MOESIN AND CLIC MODULATE Rhabdomere Morphogenesis in *Drosophila melanogaster* Photoreceptors

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Abstract

The formation of the actin-rich apical surface structures of *Drosophila* photoreceptor cells, the rhabdomeres, is a tightly regulated process involving many protein interactions. Moesin, the sole Ezrin/Radixin/Moesin (ERM) protein in *Drosophila*, serves to anchor F-actin microfilaments to the cellular membrane and is subject to many regulatory interactions. Moesin must bind to PIP$_2$ and then be phosphorylated by the Sterile20 (Ste20) kinase Slik to be activated and serve as a cytoskeletal anchor. Conversely, it is deactivated by dephosphorylation by the PP1 phosphatase PP1-87B. Here I show that the sole CLIC family protein in *Drosophila*, Clic, is also intimately involved with moesin and PP1-87B in establishing the architecture of rhabdomeres. Disruption in the function of these important proteins has adverse effects on microvilli formation within the rhabdomeres and induces a loss of epithelial integrity and organization within the photoreceptor cells. A knockdown of moesin function causes the loss of three rhabdomeres, two of which can be recovered in a Clic loss-of-function mutant background. This suggests an antagonistic role between Clic and moesin. Masking of the phenotype induced by a knockdown of PP1-87B by the Clic loss-of-function mutation suggests that Clic works upstream of PP1-87B to antagonize moesin function. Our current model is that Clic facilitates the transfer of active moesin from PIP$_2$ to a membrane protein. Once this transaction is complete, PP1-87B gains access to moesin for dephosphorylation and subsequent deactivation. Thus, I present a model of a novel role for Clic in rhabdomere morphogenesis via genetic interaction with moesin and PP1-87B.
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INTRODUCTION

I. ERM proteins

Ezrin, radixin, and moesin, known together as simply ERM proteins, are important organizers of cellular membranes. They are able to interact with many membrane associated components including transmembrane proteins, phospholipids, and filamentous actin (F-actin). The structure of ERM proteins makes them particularly well-suited for these interactions as they are comprised of two distinct regions capable of specific molecular interactions. At the amino terminus of ERM proteins is the FERM domain that allows the protein to interact with the plasma membrane or other transmembrane molecules. The carboxy terminus contains the C-terminal ERM-association domain (C-ERMAD). The C-ERMAD is capable of binding with the FERM domain or filamentous actin (F-actin) of the cytoskeleton. ERM proteins typically exist in an inactive state in which the FERM and C-ERMAD domains interact and confer a closed protein conformation. Thus, ERM proteins remain inactive unless the interaction between its distinct domains is disrupted.1,2

II. Regulation of ERM function

ERM protein function is a tightly regulated and complex process that incorporates the actions of many other protein players. Moesin, the sole ERM family protein in the fruit fly Drosophila melanogaster, is subject to many regulatory interactions. First, moesin is regulated by a conformational change in its
protein structure that allows its two bound domains to separate and participate in other interactions. The FERM domain of moesin preferentially anchors to membrane-bound phosphoinositol 4,5-bisphosphate (PIP2). This binding partially activates the protein and allows for subsequent phosphorylation, thus producing fully active moesin (Fig. 1).³ Phosphorylation of moesin is carried out by Slik, the *Drosophila* Sterile20 kinase. Once moesin is partially activated by binding to PIP2, Slik adds a phosphate group to a conserved threonine residue in the C-terminal domain. Phosphorylation by Slik allows moesin to bind F-actin and anchor the actin cytoskeleton to the cell membrane.¹,⁴ Slik is localized to the plasma membrane and brought in proximity to moesin by Sip1, the *Drosophila* orthologue of the mammalian EBP50/NHER1 protein. Sip1 forms a complex with Slik and moesin; this promotes Slik-dependent phosphorylation of moesin.⁵ Following activation, moesin may be transferred from PIP2 to a transmembrane protein.
Figure 1. Model of ERM protein activation. Upon partial activation by binding to PIP₂, ERM proteins are phosphorylated by Slik and undergo a conformational change that enables them to anchor actin microfilaments to the cell membrane. ERM proteins may be transferred to other transmembrane proteins following activation⁵ (Figure from⁵).

As the interaction between ERM proteins and PIP₂ is a necessary step in the activation of ERM proteins, regulation of PIP₂ levels also regulate ERM function. Phospholipase C (PLC) is a key regulator of PIP₂ and works to reduce PIP₂ levels by hydrolyzing the protein into two mediators, diacylglycerol (DAG) and IP₃. DAG remains bound to the membrane and IP₃ is released in this process.³

When PLC is induced to hydrolyze PIP₂, there is a marked reduction in the amount of phosphorylated ERM proteins in the cell and they are redistributed away from the plasma membrane. This is probably due to the loss of PIP₂ binding or a decrease in the amount of phosphorylated moesin in the cell.³ In Drosophila, it has been shown that the interaction of moesin with PIP₂ is necessary for correct morphogenesis of eye and wing tissues.⁶
Figure 2. Generation of phosphoinositide species. Metabolic reactions depicting the generation of the seven phosphoinositide species from phosphatidylinositol (PtdIns). PIP$_2$ circled in red$^7$ (Figure from$^7$).

PIP$_2$ levels are also regulated by type I phosphatidylinositol kinases (PIP5KIs) that synthesize PIP$_2$ from its precursor PtdIns(4)P. In Drosophila, skittles (sktl) encodes a PIP5KI. When sktl is overexpressed in Drosophila wing cells, PIP$_2$ levels increase and lead to an accumulation of phosphorylated moesin and excessive localization of phospho-moesin to the cell membrane.$^6$ Conversely, PIP$_2$ levels are decreased with overexpression of synaptojanin (synj), a protein that acts to hydrolyze PIP$_2$. $^8$

Although the FERM domain of moesin preferentially anchors to membrane bound PIP$_2$, it may also bind to other membrane-bound proteins or adaptor proteins.$^1$ In a mammalian system, ERM proteins have been shown to interact and bind with the cytoplasmic domains of CD44, CD43, and ICAM-2. Thus, these integral membrane proteins may serve to recruit ERM proteins to the cell membrane and play an important role in ERM function.$^9$ The FERM domain of
ERM proteins may also bind to EBP50, a scaffolding protein which has the potential to bind to a variety of other membrane bound proteins.\textsuperscript{10}

Moesin function is also regulated by a phosphatase that removes a phosphate from the conserved threonine residue in its C-terminal domain to inactivate the protein. The protein phosphatase responsible for most moesin inactivation is the serine/threonine phosphatase PP1-87B.\textsuperscript{11} Upon dephosphorylation, inactive moesin dissociates from the plasma membrane. Overall, ERM regulation is subject to many different protein interaction pathways and serves to anchor the actin cytoskeleton to the cell membrane by way of these specific interactions.

