ABSTRACT

The Role of Spectrin in *Drosophila* Photoreceptor Development

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Spectrin is a cytoskeletal protein that interacts with the plasma membrane, forming a scaffolding and playing an important role in maintenance of plasma membrane integrity and cytoskeletal structure. In *Drosophila,* spectrin is composed of 3 subunits: α-spectrin, β-spectrin, and β_H-spectrin. The interaction between them is important in generating the different polarized membranes in the cell. This study identified the specific effects of spectrin on *Drosophila* photoreceptor development.

In fruit flies the mosaic technique can be used to express both the mutant and the wild-type tissue in a single eye. The mosaic eyes were dissected, fixed, and mounted onto a slide and analyzed using confocal microscopy. Specific roles of α-spectrin, β-spectrin, and β_H-spectrin in photoreceptor development were identified and analyzed. This analysis can lead to the identification of the functions of spectrin in photoreceptor development, and to the potential function of spectrin in vertebrate eye development and in causing certain retinal diseases.

The Role of Spectrin in *Drosophila* Photoreceptor Development

by

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A Thesis

Approved by the Department of Biology

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Robert D. Doyle, Ph.D., Chairperson

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CHAPTER ONE

Introduction

Background

Developmental studies are essential to understanding how certain genes develop, and what effect this development has on human development. *Drosophila melanogaster*, or the common fruit fly, is a widely studied model organism for developmental studies. It is very valuable as a model organism for several reasons: it has a short generation time, usually about two weeks, high mating productivity, as it can lay up to 800 eggs in its lifetime, it is small and easy to grow in the laboratory, the mature larvae exhibit giant chromosomes called polytene chromosomes, it has only 4 pairs of chromosomes including 3 autosomal pairs and 1 pair of sex chromosomes, the males do not undergo meiotic recombination, which aids in genetic studies, genetic transformation techniques have been available since 1987, and the *Drosophila* genome was sequenced and published in 2000 which aids in genetic studies. Additionally, about 75% of known human diseases have a recognizable match in the genetic code of fruit flies (Reiter et al., 2001) and 50% of fly protein sequences have human homologues.

Spectrin

Spectrin is an essential cytoskeletal protein that is found on the plasma membrane. It forms scaffolding which allows the cell to maintain plasma membrane integrity and cytoskeletal structure (Huh et al., 2001). The changes in cell shapes, and the eventual stabilization of a specific shape, require molecular machinery that can

interact extracellularly with the cytoskeleton. An important structural element that links cell adhesion proteins in the cell membrane to the F-actin cytoskeleton is the sub-membranous Spectrin network.

In *Drosophila*, a single α-spectrin isoform combines with either of two, structurally distinct β-isoforms, β-spectrin and β-Heavy (β_H)-spectrin to produce $(\alpha\text{-}\beta)_2$ and $(\alpha\text{-}\beta_H)_2$ tetramers, respectively. In epithelial cells of *Drosophila* $(\alpha\text{-}\beta)_2$ tetramers are restricted to the basolateral membrane, while the $(\alpha\text{-}\beta H)_2$ tetramers localize to the apical membrane and the adherens junction (Dubreuil et al., 1997; Lee et al., 1997; Thomas and Williams, 1999; Thomas et al., 1998). All three spectrin subunits are essential for normal development. Together, these studies indicate that the spectrin cytoskeleton has an essential role in cell structure and morphogenesis (Thomas, 2001), making the identification of proteins that recruit and/or organize this structure of considerable interest. *Drosophila* is an attractive model system to test whether spectrins are required for cell polarity and morphogenesis. *Drosophila* contain only three members of the highly conserved spectrin family, each encoded by a single gene (Bennett, 1990), and previously identified null mutants exist for each spectrin subunit (Dubreuil and Grushko, 1998; Dubreuil et al., 2000; Thomas et al., 1998).

The development of sensory organs including eyes in animals is tightly linked to a dynamic cell capable of forming and stabilizing manifold types of differentiation. This study was interested in analyzing the functional roles of spectrins on the photoreceptor morphogenesis. Based on our preliminary results, protein null *α-* or *β-spectrin* mutant photoreceptors display severely disrupted morphogenesis with mislocalized cell polarity proteins including the Crumbs which are essential for the photoreceptor morphogenesis,

suggesting that the Spectrins are essential for the apical targeting or localization of Crumbs in photoreceptors.

