and the peripheral lens fiber cells that border the lens fulcrum are labeled blue (Figure 6A and 6B) demonstrating that Shroom3 expression occurs in these cells. However, the blue stain does not appear in the mature lens fiber cells at the center. This supports the hypothesis that Shroom3 is localized in the lens fiber cells
where apical constriction occurs, suggesting that Shroom3 is involved in the organization and development of the crystalline lens.

Figure 6. X-gal staining of E17.5 Shroom3 wild type mouse embryos. (A) Cross section of the whole lens. (B) Zoomed-in section of the lens bow region. LF denotes the lens fulcrum area. The asterisk (*) labels the posterior end of the lens fulcrum. The scale bars represent 200 micrometers.

Shroom3 Localization with Antibody Staining

The Shroom3 gene makes two known distinct mRNA isoforms which produce two different lengths of protein products (Hildebrand and Soriano, 1999). They are identical except for the N-terminus of the protein. Two different Shroom3 antibodies were used to find the location of Shroom3 protein in cryosections of wild-type E15.5 lenses. The first antibody (Shroom3-UPT131) labels both long and short isoforms of
Shroom3. This antibody was expected to immunolabel Shroom3 at the apical junctional complexes of the lens fiber cells at the lens fulcrum, however apical junctional signal was not observed. It remains possible that ideal conditions were not utilized to observe specific protein localization with this antibody. However, when utilizing an antibody that is specific to the N-terminus of the protein (Shroom3 N-terminus), it was observed that Shroom3 protein was localized in the same cells that express Shroom3 mRNA (Fig. 7 vs. Fig. 6). Furthermore, the antibody labeling demonstrates that the longest isoform of Shroom3 is localized along what appears to be the lateral junctions of lens epithelial and lens fiber cells, and not necessarily just isolated to the apical ends (Figure 7).
Figure 7. The lens bow region of an E15.5 mouse embryo lens immunostained with the Shroom3 N-terminus antibody. LE and LF denotes the lens epithelium and the lens fiber cells, respectively. The asterisk (*) labels the posterior end of the lens fulcrum. The scale bar represents 25 micrometers.

To determine if the lateral signal is junctional, co-immunolabeling with the Shroom3 and beta-catenin (a protein known to be localized at zonular adherenes cell junctions) antibodies was performed. Beta-catenin and Shroom3 are co-localized to cell junctions only within the lens epithelium and the youngest lens fibers (closed arrowheads, Fig. 8), but not within the more centrally positioned lens fibers (open
arrowheads, Fig. 8). The localization is indicative of a potential role for Shroom3-dependent shape changes occurring at this position.

Figure 8. Localization of (A) Shroom3, (B) beta-catenin, and (C) Shroom3/beta-catenin colocalization. The lens bow region of an E15.5 mouse embryo lens stained with Shroom3 N-terminus antibody. The upper bracketed region in the top panel delineates the germinative zone while the lower callout in the top panel delineates the equatorial zone. Anterior lens is to the right and posterior lens is to the left. The open arrows in the bottom two panels represent beta-catenin localization and closed arrows represent beta-catenin and Shroom3 colocalization. The scale bars represent 25 micrometers.
Apical Constriction

It was hypothesized that Shroom3 localizes to lens fiber cells and aids in facilitating morphogenesis by modifying lens fiber cell shape. To test this hypothesis, E15.5 Shroom3-deficient mouse embryos (n=4) as well as controls (n=7) were sectioned and examined histologically. Apical and basal areas of the epithelial cells and lens fiber cells in the area surrounding 25 microns of the lens fulcrum were measured in three subjectively determined zones: the germinative zone (GZ), equatorial zone (EZ), and outer cortical fibers (OCF). The germinative zone was distinguished by cells that had not begun apically constricting, elongating, or migrating around the lens bow. The equatorial zone was distinguished by cells that were located at the tip of the lens fulcrum. The outer cortical fibers were distinguished by cells that had elongated and migrated past the lens bow and, were the closest to becoming mature lens fiber cells. The means for all measurements in specific groups were calculated (Table 1, Figure 9). These measurements were compared between wild-type embryos and Shroom3 mutant embryos.