III. ERM proteins and the actin cytoskeleton

The actin cytoskeleton in the form of strands of filamentous actin (F-actin) dominates the interactions of the C-ERMAD located at the C-terminus of ERM proteins.\textsuperscript{1} Actin fibers form from small subunits of globular actin (G-actin) that polymerize and form a long protein strand of filamentous actin (F-actin). This polymerization of actin can drive cell locomotion and many other cellular processes. Actin strands or filaments are also a component of the larger, dynamic protein meshwork called the cytoskeleton that lies beneath the cell membrane and gives the cell its shape and provides structural support for the cell.\textsuperscript{12} ERM proteins have been shown to anchor these actin microfilaments to the cell membrane and play a role in organizing cell polarity and morphogenesis, both essential processes for normal structural development.\textsuperscript{2} Actin filaments are also used by the cell to construct complex surface structures such as intestinal brush border cell microvilli.
that increase cell surface area and increase absorption efficiency.\textsuperscript{13} During mitosis, moesin may cause actin microfilaments to align parallel to the cell membrane to provide the cell with a stiff cortical meshwork to divide properly. Disruption of this interaction between actin filaments and moesin is problematic and can lead to a loss of structural integrity.\textsuperscript{14}

In \textit{Drosophila}, moesin activity is required for the formation of actin rich structures like the cytoskeleton, but is also important in other specialized structures comprised of actin including the cortex of ovarian nurse cells and the rhabdomeres of photoreceptors.\textsuperscript{2}

\textbf{IV. \textit{Drosophila} photoreceptors}

The \textit{Drosophila} compound eye contains many discrete units of cells called ommatidia. The eight photoreceptor cells in each ommatidium in the \textit{Drosophila} compound eye each contain a light-receptive, subcellular structure called a rhabdomere. Each rhabdomere is a collection of densely packed microvilli.\textsuperscript{15} Each individual microvillus contains at least two distinct actin filaments\textsuperscript{16} that are anchored to the plasma membrane by the solitary ERM protein in \textit{Drosophila}, moesin.\textsuperscript{17} In each ommatidium, there are eight photoreceptor cells (R1-R8) that each produce an actin-rich rhabdomere. R7 and R8 make the central rhabdomeres and are located at different depths in the ommatidium, while R1-R6 make the rhabdomeres of the periphery (Fig. 3).\textsuperscript{15} During development, the apical tips of the photoreceptor cells are involuted and the apical surfaces of these cells turn inward to the center of the ommatidium. Adherens junctions are maintained after
involution to form the seams of the pocket created by the photoreceptor cells. Later, these involuted apical surfaces undergo rapid growth to form the light-receptive rhabdomeres. Therefore, the rhabdomeres are the major constituents of the apical surface of the photoreceptor cells. The fact that rhabdomeres are located apically may be important for directing polarized membrane interactions that work to maintain integrity of the rhabdomere structure.\textsuperscript{18} Also, the distinct apical-basal polarity of the photoreceptor cells and corresponding rhabdomeres allows for prediction of microvilli orientation and straightforward assessment of changes in epithelial organization. Studies have shown that moesin plays a major role in this process of organizing the photoreceptors and forming the corresponding rhabdomeres. When moesin function is disrupted, actin cytoskeleton organization and apical differentiation of these cells is impaired and rhabdomeres appear abnormal.\textsuperscript{17}

\textbf{Figure 3. Cross-section of a wild-type ommatidium.} An electron micrograph depicting a cross section of a normal, wild-type ommatidium with rhabdomeres R1-R7 visible\textsuperscript{19} (Figure from\textsuperscript{19}).
V. Moesin and cancer progression

The aspect of cancer pathology that leads to the most deaths from the disease is metastasis of cancerous cells. This is the process by which the cancerous cells spread and invade another part of the body separate from the primary site. In order for cancer cells to metastasize and spread to other tissues, certain changes in the cell must occur. These changes may include a loss of adhesion properties, increased motility, or a loss of epithelial integrity via changes in the actin cytoskeleton in the cancerous cell. Research focused on achieving a better understanding of the process of cancer metastasis could therefore be valuable for treating cancer patients in the future.

Moesin is an important player in regulation of the processes compromised in cancer metastasis, including maintenance of the structural integrity of epithelial cells. Moesin is also known to regulate downstream effectors that have been implicated in processes promoting metastasis. Therefore, its function and molecular interactions may also have important implications for cancer research. In Drosophila, moesin has been proposed to antagonize the activity of the small GTPase Rho1 as a reduction in Rho1 gene dosage partially rescues a loss-of-function moesin phenotype. This antagonistic interaction between the Rho pathway and moesin is thought to promote epithelial integrity and maintenance of apical-basal polarity. Further, in a loss-of-function moesin phenotype, cells show an increase in migratory behavior consistent with an increase in Rho pathway activity. This is consistent with previous research showing that increased Rho
activity can cause invasive and metastatic phenotypes in epithelial cells. Thus, moesin may work in a negative feedback loop with Rho1 to inhibit migratory and invasive behaviors that are hallmarks of metastatic cells. Moesin has also been shown to be a key player in the maintenance of epithelial organization and apical-basal polarity in *Drosophila*. In mutants with reduced moesin activity, epithelial cells of wing discs lost their ability to remain integrated in the epithelium and were extruded basally. Normally, these cells underwent apoptosis after extrusion. If, as in the case of cancerous cells, these cells had been able to resist cell death, their increased motile properties would have increased their transformation towards cancerous phenotypes.

VI. **Moesin and chloride intracellular channels (CLICs)**

Adding to its many interactions with other proteins, moesin has been shown to exhibit significant interaction with the sole chloride intracellular protein (CLIC) in *Drosophila*, Clic. This conclusion is based upon unpublished research produced by Dr. Mark Berryman, Dr. Soichi Tanda, and Regan Price of the Ohio University Department of Biological Sciences. The first of two experiments was a phosphoprotein assay using extracts from *Drosophila* 3rd instar larval salivary glands. This experiment showed much higher levels of phospho-moesin in the cells with a Clic\textsuperscript{109} loss-of-function mutation than in wild-type cells. This suggests that Clic works to antagonize activation of moesin *in vivo* by indirectly increasing its dephosphorylation.
The second experiment was a co-immunoprecipitation (Co-IP) assay that also used extracts from *Drosophila* 3rd instar larval salivary glands. A Clic-GFP fusion protein construct was expressed in this tissue and precipitated with rabbit anti-GFP antibodies. The pull down of the Clic-GFP construct also precipitated moesin protein and revealed that moesin and Clic exhibit physical interaction *in vivo*. It is unclear whether moesin is directly binding to Clic or if it binds via a bridging protein or as part of a larger protein complex.

Although these previous, and yet unpublished, results show a clear physical and genetic interaction between Clic and moesin in *Drosophila*, the nature of the interaction is largely uncharacterized. Thus, my focus in the present study will be to determine through what mechanism these two proteins interact and the implications it has for the integrity of the actin-rich rhabdomeres in *Drosophila* photoreceptors.

**VII. Chloride intracellular channels (CLICs)**

The discovery of p64, the founding member of the p64/CLIC protein family, was made in 1987 from bovine tracheal epithelium and kidney tissues. Since this initial discovery, other members of the CLIC family of proteins have been discovered in humans, mice, fruit flies, worms, plants, and a variety of other organisms. CLIC proteins appear to be widely expressed in multicellular organisms and are known to function as anion channels, as well as regulating secretion, apoptosis, and cell division. In vertebrates there are six known CLIC
family members: CLIC1-6. The fruit fly *Drosophila melanogaster* has only one CLIC protein: Clic. CLIC proteins can be distinguished from other ion channels in that they may exist in two different forms within the cell. They may be in a solid globular form in the cytoplasm, or they may be integral membrane proteins that function as ion channels. In addition to possible ion channel activity, CLICs may function in oxidation-reduction (redox) reactions, as protein chaperones, or as regulators of the actin cytoskeleton. In *Drosophila*, it is known that Clic is present in hemocytes and retinal tissues and it is speculated to function in a protective role during cellular apoptosis and the cell’s response to oxidative stress. This is possibly due to the presence of a thioredoxin motif similar to that of the glutathione-S-transferase (GST) family of proteins. In a mammalian system, evidence suggests that CLIC5 interacts significantly with the microvillus actin cytoskeleton to localize CLIC5 to its proper region in polarized epithelial cells. Furthermore, CLIC5 and CLIC4 have been shown to be components of actin-rich microvilli structures in human placental cells. CLIC5 was shown to interact in a protein complex that included actin and actin associated proteins.