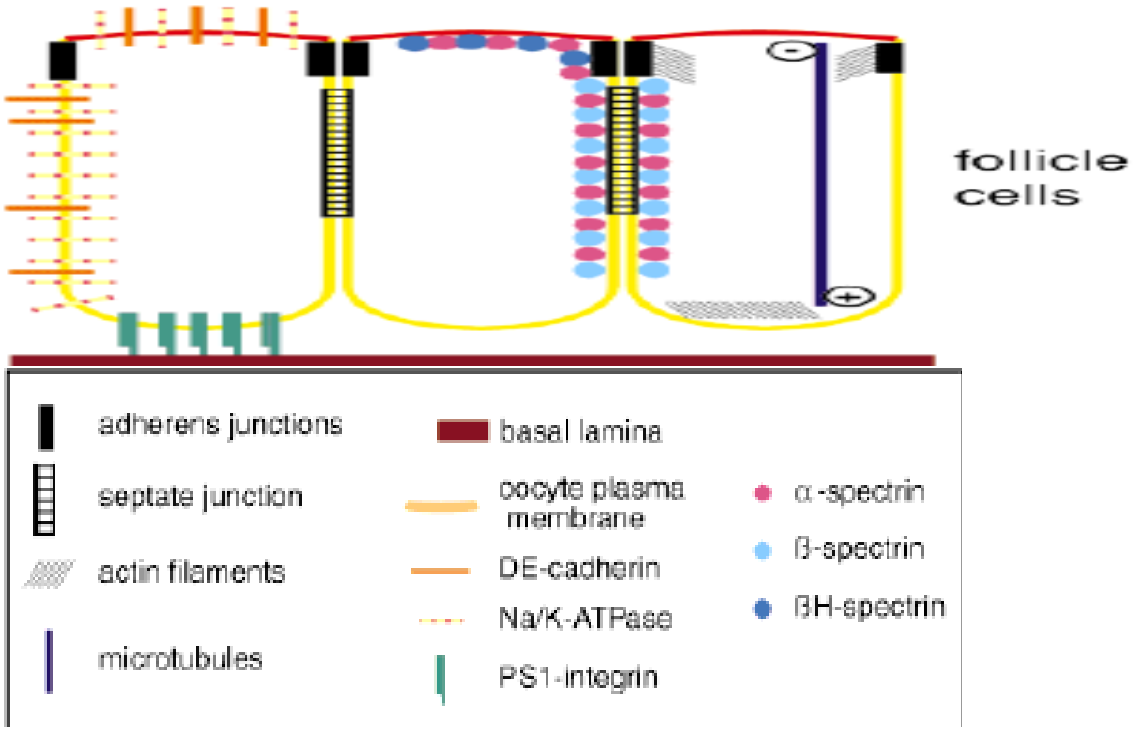

Figure 1. Polarized follicle epithelium. The apical membrane is red and the basolateral membrane is yellow. α-spectrin is found throughout the cell, β-spectrin is found only in the basolateral membrane and β_H-spectrin is only found in the apical membrane (Muller, 2000).

Cell Polarity

Genetic control of apical-basal cell polarity is essential for epithelial morphogenesis and cell division during cell fate specification. Apical-basal cell polarity is also extremely important for the development of specialized subcellular structures like the ones in photoreceptor cells (Fig. 2). Certain evolutionarily conserved proteins play an important part in maintaining polarity in epithelial cells. These cell polarity proteins consist of two heterotrimeric cassettes, Crumbs-Stardust-Dpatj (Crumbs complex), and Par-6-aPKC-Baz (Par-6 complex) that are found in the apical cell membrane. It has been

shown that these two complexes are the major forces behind establishing apical-basal cell polarity (Hurd et al., 2003; Lemmers et al., 2004; Nam and Choi, 2003; Sotillos et al., 2004).