Table 1 and Figure 9 display the average widths of these three zones in the wild type lenses compared to the mutant lenses. Table 2 displays the ratios of apical widths to basal widths in the wild type lenses compared to the mutant lenses. A statistically significant difference in apical constriction was observed between the wild type and mutant lenses in the EZ and the OCF. The apical and basal widths of the lens fiber cells are similar in the GZ because the cells are not yet undergoing apical constriction. This makes the apical-basal width ratios close to 1 in both the wild type embryos and the
mutant embryos (Table 2). The apical widths are also similar between the wild-type (average 4.062 microns) and the mutant (average 3.864 microns).

In the EZ, the lens fiber cells have undergone most of their apical constriction. The basal portions remain the same width while the apical widths decrease about 75%. There is a statistical difference between the apical widths of the wild type (average 1.013 microns) compared to the mutant (average 1.552 microns) with a \( p\)-value of 7.671E-24.

The lens fiber cells in the OCF have completely undergone apical constriction. There is a statistical difference between the apical widths of the wild type (average 1.047 microns) compared to the mutant (average 1.459 microns) with a \( p\)-value of 4.511E-15. As the lens fiber cells migrate through the EZ and the OCF, the wild type apical-to-basal width ratios decrease to approximately 20% whereas the mutants are closer to 30%. There is a statistically significant difference between the apical widths of the control lenses, and lenses with a mutant Shroom3 phenotype in the equatorial zone and outer cortical fibers, but not in the germinative zone. It was observed that there is still significant apical constriction occurring, but not as drastic as with the participation of Shroom3.

Table 1. Average apical and basal widths among wild type and Shroom3 null embryos

<table>
<thead>
<tr>
<th></th>
<th>GZ</th>
<th></th>
<th>EZ</th>
<th></th>
<th>OCF</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Apical</td>
<td>Basal</td>
<td>Apical</td>
<td>Basal</td>
<td>Apical</td>
<td>Basal</td>
</tr>
<tr>
<td>Wild Type</td>
<td>4.062</td>
<td>4.345</td>
<td>1.013</td>
<td>4.729</td>
<td>1.047</td>
<td>4.721</td>
</tr>
<tr>
<td>Mutant</td>
<td>3.864</td>
<td>4.035</td>
<td>1.552</td>
<td>4.650</td>
<td>1.459</td>
<td>4.660</td>
</tr>
<tr>
<td>( p)-value</td>
<td>0.977</td>
<td>0.998</td>
<td>7.671E-24</td>
<td>0.944</td>
<td>4.511E-15</td>
<td>0.843</td>
</tr>
</tbody>
</table>
Figure 9. Average widths of apical and basal ends of lens cells. The gray bars represent wild-type Shroom3 embryos and the blue bars represent the Shroom3-null embryos. The standard deviation is represented by the error bars. GZ: germinative zone, EZ: equatorial zone, OCF: outer cortical fibers. NS = Not significant. Asterisk (*) = p-value < 0.001

Table 2. Ratio of average apical width to average basal width in different zones among wild type and Shroom3 null embryos

<table>
<thead>
<tr>
<th>Zone</th>
<th>Wild Type</th>
<th>Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>GZ</td>
<td>0.935</td>
<td>0.958</td>
</tr>
<tr>
<td>EZ</td>
<td>0.214</td>
<td>0.334</td>
</tr>
<tr>
<td>OCF</td>
<td>0.222</td>
<td>0.313</td>
</tr>
</tbody>
</table>

The overall cell shape was outlined and assessed. Figure 10 shows the differences in cell shape in the three different zones of the wild type and mutant lenses. The cells in the GZ appear to be a similar shape and size in the wild type and mutant embryos.
lens fiber cells undergo differentiation through the EZ and the OCF, it is more apparent that the wild type embryo cells are more apically constricted and elongated than the mutant embryo cells.