**VIII. CLICs and actin**

Further research on a splice variant of mammalian CLIC5, CLIC5A, showed this variant protein was capable of inserting into an artificial membrane and acting as a chloride channel in vitro. CLIC5A was also able to interact with ezrin, an ERM protein present in mammalian systems. Polymerization of G-actin to F-actin is required for the formation of the CLIC5-actin protein complex involved in
microvilli assembly. This research demonstrated that a member of the CLIC family is closely associated with actin-binding proteins, and its activity is linked to actin-rich structure formation.26

Knowledge of the interaction of CLIC5 with the actin cytoskeleton has been further reinforced in a study on mouse hair cell stereocilia. Mice that are homozygous for a recessive mutation of the Clic5 gene called jitterbug (jbg) are hearing impaired or completely deaf. This effect occurs because CLIC5 is essential for proper function and formation of stereocilia of hair cells in the inner ear. Stereocilia are large actin-rich microvilli that respond to sound waves and head movements to allow the mice to hear and to balance their bodies. The cochlea of CLIC5 deficient mice was dissected to reveal deformed and disorganized stereocilia (Fig. 4).27

As sound waves enter the ear, the highly organized stereocilia of the hair cells bend and this deformation of the membrane results in the opening of ion channels and depolarization in the hair cells. This depolarization leads to signal transduction
to the brain and the perception of sound. In wild-type mice, CLIC5 was localized at the base of the stereocilia. In CLIC5 null mice, levels of the actin-binding protein radixin and moesin (both ERM proteins) were reduced. A decrease in the concentration of linker ERM proteins was hypothesized to lead to destabilization of the actin core in the stereocilia and the observed deformities. This study provided further evidence that CLIC proteins were involved in the organization and stability of the actin cytoskeleton, possibly through the interactions with known actin-binding proteins such as ERM.

Previous research in this laboratory has been done linking the sole CLIC family protein in *Drosophila*, Clic, to actin cytoskeleton dynamics. Clic has been implicated in filopodia formation in larval hemocytes (blood cells). Genetic results suggest that Clic acts downstream of Cdc42, a small GTPase required for proper formation of actin-rich filopodia and lamellipodia. This observed genetic interaction of Clic and Cdc42 further suggests that Clic has important implications for cytoskeletal remodeling and actin-rich structure formation.

**IX. CLICs and cancer**

In humans, members of the CLIC family have been linked to nasopharyngeal carcinoma, hepatocellular carcinoma, colorectal cancer, breast cancer, and gastric carcinoma. These studies suggest that the CLIC family of proteins may be a useful biomarker for cancer and that they may function directly in cancer progression, but the mechanism for this is not well understood.
Chen et al. (2007) found that CLIC1 protein was significantly upregulated in patients diagnosed with gastric cancer. Further, in most cases CLIC1 expression in gastric tissues gradually rose as the cancer progressed from stage 1 to 4, and high expression of CLIC1 was also strongly correlated with lymph node metastasis of the cancerous tissues.\textsuperscript{33} CLIC1 overexpression was also observed in hepatocellular carcinoma and colorectal cancer.\textsuperscript{30,31} Another study found that CLIC1 is overexpressed in nasopharyngeal carcinoma (NPC) cell secretions. CLIC1 can be detected in the plasma of healthy individuals, but levels are significantly higher in individuals with NPC. This suggests that CLIC1 may be a functional biomarker for NPC, as elevated CLIC1 plasma levels can be detected in the early stages of the cancer.\textsuperscript{29}

Other studies have looked for possible cancer biomarkers as early indicators of cancer progression and as a means to improve patient treatment. A study done on hormone receptor-negative breast cancer sought to find biomarkers in the cancerous tissues that would indicate the likelihood of cancer metastasis. Results showed that elevated levels of CLIC5, another CLIC family protein, were one of 14 prognostic candidates that likely identify cases that will not have metastatic relapse. Therefore, CLIC5 could be an important biomarker for this type of cancer, especially in relation to its progression.\textsuperscript{32}

In addition to being a valuable biomarker for cancer, it is possible that CLIC proteins may be a viable target for drugs designed to combat cancer progression. However, before development of such a drug could be feasible, a complex and
dynamic understanding of the mechanisms by which CLICs contribute to cancer progression must be reached. There are two main processes involved in cancer: proliferation and metastasis. CLICs could be involved in either of these processes. It is known that the actin cytoskeleton is involved in cellular motility and that disruption of its various interactions contributes to cancer progression. As evidence points to an interaction between CLICs and the actin cytoskeleton, further elucidation of this interaction may be valuable to understanding the mechanism by which CLICs contribute to cancer progression.
EXPERIMENTAL DESIGN

I. Overview of rhabdomere formation

Rhabdomeres, the light sensitive apical surfaces of the photoreceptor cells of the *Drosophila* compound eye, accommodate more than 90% each photoreceptor’s plasma membrane in a closely packed stack of about 60,000 microvilli. Microvilli extend from the surfaces of cells including fibroblasts, epithelial cells, myoblasts, and macrophages. Probably the most well characterized are the microvilli that compose the apical brush border of the epithelium lining the lumen of the intestine that serve to increase cell surface area and therefore increase nutrient absorption efficiency. These microvilli consist of a bundle of actin filaments linked together by actin bundling proteins. In *Drosophila*, the primary actin bundling protein in microvilli is fascin, coded for by the *singed (sn)* gene. The actin filaments are nucleated near the plasma membrane and elongation of these filaments occurs by addition of G-actin monomers to the membrane-associated ends of the filaments. Most of rhabdomere morphogenesis occurs during the latter half of pupal development, when abundant vesicular traffic expands the photosensitive membrane. Rhabdomere microvilli do not project as freestanding individuals; they associate with other neighboring microvilli over their entire length. The central microvilli in the rhabdomere grow longer than their more lateral neighbors creating a convex front at the center of the ommatidium. As the microvilli grow during development, they do not extend away from the photoreceptor cell, but rather they grow back into the photoreceptor cell.
cytoplasm. The microvilli terminate on this end at a distinctive rhabdomere base that marks the boundary between the rhabdomere and the photoreceptor cell cytoplasm. The rhabdomere base is defined by a specialization of the cortical actin cytoskeleton called the rhabdomere terminal web (RTW). This is a protrusion of actin microfilaments beginning at the base of the rhabdomere that extend deep into the cell cytoplasm. Moesin, the membrane/cytoskeletal-linking ERM protein discussed previously, is concentrated at the rhabdomere base and it is known that mutations of moesin disrupt rhabdomere morphogenesis, presumably by failure to properly anchor actin microfilaments.35

II. **Drosophila as a model system**

The fruit fly *Drosophila melanogaster* is an attractive system for studying protein interactions for a number of reasons. Importantly, manipulations and conclusions made about disease genesis and progression in *Drosophila* can be applied to human systems. Many basic biological and physiological properties are conserved between humans and *Drosophila*, and nearly 75% of human disease-causing genes are believed to have a functional homolog in the fly.36 This similarity allows important work to be done in the area of disease prevention and development of novel treatments. Another attractive aspect of using *Drosophila* is the variety of gene manipulations and genetic crosses that can be made for study. Genes can be added or subtracted with mutations, or gene product levels may be altered with RNA interference (RNAi). The GAL4/UAS system may also be used to induce genetic changes in specific cell types in order to produce complex
individuals in which gene interactions can be accurately and reliably observed.\textsuperscript{37} This work is made easier by the availability of many mutant lines of flies that cover most of the known genes of this organism.\textsuperscript{36}