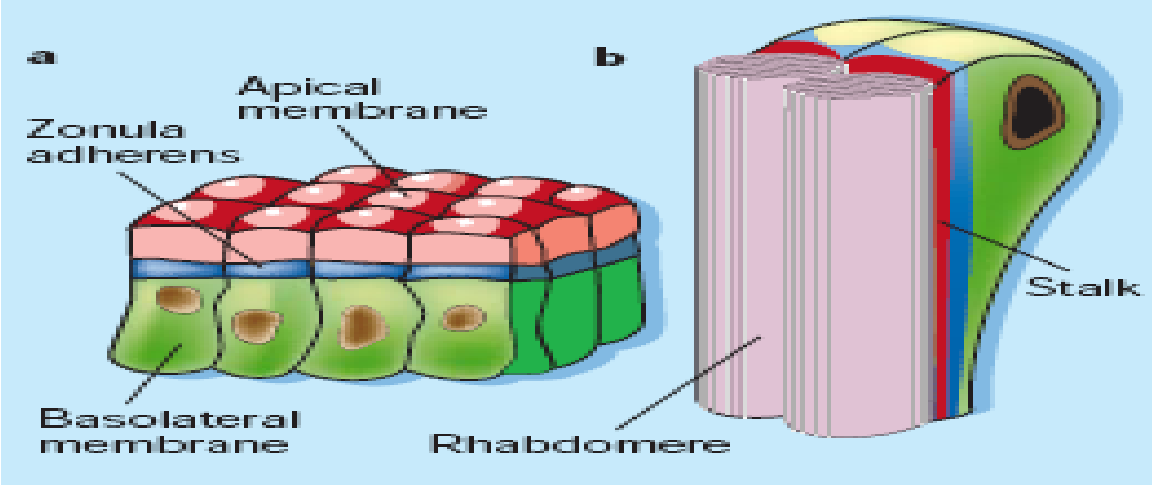

Figure 2. The determinants of cellular polarity). Reprinted by permission from Macmillan Publishers Ltd: [Nature] ((Pichaud and Desplan, 2002), copyright (2002)
a) epithelial cells. The apical membrane is red, the Zonula Adherens or Adherens Junction is blue and the basolateral membrane is green, Crumbs, Stardust, discs lost, and β_H-spectrin are found in the apical region. Armadillo is found in the blue region. β-spectrin and Na+/K+/ATPase are found in the green region. α-spectrin is found throughout the cell. b) photoreceptor cells. the rhabdomere is purple, and the other regions are the same color.

Previous Research on Spectrin

Spectrin forms a heterotetramer with two α-spectrin subunits and two β-spectrin subunits. These tetramers interact with actin, forming a hexagonal mesh (Deng et al., 1995). Spectrin is often associated with actin filaments (Kiehart, 1990) and contains an ankyrin-binding site (Pesacreta et al., 1989) within its 106 amino acid repeating motif (Byers et al., 1989). Spectrin was originally studied in vertebrate erythrocytes (Speicher and Marchesi, 1984). In erythrocytes spectrin mutants often causes hereditary defects such as hereditary elliptocytosis and hereditary spherocytosis (Dubreuil et al., 1987).

However, spectrin, which is ubiquitously expressed in the plasma membrane of most cells, was identified in non-erythroid cells (de Cuevas et al., 1996; Wasenius et al., 1989; Wasenius et al., 1985). However, the study of spectrin in vertebrate nonerythroid tissue is extremely difficult because there are two alpha spectrin isoforms and five beta spectrin isoforms in vertebrates, while in invertebrates there is only one alpha spectrin and two beta spectrin isoforms, β-spectrin and β_H-spectrin. It is still valuable to study the invertebrate spectrins, because of their conserved structure (Dubreuil et al. 1989; Byers at al., 1987). *Drosophila* β_H-spectrin is only localized in the apical domain of the membrane, while β-spectrin is only localized in the basolateral domain of the membrane (Thomas and Kiehart, 1994; Fig. 1). This allows for the study of the specific interactions between spectrin and the plasma membrane.

The *C. elegans* homologue of β-spectrin is encoded in the *unc-70* gene. *Unc-70* mutants develop slowly and adults are paralyzed and dumpy, showing that β-spectrin is important in anchoring proteins at cell membranes (Lee et al. , 1993). Current research on *Drosophila* spectrin focuses on neuronal spectrin. Presynaptic spectrin has been shown to disrupt presynaptic neurotransmitter release at the NMJ (Pielage et al. , 2005) and elimination of essential cell-adhesion molecules leading to disruption of synapse stability at the NMJ (Featherstone et al. , 2001). Postsynaptic spectrin has been shown to disrupt the synaptic connections formed by the NMJ (Pielage et al., 2006). Although *Drosophila* spectrin has an ankyrin-binding site, it has been shown that β-spectrin functions upstream of ankyrin (Das et al. , 2006) in the cytoskeletal assembly pathway, functions independently of ankyrin in maintaining axon connections in the *Drosophila* CNS (Hulsmeier et al. , 2007), and that ankyrin and β-spectrin accumulate independently

of α-spectrin in *Drosophila* (Garbe et al. , 2007). Like previous studies, α-spectrin and β-spectrin were shown to have distinct functions in axonal pathfinding (Dubreuil and Yu, 1994), and β-spectrin appeared to function independently of α-spectrin.