Figure 10. (A) Lens bow region (anterior lens is at the top and posterior lens is at the bottom) of an E15.5 mouse lens. Designated zones for cell shape analysis. GZ: germinative zone, EZ: equatorial zone, and OCF: outer cortical fibers. (B) Staining with Shroom3 antibody. (C) Tracing of individual cell shapes in the different regions. Scale bars in panel B represent 1 micrometer. Scale bars in panel C represent 5 micrometers.
Actin and non-muscle myosin localization

F-actin and non-muscle myosin form the complexes that contribute to the forces along the membrane causing apical constriction. Figure 11 shows fluorescent intensity of f-actin and non-muscle myosin staining in control and Shroom3 null embryos. The open arrows represent areas exhibiting less fluorescent intensity and the closed arrows represent those with greater fluorescent intensity. The intensity is directly correlated with the amount of protein present. Figure 11A shows more intense fluorescence in the control along the fulcrum, and therefore more protein localization. Whereas Figure 11B shows more intense fluorescence in the Shroom3 null along the basal membrane. The signal intensities at the lens fulcrum are similar for non-muscle myosin localization compared to the f-actin. The signal intensities of non-muscle myosin at the basal membrane appear to be only slightly stronger in the Shroom3 null compared to the control.
Figure 11. Staining for F-actin (A, B) and non-muscle myosin (C, D) in wild type and Shroom3 null mice embryo lenses. Closed arrows = more protein present in similar areas of the wild type compared to the mutant. Open arrows = less protein present in similar areas of the wild type compared to the mutant. The scale bars represent 25 micrometers.
Chapter 4: Conclusions and Discussion

The purpose of this research was to analyze the localization of the protein Shroom3, and compare the degree of apically constriction of lens fiber cells in control and Shroom3-deficient mice embryos during the organization and development of the embryo lenses. It has previously been shown in the lens placode that Shroom3 is a crucial component in the pathway that leads to apical constriction (Appendix A). To determine if this is also the case in the lens fiber cells within the lens bow of developing lenses, the location of Shroom3 expression was first evaluated, followed by an analysis of the degree of apical constriction. It was concluded that Shroom3 is localized to the epithelial cells and differentiating lens fiber cells in the developing lens. It was also concluded that Shroom3 has an influential role in the apical constriction pathway by showing a decrease in apical constriction in Shroom3-deficient mice embryo lenses.

Due to the location of Shroom3 expression along the lateral membranes, it is thought that Shroom3 is an important influence for apical constriction and elongation of differentiating lens fiber cells. If Shroom3 expression was not deleted in the developing lens and instead expression was increased, it is hypothesized that the lens cells would further apically constrict and further elongate.
Localization of Shroom3

It can be concluded from these experiments that Shroom3 is localized in the lens epithelium and lens fiber cells. It has already been shown that Shroom3 is present in the lens placode during apical constriction earlier in lens development (Plageman et al., 2010). Shroom3 is expressed in other tissues undergoing apical constriction, suggesting that the lens also requires apical constriction for proper development (Das et al., 2014; Brodland et al., 2010; Plageman et al., 2011; Kim et al., 2013; Loebel et al., 2016).

Shroom3 expression was evaluated using two different methods. One procedure was using an x-gal stain. Mouse embryos with the knock-out allele express beta-galactosidase protein, encoded by the lacZ gene, instead of expressing Shroom3. This staining procedure showed Shroom3 staining in the lens epithelial cells as well as the differentiating lens fiber cells. However, there was no staining of the central mature lens fiber cells. The mature lens fiber cells are completely differentiated and have already completed apical constriction.

This staining process only showed which cells express Shroom3, but not necessarily where Shroom3 is expressed within those cells. Two different Shroom3 antibodies were utilized to determine the location of Shroom3 protein within the differentiating lens cells. The Shroom3-131 antibody was inconclusive for Shroom3 expression in the differentiating lens fiber cells undergoing apical constriction along the lens fulcrum. The N-terminus Shroom3 antibody was also used which differs in that it only detects the longest isoform of Shroom3 (Muccioli et al., 2016). It was discovered
that this antibody found Shroom3 expression along the lateral membranes, not necessarily just at apical junctions, of the differentiating lens fiber cells. Previous research on Shroom3 localization has not used this type of antibody and has only found Shroom3 to be localized at apical junctions. Although this localization is different than expected, the data from the Shroom3 antibody staining is consistent with the x-gal staining in that Shroom3 is localized in the lens epithelial cells and differentiating lens fiber cells and not in the central mature lens fiber cells. This could mean that the longest isoform of Shroom3 may not be specific to the apical domain. However, antibodies that detect both long and short isoforms have previously detected apical localization. One reason for this discrepancy could be that the N-terminus antibody is better at detecting the long isoform than the other antibodies.