Further, \textit{Drosophila} have much less redundancy in many genes than mammalian systems. \textit{Drosophila} has only one CLIC gene, called \textit{Clic}, which allows researchers to study a CLIC null mutant more easily. When there are multiple genes from the same family, as in mammals, other family members may compensate for a lack of function in one member. This redundancy complicates genetic analysis. Similarly, \textit{Drosophila} have only one ERM protein, moesin, which makes study of downregulation of ERM function in the fly greatly simplified in comparison to mammals.\textsuperscript{1}

While \textit{Drosophila} as a whole is an extremely useful model system to work with, the \textit{Drosophila} compound eye and included rhabdomeres are particularly well suited to study the interactions of moesin and \textit{Clic} in relation to F-actin. The sequential development of the compound eyes of \textit{Drosophila} makes it possible to model certain mechanisms of disease genesis\textsuperscript{38} and the high density of F-actin that composes the microvilli in the rhabdomeres makes it an ideal structure in which to study the interaction of various proteins with F-actin.
III. Research questions and hypotheses

The focus of my research to date has been to determine the mechanism for the genetic interaction of moesin and Clic proteins in relation to F-actin in *Drosophila* rhabdomeres. To address this question, I focus mainly on the morphogenesis of rhabdomeres from their respective photoreceptor cells in varying genetic backgrounds. My approach uses visual analysis of the phenotypic manifestations of the genetic interactions at work in each engineered genotype based on high magnification electron micrographs of cross-sections of the *Drosophila* compound eye.

I hypothesize that Clic is involved in formation of the projections of microvilli from the photoreceptor cells to form functional rhabdomeres. I predict that Clic works in the formation of these structures via interaction with a PP1 phosphatase responsible for dephosphorylating and thus deactivating wild-type moesin. I studied these genetic interactions by observing rhabdomere structure in individuals that have either a wild-type or loss-of-function Clic background, while manipulating gene dosage of moesin, the PP1 phosphatase, and other important protein players within this system.

If Clic is involved in rhabdomere morphogenesis, I predict that its manipulation may result in a loss of epithelial integrity in these cells, and possibly a disruption of rhabdomere morphogenesis. If Clic promotes formation of rhabdomeres via cooperation with moesin, I would expect that the absence of Clic would cause a further reduction in the size and regularity of the rhabdomere
structure due to loss of moesin function. If Clic hinders formation of rhabdomeres via antagonism of moesin, I expect that the absence of Clic would cause restoration of the malformed rhabdomeres.
MATERIALS AND METHODS

I. Manipulation of gene expression levels

In order to increase or decrease expression levels of moesin and other proteins known or predicted to play a role in rhabdomere morphogenesis, I utilized the GAL4-UAS system.\(^3\) This system is based on the production of two independent transgenic lines. The first line expresses GAL4, a transcriptional activator from yeast, under a tissue- or cell-specific promoter. This is referred to as the driver line. The second line has a transgene that can be induced via GAL4 protein binding to sites called upstream activation sequences (UAS). This line is called the responder line. Crossing these two lines results in the production of a hybrid organism that expresses the gene of interest in a manner dependent on the promoter chosen in the driver line.\(^3\) This process is illustrated in *Drosophila* in Figure 5.

This system is useful in *Drosophila* because there are no endogenous UAS sequences or GAL4 proteins to complicate analysis. This insures that we are only altering the expression levels of the protein of interest. Also with this system, we can manipulate gene expression only in specific tissues in the *Drosophila* by using tissue-specific promoters of GAL4 expression. Since changes in expression levels of some genes can result in lethality if manipulation occurs in all cells, this is a very useful tool for studying those essential genes.
In my research I used the GAL4 driver called Sevenless GAL4 (sev-GAL4).

The *sevenless* gene codes for a surface transmembrane protein and is highly involved in morphogenesis of the *Drosophila* compound eye. *Sevenless* expression in the R7 cell within each ommatidium of the compound eye has been confirmed and studied extensively; however, its expression is not limited to R7 cells. *Sevenless* expression has been detected in the R1 and R6 pair, the R3 and R4 pair, and four cone cell precursors.\(^40\)

This system can be used to increase levels of a specific protein with promoter dependent expression by creating a transgene containing a UAS site and an attached wild-type or constitutively active form of a gene of interest. Conversely, the GAL4-UAS system can be used to decrease protein levels via the RNA interference (RNAi) pathway. RNAi is a means of post-transcriptional gene silencing that targets and degrades specific messenger RNA (mRNA) molecules.
before they can be translated into proteins. To use this pathway to modulate gene expression, an RNAi sequence that corresponds to a specific gene is engineered and paired with the UAS site described previously. When this sequence is expressed in the presence of GAL4, it forms a double stranded RNA (dsRNA) molecule that is processed into a small interfering RNA (siRNA) by the protein Dicer. This siRNA is complementary to the mRNA sequence for a specific gene of interest. Thus, the siRNA binds to the complementary mRNA and targets it for degradation by the RNA induced silencing complex (RISC). This process of gene knockdown decreases the level of mRNA in the cell and therefore decreases the amount of protein present within the cell. When this system is used, it is typical to also overexpress Dicer protein to effectively convert the excess dsRNA formed to siRNA.41 This method was used to manipulate the gene dosage of moesin and other important protein players in rhabdomere morphogenesis.

**Figure 6. RNAi pathway.** The RNAi pathway used to post-transcriptionally silence gene expression. Dicer recognizes and cleaves dsRNA creating siRNA. The RISC complex then used the siRNA template to target specific mRNAs for degradation42 (Figure from 42).
II. Maintenance of fly cultures

Fly stocks were maintained at 22ºC. Food was prepared using 2280g Jazz mix, 360g yeast, 120g soy powder, and 12L of water (see section VB.). Fly crosses were cultured at 22ºC until the middle third instar stage (about day 5) and then moved to 28ºC to maximize expression of the GAL4-UAS system. Crosses with UAS-Moe<sup>TD</sup> were kept at 22ºC due to poor viability at 28ºC. Adult flies with appropriate genotypes were maintained at 22ºC for about 5 days before fixation. Flies were genotyped based on dominant mutant markers: Bar (B) for the X chromosome, Curly (Cy) for the second chromosome, and Stubble (Sb) or Ultrabithorax (Ubx) for the third chromosome.

III. Fixation, staining, and embedding of compound eyes

The heads of anesthetized Drosophila were removed from the bodies and then the proboscis and antennae were removed. The heads were fixed in 2.5% glutaraldehyde and 2.5% paraformaldehyde in a 0.1M cacodylate buffer at pH 7.4. All solutions were made in a 0.1M cacodylate buffer. Initial fixation was done at room temperature for at least 4 hours to overnight. Then the heads were fixed again for at least 6 hours to overnight in fresh fixative containing 1% tannic acid. The heads were then rinsed in a cacodylate buffer for 10 minutes then post-fixed in 2% osmium tetroxide for at least 15 minutes to 2 hours. After a brief rinse in cacodylate buffer, the heads were treated with 2% aqueous uranyl acetate in the dark, at room temperature for 2-4 hours. Osmium tetroxide and uranyl acetate
“stain” the tissues by adding heavy metals to make specific structures electron dense to provide contrast with transmission electron microscopy (TEM). The heads were then rinsed again in cacodylate buffer, dehydrated in an ethanol series, and immersed in propylene oxide for 15-30 minutes to remove residual ethanol.

Following fixation, staining, and dehydration, heads were embedded in either Epon/Araldite or Spurr’s low viscosity resin (see section VB.). The Epon/Araldite resin mixture consisted of Epon 812 (13.1g), Araldite 502 (10g), DDSA (27.7g), and DMP-30 (1.5ml) or BDMA (1.75ml). The tissues were gradually infiltrated with resin by immersing in 50% propylene oxide/50% resin overnight, then 100% resin at room temperature for 2 hours. Resin was then polymerized for 48 hours at 60°C. The Spurr’s low viscosity resin mixture consisted of ERL 4221 (18ml), DER 736 (14ml), NSA (48ml), and DMAE (0.6ml). The heads were put through a graded series of propylene oxide and Spurr’s resin for up to 5 days according to kit directions. Resin was then polymerized for 8-12 hours at 70°C. Both resin mixtures were mixed thoroughly and allowed to degas for at least 1 hour before use.

IV. Tissue sectioning and microscopy

Embedded heads were trimmed so that the surface of one of the compound eyes was oriented toward the surface of the resin block. The surface was sliced until the lens was exposed and cut into slightly. Semi-thin sections about 0.5-0.7µm thick were cut with glass knives using a Reichert Ultracut E Ultramicrotome. Tissue sections were mounted on microscope slides then stained
with 0.7% toluidine blue in 0.7% borax and covered with coverslips. Tissue sections were examined using a Nikon Labphot-2 microscope with 40X and 63X oil immersion objective lenses. Photomicrographs were taken with a Nikon DXM-1200 digital camera using the Nikon Act-1 software program. Ultrathin sections 60-80nm thick were cut with a Diatom diamond knife using a Reichert Ultracut E Ultramicrotome. Tissue sections were mounted on either naked or formvar-coated grids, stained with 2% uranyl acetate and lead citrate, then examined using a JEOL 1010 transmission electron microscope operated at 80kV. Electron micrographs were taken at 1,000X-80,000X using a Gatan digital camera mounted within the microscope.

V. Materials

A. Fly stocks

Below is a table of the fly stocks used in my experiments. In some cases, these stocks were combined to generate the correct genetic background, to allow for genotyping of adult flies, or to generate the appropriate genotype of interest.

<table>
<thead>
<tr>
<th>Abbreviated Genotype</th>
<th>Formal Genotype</th>
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<tbody>
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<td>Bloomington Drosophila Stock Center</td>
</tr>
<tr>
<td>w Clic^{109}</td>
<td>w* Clic^{109}</td>
<td>S. Tanda &amp; M. Berryman, personal communication</td>
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<td>Richard Fehon&lt;sup&gt;4&lt;/sup&gt;</td>
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**B. Chemical reagents**

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<td>Sodium citrate</td>
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**C. Specialized supplies**

| 3 mm grids coated with formvar and carbon | Electron Microscopy Sciences |
RESULTS

I. Moesin is essential for rhabdomere morphogenesis.

Karagiosis and Ready showed that *Drosophila* lacking moesin protein fail to undergo photoreceptor differentiation by examining flies with the $Moe^{G0323}$ allele, which was thought to be genetically null.\textsuperscript{17} They also expressed $UAS-Moe^{RNAi}$ using a $hsp70$-GAL4 driver in flies to induce downregulation of moesin during eye development. Using this system, they found that the rhabdomeres of those flies with a moesin knockdown by RNAi typically have reduced and irregular microvilli formation, severely disrupted apical membranes, and the rhabdomere terminal web (RTW) was replaced with abnormal F-actin accumulations.

In order to investigate moesin’s role in rhabdomere morphogenesis and to form a base for comparison of the effect of other protein manipulations in this system, it was first necessary to replicate the results of these experiments using $UAS-Moe^{RNAi}$. Wild-type control flies with genotype $w^{1118}$ were examined and compared to flies with a knockdown of moesin function using the GAL4 system and RNAi. Male flies with the following genotype were engineered and selected based on the presence or absence of genetic markers: $w, UAS-Dcr2/Y; sev-GAL4/+; UAS-Moe^{RNAi}/+$. Unlike Karagiosis and Ready\textsuperscript{17} who used a $hsp70$-GAL4 driver to drive expression of the $UAS-Moe^{RNAi}$ construct, this study utilized the *sevenless*-GAL4 ($sev$-GAL4) driver. This allowed cell specific rather than time specific knockdown of moesin. As stated above, *sevenless* is known to be expressed in the R7 cell and has been detected in the R1 and R6 pair, the R3 and R4 pair of photoreceptor
In the flies with a moesin knockdown, each discrete ommatidium contained only 4 normal, visible rhabdomeres as compared to 7 visible rhabdomeres in the wild-type specimen (Figs. 7 & 8). Photoreceptor cell bodies in the MoeRNAi specimens were still distinguishable based on boundaries maintained by adherens junctions but the R7 rhabdomere and the R3 and R4 pair rhabdomeres were missing or malformed. The remaining rhabdomeres R1, R2, R5, and R6 were largely unaffected (Fig. 7). Notably, changes induced in each ommatidium were highly consistent throughout the compound eye of each individual specimen. This result is consistent with previous data describing sevenless expression in the R7 cell and R3 and R4 pair, however a lack of the moesin knockdown phenotype observation in the R1 and R6 pair suggests that sevenless may not be expressed in these photoreceptor cells or the level of expression might be lower than previously thought. This may also be a result of differing levels of expression between endogenous sevenless and the sev-GAL4 transgene.

These results showing a loss, reduction in size, or abnormal microvilli formation in affected rhabdomeres in flies with a moesin knockdown by RNAi mimic the results of Karagiosis and Ready. These results implicate moesin in the genesis and organization of membrane associated cytoskeletal components, namely F-actin, in vivo. Moesin may also play a significant role in the morphogenesis and organization of the membrane skeleton that supports the array of microvilli in the apically specialized structure, the Drosophila rhabdomere.
Figure 7. Electron micrographs of wild-type vs. MoeRNAi ommatidia.
Left: Electron micrograph cross-section of an adult wild type w1118 ommatidium. Rhabdomeres R1-R7 are visible and in regular arrangement.
Right: Electron micrograph cross-section of an adult MoeRNAi ommatidium. Rhabdomere R7 is missing. The R3 and R4 pair of rhabdomeres are visible but have marked abnormal microvilli formation and are reduced in size. Scale bars, 1µm.

Figure 8. Light micrograph of wild-type ommatidia. Light micrograph of a cross-section of an adult wild type w1118 ommatidium. Rhabdomeres R1-R7 are visible and in regular arrangement in each ommatidium. Scale bar, 25µm.
II. Clic plays a role in rhabdomere morphogenesis.

The CLIC family proteins are widely expressed in multicellular organisms and are known to function as anion channels as well as in regulating secretion, apoptosis, and cell division. In addition to these functions, research has also demonstrated that a member of the CLIC family, CLIC5, is closely associated with actin-binding proteins, and its activity is linked to actin-rich structure formation. The known interaction of CLIC family members with actin-rich structures and biochemical evidence linking it to moesin function led the present study to explore the role of Clic within actin-rich Drosophila rhabdomeres, a system in which Clic function is largely unknown.