Shroom3 expression and localization is coincident with cell shape. It is believed Shroom3 is an important inducer of apical constriction at the lens fulcrum. Localization of Shroom3 at the apical aspect of the differentiating lens fiber cells supports this idea. It is also logical that Shroom3 is no longer expressed in mature lens fiber cells because they are essentially sedentary and no longer need to undergo apical constriction. However, expression in the lateral membranes of the elongated, skinny lens fiber cells does not initially support this. Another reason that the long isoform of Shroom3 might be expressed in the lateral membranes is that this portion also requires a change in cell shape. The apical ends of the lens fiber cells are not the only area that changes shape or size. The lens fiber cells are generally narrow for much of the length of the cells rather than just at the apical portion. Therefore, Shroom3-dependent contractile events may
occur along the lateral junctions. As the lens fiber cells elongate and migrate around the lens fulcrum, it is possible that there is a similar change at the lateral membrane, but to a lesser degree, to allow for the elongation and bending of the entire cell. Since this is a novel idea that hasn’t been studied before, additional research is necessary.

Cell Shape

Our results show the Shroom3 null embryos constrict less at the apical aspect of the differentiating lens fiber cells than the controls. This decrease in apical constriction results in more columnar-shaped cells rather than conical-shaped cells. Columnar-shaped cells don’t allow the tissue as a whole to bend which is necessary for the proper development of the tissue. Since the Shroom3 null mice embryos still undergo apical constriction, but to a lesser degree, it can be concluded that other proteins and pathways are involved. These results have been found in other parts of the body, including the lens placode, but never at the lens fulcrum.

The shape change induced from apical constriction is very important for the development of the lens fulcrum as well as the maintenance of the entire shape of the lens. If these cells don’t apically constrict and follow the precise process of lens development, it is thought that the lens fulcrum could become disorganized resulting in a disorganization of all the following lens fiber cells. In some of the Shroom3 mutant cryosections, it appears as if the differentiating lens fiber cells are pulled away from the lens epithelium, causing the lens fulcrum to not have such a “tight bend”. This could cause the entire structure of the lens to form incorrectly which could possibly result in
lens opacities and congenital cataracts. Lens opacities and cataracts can even affect the final refractive error of the eye, possibly causing significant uncorrectable deficits in vision. It currently is not known if disrupting the expression of Shroom3 will cause cataracts, but it is a distinct possibility. Since the role of Shroom3 has been discussed in apical constriction, which aids in the creation of a very specific and organized structure of the development lens, a disruption in this process could very well cause disorganization of the lens fiber cells and result in cataracts. Currently Shroom3 can’t be evaluated after birth because the systemic malformations cause death of the embryo before gestation is complete. One possible way to get around this obstacle would be with a conditional, tissue-specific knock out of Shroom3 to avoid embryonic death. If a Shroom3 allele deletion could occur in only the lens, the rest of the embryo could develop normally while the lens develops with the Shroom3 mutations.

*Actin-myosin complexes and localization*

Actin and myosin are components of the lens fiber cells that are crucial to maintaining the cellular integrity. These components form a lattice which strengthens the whole lens and helps maintain its shape. Activation of Rock leads to the phosphorylation of non-muscle myosins and then activation. This leads to the connection of non-muscle myosins to filamentous actin (F-actin), producing actin-myosin complexes. These complexes are able to interact with myosin light chain kinase (MLCK) and ATP to produce a contracting force within cells.
It is critical that the actin cytoskeleton complexes are carefully controlled during lens morphogenesis to ensure that all the cells are developing properly and will initiate the next steps in the pathway to occur correctly. These complexes are arranged with adherens belts and stress fibers organized throughout the epithelial cells. Tropomodulin and tropomyosin are proteins that aid in regulating actin cytoskeleton. Tropomodulin is expressed in post-miotic differentiated lens fiber cells and arranged on the membranes as the cells elongate, aiding in stabilization of actin filaments (Fischer et al., 2000). Tropomyosin binds along actin filaments and regulates the stability and location of actin filaments (Fischer et al., 2000).