In order to investigate the role of Clic within this system, flies with a loss-of-function Clic mutation, Clic109, with the following genotype were examined: w Clic109/ Y. In these mutants, rhabdomeres R1-R7 are all visible in a semi-regular arrangement. However, the shape of the rhabdomeres in these mutants is abnormal. Normal rhabdomeres take on a rounded shape (Fig. 7) because the microvilli that make up the rhabdomere elongate between two tensile surfaces during morphogenesis. The base and tip of the rhabdomere form a curved shape due to a balance between the forces of microvilli extension and the tensile sheets bounding the microvilli. In the Clic109 mutants, rather than taking on a normal rounded shape, the rhabdomeres are relatively squared (Fig. 9). This abnormality implicates Clic in regulating some facet of rhabdomere morphogenesis.
Figure 9. Electron micrograph of a Clic\textsuperscript{109} mutant ommatidium. All rhabdomeres R1-R7 are visible but malformed. Instead of wild-type (Fig. 7) rounded rhabdomeres, these rhabdomeres appear relatively squared. Scale bar, 1µm.

III. Clic antagonizes moesin function

Given the evidence for an essential role of moesin in rhabdomere morphogenesis\textsuperscript{17} and suggestion that Clic has role in this process, we next investigated the genetic interaction of these two proteins in a single individual. Flies were engineered with a knockdown of moesin via RNAi in a genetic background with increased levels of wild-type Clic: \textit{w, UAS-Dcr2/Y; sev-GAL4/UAS-Clic\textsuperscript{WT}; UAS-Moe\textsuperscript{RNAi}/+}. These mutants were compared to flies with a knockdown of moesin via RNAi and the Clic\textsuperscript{109} loss-of-function mutation: \textit{w Clic\textsuperscript{109}, UAS-Dcr2/Y; sev-GAL4/+; UAS-Moe\textsuperscript{RNAi}/+}. The Moe\textsuperscript{RNAi} flies with increased Clic had a phenotype closely resembling Moe\textsuperscript{RNAi} alone, but the affected rhabdomeres, R7, R3, and R4, display more severe disorganization and an overall decrease in microvilli (Fig. 10). This result suggests that Clic is acting in a manner
that is antagonistic to moesin function, as the $Moe^{RNAi}$ phenotype is more severe when Clic is present in excess.

Conversely, in the $Moe^{RNAi}$ flies with the $Clic^{109}$ loss-of-function mutation, the phenotype more closely resembles wild-type than specimens with $Moe^{RNAi}$ alone. In these individuals, R3 and R7, which are lost in $Moe^{RNAi}$ flies, form relatively normally. R4 is not restored (Fig. 10). This partial rescue of the $Moe^{RNAi}$ phenotype when Clic is absent again suggests that Clic’s role in rhabdomere morphogenesis is antagonistic to moesin function.

**Figure 10.** Electron micrographs of $Moe^{RNAi} Clic^{WT}$ vs. $Moe^{RNAi} Clic^{109}$ ommatidia.

Left: Electron micrograph cross-section of an adult $Moe^{RNAi}$ ommatidium in a genetic background with additional wild-type $Clic$ function. Rhabdomeres R7, R3, and R4 are severely malformed and display markedly disorganized microvilli organization.

Right: Electron micrograph cross-section of an adult $Moe^{RNAi}$ ommatidium in a genetic background with the $Clic^{109}$ loss-of-function mutation. Rhabdomeres R7 and R3 are restored compared with $Moe^{RNAi}$ alone suggesting an antagonistic role of Clic in relation to moesin in rhabdomere morphogenesis.

Scale bars, 1µm.
IV. Phosphomimetic moesin phenotype is partially rescued by additional Clic

Moesin, the sole ERM protein in *Drosophila*, is partially activated by phosphorylation. Phosphorylation of moesin is carried out by Slik, the *Drosophila* Sterile20 kinase, which adds a phosphate group to a conserved threonine residue in the C-terminal domain. However, there exists a phosphomimetic form of moesin, *Moe*\textsuperscript{TD}, which mimics a state of constant activation by phosphorylation without the need for the addition of a phosphate group; this form is greatly enriched at the plasma membrane. To observe the effects of the constitutively active *Moe*\textsuperscript{TD} allele, flies with the following genotype were engineered and examined: *w/Y; sev-GAL4/UAS-Moe*\textsuperscript{TD}. These mutants display an external rough eye phenotype (Fig. 11) and have aberrant growth of microvilli in all rhabdomeres, R1-R7. Some rhabdomeres have even bifurcated into two distinct units as a result of the presence of overactive moesin (Fig. 12). As the *Moe*\textsuperscript{TD} allele was expressed using the *sev-GAL4* driver, it is unclear why there are observable abnormalities in the R2 and R5 rhabdomere pair, as there is no evidence that *sevenless* is expressed in these photoreceptor cells.
Figure 11. Surface structure of a wild-type vs. a $Moe^{TD}$ compound eye. Left: Surface structure of a wild-type adult fly compound eye raised at 22°C. Right: Surface structure of a $Moe^{TD}$ adult fly compound eye raised at 22°C. The eye appears rough and abnormal.

I also observed the effects of the $Moe^{TD}$ allele in a background with increased wild-type $Clic$ in flies with the following genotype: $w/Y; sev\text{-}GAL4/\text{UAS-}Moe^{TD}$, $UAS-\text{Clic}^{WT}$. All of the phenotypes observed with $Moe^{TD}$ alone, external rough eye, aberrant rhabdomere growth, and overall disorganization, were partially rescued by wild-type $Clic$ overexpression. In double mutants, rhabdomere size and shape closely resembles wild-type, including no overgrowth of microvilli (Fig. 12).

Conversely, flies were engineered with the $Moe^{TD}$ allele in a background with the $Clic^{109}$ loss-of-function mutation with the following genotype: $w \ Clic^{109}/Y; \ sev\text{-}GAL4/\text{UAS-}Moe^{TD}$. The phenotypic abnormalities induced by the $Moe^{TD}$ allele are enhanced in these individuals. Discrete rhabdomeres, besides the enlarged and
malformed R7, cannot be distinguished because of highly aberrant microvilli overgrowth (Fig. 13).

**Figure 12.** Electron micrographs of $Moe^{TD}$ vs. $Moe^{TD} \text{Clic}^{WT}$ ommatidia. Left: Electron micrograph cross-section of an adult $Moe^{TD}$ ommatidium. Rhabdomeres R1-R7 are visibly enlarged and malformed and some rhabdomeres have bifurcated into separate units. Right: Electron micrograph cross-section of an adult $Moe^{TD}$ ommatidium with added wild-type Clic function. Rhabdomeres appear more normalized as in wild-type flies (Fig. 7) and arrangement is more regular. Thus, additional Clic function partially rescues the $Moe^{TD}$ phenotype. Scale bars, 1µm.

**Figure 13.** Electron micrograph of a $Moe^{TD}$ Clic$^{109}$ ommatidium. Microvilli growth is aberrant and only the R7 rhabdomere is distinguishable. Scale bar, 1µm.
V. Kinase mediated moesin activation is independent of Clic

Hipfner et al. determined that the *Drosophila* Sterile20 (Ste20) kinase Slik acts on cytoskeletal organization by regulating moesin via phosphorylation. This study showed that photoreceptor cells in *Slik* mutants have sections of apical membrane (corresponding to the rhabdomere) that are devoid of microvilli, presumably from a decrease in the amount of phosphorylated moesin. This result suggested that Slik is required for normal phosphorylation of moesin. The present study sought to investigate whether there was an interaction between Clic and Slik. Since Clic appears to function antagonistically to moesin, it is possible that Clic exerts its effect by inhibition of Slik, moesin’s primary kinase and activator in *Drosophila*. To test this hypothesis, flies were engineered with increased Slik function in the eye with the following genotype: *y w, UAS-Slik17B/Y; sev-GAL4/+*. As expected, rhabdomeres in the ommatidia of these specimens are enlarged and fill the empty space within the ommatidium (Fig. 14). This overgrowth of microvilli is consistent with an increase in moesin activity resulting from excess Slik. To assess the genetic interaction between Slik and Clic, flies were engineered with increased Slik function and the *Clic* loss-of-function mutation with the following genotype: *y w Clic*, *UAS-Slik17B/Y; sev-GAL4/+*. These individuals show a slight enlargement of rhabdomeres compared with *Slik17B* alone. Rhabdomeres take on the characteristic *Clic* squared phenotype, but are enlarged and fill the empty space within the ommatidium (Fig. 14). This result reinforces our hypothesis that Clic is a moesin antagonist, as the loss of Clic results in more
excessive microvilli growth than in those individuals with Slik17B alone. This excessive growth of microvilli is consistent with an accumulation of active moesin. It is unlikely that Clic exerts this function via direct interaction with Slik, based on the subtle change in phenotype from Slik17B alone to Slik17B in the Clic109 genetic background. This result suggests that Slik function is independent of Clic function in regard to moesin activation and that these proteins do not directly interact in Drosophila photoreceptors.

Figure 14. Electron micrographs of Slik17B vs. Slik17B Clic109 ommatidia.
Left: Electron micrograph cross-section of an adult Slik17B ommatidium. Rhabdomeres are enlarged and fill the empty space with the ommatidium.
Right: Electron micrograph cross-section of an adult Slik17B ommatidium in a genetic background with the Clic109 loss-of-function mutation. Rhabdomeres take on the characteristic Clic109 squared phenotype but remain enlarged and fill the empty space within the ommatidium.
Scale bars, 1µm.
VI. Phosphatase mediated moesin deactivation is dependent on Clic

Kunda et al. (2012) identified the PP1 phosphatase PP1-87B as the major regulator of moesin dephosphorylation in *Drosophila* cultured cells, as confirmed by both immunofluorescence and western blotting. Since Clic appears to be an antagonist to moesin, we hypothesized that Clic may exert its function by way of an interaction with the phosphatase PP1-87B. One of the mammalian homologs of *Drosophila* Clic, CLIC5A, has a predicted binding site in its structure for one of the catalytic subunits of the PP1 phosphatase PP1CA (M. Berryman, personal communication). This further suggests that Clic may be interacting with the PP1-87B phosphatase to exert its antagonistic effect on moesin. To test this hypothesis, flies were engineered with a knockdown of PP1-87B function via the GAL4 system and RNAi with the following genotype: *w, UAS-Dcr2/Y; sev-GAL4/+; UAS-Pp1-87B RNAi/+*. Rhabdomere structure within the ommatidia of these individuals was abnormal. Rhabdomeres appear enlarged and there is less open space between the individual rhabdomeres than normal. There is clearly an overgrowth of microvilli causing crowding of the rhabdomeres in these specimens (Compare Fig. 7 and Fig. 15). This aberrant growth of microvilli is consistent with an excess of active moesin induced by a loss of PP1-87B, the protein responsible for its dephosphorylation and subsequent inactivation. To test the interaction between the PP1-87B phosphatase and another probable moesin antagonist, Clic, flies were engineered with a knockdown of PP1-87B and the *Clic*¹⁰⁹ loss-of-function mutation with the following genotype: *y w Clic*¹⁰⁹, *UAS-Dcr2/Y; sev-
In these individuals with a deficit in one known and one probable antagonist to moesin, I expected microvilli overgrowth to be extreme and more severe than in individuals with a knockdown of PP1-87B alone. Interestingly, microvilli overgrowth is not extreme but milder in the double mutant than PP1-87B RNAi alone. Rhabdomeres take on the characteristic Clic109 squared phenotype but they are more normally sized and distributed within the ommatidium (Fig. 15). This result suggests that the antagonistic functions of these two proteins in relation to moesin are not independent. In the double mutants, the Clic109 phenotype masks the PP1-87B knockdown phenotype, suggesting that the action of Clic is upstream of PP1-87B function.
Figure 15. Electron micrographs of \textit{PP1-87B}^{\text{RNAi}} vs. \textit{PP1-87B}^{\text{RNAi}}\text{ Clic}^{109} ommatidia.

Left: Electron micrograph cross-section of an adult \textit{PP1-87B}^{\text{RNAi}} ommatidium. Rhabdomeres appear enlarged and microvilli overgrowth leaves little open space within the ommatidium as compared to wild-type individuals.

Right: Electron micrograph cross-section of an adult \textit{PP1-87B}^{\text{RNAi}} ommatidium in a genetic background with the \textit{Clic}^{109} loss-of-function mutation. Rhabdomeres are more normally sized and distributed within the ommatidium with milder microvilli overgrowth.

Scale bars, 1\(\mu\)m.
DISCUSSION

I. Overview

I have shown that Clic is involved in rhabdomere morphogenesis in the *Drosophila* compound eye. My results suggest that Clic antagonizes moesin function via an interaction with its principal phosphatase, PP1-87B. Restoration of the R3 and R7 rhabdomeres in *MoeRNAi* specimens with the *Clic*<sup>109</sup> loss-of-function genetic background implicates Clic as a moesin antagonist. Further, evidence showing that Slik function is independent of Clic and the masking of the *PP1-87B<sup>RNAi</sup>* phenotype in the *Clic*<sup>109</sup> loss-of-function genetic background mutation implicates Clic as a necessary upstream effector for PP1-87B function. The extent to which actin-rich microvilli, that comprise rhabdomeres, grow is influenced by Clic’s interaction with moesin and PP1-87B.

II. A new role for Clic in rhabdomere morphogenesis

CLIC family proteins have been implicated in various important cellular functions including membrane trafficking, cytoskeletal function, apoptosis, cell cycle control, mitosis, and cellular differentiation. However, the mechanism by which CLICs perform these functions is not well understood. Even less is known about the role of the single *Drosophila* CLIC family protein, Clic. Here I present a model for a novel role of Clic in mediating rhabdomere morphogenesis in *Drosophila* photoreceptor cells by regulating moesin function via an interaction with the PP1 phosphatase PP1-87B.
By comparing the extent of microvilli growth that forms the rhabdomeres in varying genetic backgrounds, I found that Clic genetically interacts with moesin and PP1-87B to modulate rhabdomere morphogenesis. Observation of abnormal rhabdomere structure in Clic loss-of-function mutants from a rounded to squared shape gave the first indication that Clic played a role in rhabdomere formation (Fig. 9). This squared rhabdomere phenotype may be a result of a disruption in the tensile forces that cause curvature in a normal rhabdomere. Normal rhabdomeres take on a rounded shape because the microvilli that make up the rhabdomere elongate between two tensile surfaces during morphogenesis. A loss of Clic may induce a disruption of these balanced tensile forces, thereby causing the observed phenotype.

An essential role for moesin in rhabdomere morphogenesis shown in previous research was confirmed by the present study. A knockdown of moesin function by RNAi and the GAL4 system using the sevGAL4 driver induced a loss of the rhabdomeres of R7 and R3/R4 photoreceptor cells (Fig. 7). These results implicate moesin in the genesis and organization of membrane cytoskeletal components in vivo. In light of research showing that sevenless expression has been detected in the R7 cell, the R1 and R6 pair, and the R3 and R4 pair, I expected to see a loss of all rhabdomeres in the MoeRNAi specimens except for the R2 and R5 pair. The retention of the R1 and R6 pair in these specimens suggests that sevenless may not be expressed in these photoreceptor cells or the level of its expression might not be sufficient to alter the phenotype. Additional evidence showing a partial rescue of
the \textit{Moe}^{RNAi} phenotype induced by the \textit{Clic}^{109} loss-of-function mutation suggests a role for Clic as a moesin antagonist. In these double mutants, I observed retention of the R3 rhabdomere that is lost in \textit{Moe}^{RNAi} alone strains. This presents strong evidence that Clic works in opposition to moesin function during rhabdomere morphogenesis.