The location of f-actin was found using the phalloidin stain. In the control embryos, the staining shows f-actin localization in the cell membranes with more intense staining at the apical ends at the lens fulcrum. Removal of Shroom3 shifts the location of f-actin along the membranes with more intense staining at the basal aspects. The results support this hypothesis by showing a shifted phalloidin staining of the Shroom3 null mice embryos. Figures 10A and 10B show another example of the staining pattern for f-actin. Figure 10B shows a decrease in f-actin localization at the lens fulcrum compared to the control 10A. Since f-actin and non-muscle myosin are closely involved in apical constriction, they have similar localization patterns in the control compared the Shroom3 null. Figure 10D shows more diffuse staining near the lens fulcrum compared to the localized staining in the control (Figure 10C).

The localization of actin and myosin is important in the pathway for apical constriction. Since Shroom3 is necessary for the localization of actin and myosin, but not
necessarily the expression of them, it can be concluded that the absence of Shroom3 would result in disorganization of f-actin ad myosin rather than an absence of them. This is exactly what was found in the results. The signal intensities in Figure 10 show that f-actin and non-muscle myosin are still expressed in Shroom3 null embryos, but not in the same distribution as the control embryos. This supports the idea that Shroom3 is involved in multiple processes leading to apical constriction.

**Potential future studies**

Further research will provide a better understanding of the components involved in the apical constriction of lens fiber cells. One aspect of the pathway that is likely to be important is the activation of Rock during Shroom3-mediated apical constriction. Previous research has shown that this activation of Rock can be dependent on Rho (Plageman et al., 2011b) or independent of it (Haigo et al., 2003). Since Rock and myosin interactions are key components in multiple pathways throughout the body, a further evaluation of the signaling, activation, and other contributors is required.

Another pathway to further examine would be the influence of FGF. It has been shown that the retina secretes FGF which travels to the developing lens and causes lens fiber cell elongation, migration, differentiation, and survival. FGF has also been shown to have a role in the process of apical constriction. Shroom3 requires FGF signaling in order to regulate non-muscle myosin activity in specific cells during the development of zebrafish embryos (Ernst et al., 2012). However, it is unknown whether FGF signaling is involved in the localization of Shroom3.
Improvements could be made in this research. Additional analysis of Shroom3 with a larger sample size is necessary to replicate these results and support these findings. The use of a computer program to measure the lens fiber cell widths would also provide a more accurate data analysis.

**Conclusion**

It has previously been shown that Shroom3 is a crucial component in the apical constriction in the lens placode. The purpose of this research was to analyze the localization of Shroom3 in the lens and examine its association with cell shape changes. It was found that Shroom3 localized in the lens epithelium and lens fiber cells using two different methods. Shroom3 mutants constrict less at the apical aspect of the differentiating lens fiber cells compared to controls. Lens fiber cells still apically constrict, but to a lesser degree, so other proteins and pathways must be involved. Shroom3 facilitates apical constriction by regulating F-actin and myosin apical localization.

Shroom3 mutations can cause numerous disruptions throughout the body, but most importantly for this research, in the developing lens. Cells of mutants are more columnar than wedge-shaped, which doesn’t allow the tissue to bend as necessary for proper lens development and lens maintenance. The lens bow can become disorganized causing a cascade of disruptions of all the subsequent cells, possibly resulting in opacities and congenital cataracts. There are also significant systemic abnormalities resulting in the death of the embryo before birth.
References


Appendix A: Shroom3 pathway to apical constriction within the lens placode

Figure 12. A summary schematic of the pathways involved in the beginning of eye and lens morphogenesis of the mouse (Chauhun, 2015). The red box summarizes intrinsic and extrinsic forces previously discussed that induce invagination. Shroom3 and other factors previously discussed are shown on the left side of the red box and lead to apical constriction.