My results suggest that the mechanism by which Clic antagonizes moesin is via Clic’s interaction with the PP1 phosphatase PP1-87B, the principal deactivator of moesin in \textit{Drosophila}.\textsuperscript{11} Evidence showing a suppression of the \textit{PP1-87B}^{RNAi} phenotype characterized by aberrant microvilli growth in the \textit{Clic}^{109} loss-of-function genetic background suggests co-dependency of these two proteins for normal moesin regulation. PP1-87B is a known antagonist of moesin and Clic has probable antagonistic function, but these functions are not independent in regard to moesin function. The observed masking of the \textit{PP1-87B}^{RNAi} phenotype by a loss of Clic suggests that Clic functions upstream of PP1-87B to antagonize moesin function.

\textbf{III. Clic may facilitate the transfer of phosphorylated moesin from PIP\textsubscript{2} to membrane bound proteins}

In light of my evidence implicating Clic in rhabdomere morphogenesis by antagonism of moesin via an interaction with PP1-87B, as well as previous studies, I propose the following model. Previous research has shown that ERM proteins may bind to the cytoplasmic domains of membrane proteins following activation initiated by PIP\textsubscript{2} binding. CD44, CD43, and ICAM-2 are some of the membrane
proteins known to exhibit this interaction with ERM proteins.\textsuperscript{9} Other research has implicated CLIC family proteins in membrane trafficking function.\textsuperscript{24} The results of the present study along with these pieces of information imply a role for Clic as a facilitator of the transfer of active, phosphorylated moesin from PIP\textsubscript{2} to a membrane protein.

I propose that PP1-87B may only function to dephosphorylate and thereby deactivate moesin when moesin is bound to a membrane protein rather than PIP\textsubscript{2}. Clic may work upstream of PP1-87B function by facilitating the transfer of phosphorylated moesin from PIP\textsubscript{2} to a membrane protein. Thus, Clic makes PP1-87B’s substrate accessible. Transfer of phosphorylated moesin by Clic frees up PIP\textsubscript{2} binding sites that are required for further moesin activation and contributes to turnover of moesin in the microvilli of the rhabdomeres. In this way, Clic exerts its antagonistic effect on moesin by interfering with moesin turnover and modulates rhabdomere morphogenesis (Fig. 16).
IV. Transfer by Clic of phosphomimetic Moe\textsuperscript{TD} to a membrane bound protein facilitates its degradation

Although evidence for the proposed model above is strong, a concrete identity for the membrane protein that Clic transfers moesin to is unclear. However, the observations of Moe\textsuperscript{TD} phenotypes suggest candidates for membrane protein X that is required for moesin activation (Fig. 16).

The results of the present study showing rescue of the Moe\textsuperscript{TD} phenotype by additional Clic function provide further evidence for Clic’s antagonistic function in regard to moesin but the mechanism for this rescue is unclear (Fig. 12).
According to the proposed interaction model, Clic is thought to regulate moesin function by making PP1-87B’s substrate available. The problem arises in that Moe\textsuperscript{TD} lacks a removable phosphate group and cannot be deactivated by dephosphorylation facilitated by Clic and PP1-87B.

According to unpublished data by Mark Berryman of the Ohio University Department of Biomedical Sciences, mammalian ERM proteins genetically interact with the protein tyrosine phosphatase PTPRQ. Based on these data, the cytoplasmic domain protein sequence of rat PTPRQ was submitted to a BLASTp search via UniProt.org against known \textit{Drosophila melanogaster} proteins.\textsuperscript{43} The following \textit{Drosophila} proteins were found to have the highest homology with rat PTPRQ: PTP10D (e=5\textsuperscript{-83}), PTP4E (e=6\textsuperscript{-82}), Lar (e=4\textsuperscript{-67}), PTP99A (e=7\textsuperscript{-59}), PTP69D (e=5\textsuperscript{-57}). From this result, it can be inferred that moesin may interact with any of these proteins in \textit{Drosophila} and one or more of these proteins may be membrane protein X in the proposed model.

A further search of FlyBase.org showed that some of these proteins have known interactions with E3 ubiquitin ligase proteins.\textsuperscript{44} Interaction of Moe\textsuperscript{TD} with a ubiquitin ligase could cause polyubiquitination of the phosphomimetic moesin and thereby its subsequent degradation independent of its phosphorylation. It is by this mechanism that I propose an up-regulation of Clic is able to rescue the Moe\textsuperscript{TD} phenotype. In my model, Clic facilitates the transfer of Moe\textsuperscript{TD} from PIP\textsubscript{2} to one of the membrane proteins listed above. Once bound to this specific membrane protein, interaction with a ubiquitin ligase and polyubiquitination leads to Moe\textsuperscript{TD}
degradation. In the $Clic^{109}$ mutant background, Moe$^{TD}$ remains bound to PIP$_2$ or to some other membrane protein(s) and is thereby protected from degradation.

**FUTURE DIRECTIONS**

My research focused primarily on Clic’s interaction with moesin and its primary phosphatase PP1-87B. This leaves a broad spectrum of actin regulatory proteins to be tested in *Drosophila* rhabdomere morphogenesis. The phenotype induced by the $Clic^{109}$ loss-of-function mutation that conferred squared rather than rounded rhabdomeres is still not well understood. It is clear from this result that Clic is involved in establishing the rhabdomere architecture but the mechanism responsible for this squared phenotype is unclear. Perhaps Clic interacts with actin bundling proteins such as Fascin coded for by the *singed (sn)* gene to establish the wild-type rounded rhabdomere.

Some other, yet unexplored proteins of interest include Skittles, Pten, and Synaptojanin, three important regulators of PIP$_2$ levels, as PIP$_2$ binding is necessary for moesin activation.$^{45}$ Also, it may be interesting to test other moesin alleles such as Moe$^{TA}$, which cannot be phosphorylated, or Moe$^{TN}$, which is unable to bind to PIP$_2$.\textsuperscript{6} Testing these two moesin alleles may illuminate how the phosphorylation of moesin affects its interaction with other proteins involved in rhabdomere morphogenesis and the importance of PIP$_2$ binding in moesin activation within this system. It may also be fruitful to test another PP1
phosphatase known to regulate moesin, Flapwing, to determine how manipulation of this protein level affects rhabdomere morphogenesis.\textsuperscript{46}

This research has led to the proposal for a novel mechanism for Clic involvement in rhabdomere morphogenesis but in order to confirm if these interactions are valid, further biochemical testing is necessary. The present study exclusively studied genetic interactions to propose a model for Clic’s role in establishing rhabdomere structure. Thus, it would be helpful to determine if and how these proteins are physically interacting via co-immunoprecipitation (CoIP) assays. For example, this type of testing is necessary to determine the identity of membrane protein X in my proposed model. If biochemical evidence can confirm that Clic is interacting with one of the \textit{Drosophila} homologs of PTPRQ and that the homolog is, in fact, interacting with a ubiquitin ligase, the proposed model would be strengthened and Clic’s function in rhabdomere morphogenesis would be more wholly understood.
Bibliography