Previous *Drosophila* spectrin research has also focused on cell polarity. Epithelial cells are organized into separate cell layers which are not point-symmetrical, and these separate layers form the apical membrane, the basolateral membrane, and the adherens junction, or zonula adherens, which serves as a junction between the other two cell layers (Fig . 3). Research has shown that α-spectrin is required for ovarian follicle monolayer integrity in *Drosophila* (Lee et al. , 1997) and β_H-spectrin is required for apical-basal polarity in *Drosophila* follicular epithelium (Conder et al. , 2007). But apical spectrin (β_H-spectrin) is essential for morphogenesis but not apicobasal polarity in *Drosophila* epithelial cells (Zarnescu and Thomas, 1999). Like the previous studies on neuronal spectrin, β-spectrin has been shown to function independently of α-spectrin to polarize the Na+/K+/ATPase in epithelial cells (Dubreuil et al. , 2000) and that α-spectrin is not essential for Na/K/ATPase maintenance and distribution in blowflies (Baumann, 1997). Spectrin was also shown to severely affect membrane transports, with phenotypes that are similar to knocking out the transporters themselves (Dubreuil 2006), and are essential to the stabilization of polarized membrane domains (Williams et al. , 2004).

Drosophila Eye Development

The *Drosophila* eye serves as an excellent model to study the function of cell polarity genes in the apical membrane of photoreceptor cells (Fig. 4). In *Drosophila* retinal development, photoreceptor morphogenesis is the final stage of photoreceptor development that occurs during pupal stage.

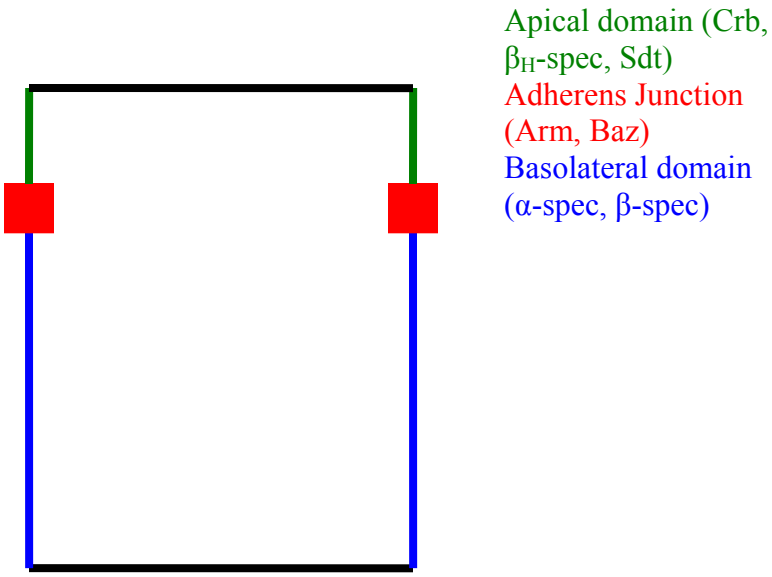

Figure 3. Polarized epithelial cell. The apical membrane is green, the adherens junction is red, and the basolateral membrane is blue. α-spectrin is found throughout the cell, β-spectrin is found only in the basolateral membrane and β_H-spectrin is only found in the apical membrane.

Epithelial cells in the eye imaginal disc differentiate as photoreceptor neurons in the late larval stages. About 800 ommatidial clusters comprising of 8 photoreceptor cells (R1-R8) are generated in the eye disc epithelium during the third instar larval stage, but morphogenesis of photoreceptor cells takes place mainly during the following pupal stage. By 37% pupal development, the apical region of each photoreceptor cell is involuted by 90°, which reorients the apical side toward the center of the cluster (Kumar and Ready, 1995; Longley and Ready, 1995). In photoreceptor cells, Crumbs complex proteins are localized immediately apical to AJs (Fig. 2). At 55% pupal development, when the rhabdomeres begin to develop from the apical surface of photoreceptor cells, Crumbs complex proteins are positioned to the region called the rhabdomere stalk, which links the rhabdomere with the AJ. During this time, developing rhabdomeres undergo

dramatic vertical extension from the distal region of photoreceptor cells to the proximal base of the retina. The process of morphogenesis, occurring at this terminal differentiation stage, is critically dependent upon maintenance of apical-basal polarity in the photoreceptors.

Photoreceptors, which consist of a stalk and a rhabdomere, also have these polarized cell structures with the apical membrane, basolateral membrane, and adherens junction, but they appear to be epithelial cells turned 90° (Pichaud and Desplan, 2002; Fig. 4). *Drosophila* β_H-spectrin has been shown to interact with Crumbs, the *Drosophila* homologue of human CRB1/RP12, which is responsible for retinal dystrophies including retinitis pigmentosa, to form the stalk membrane in the apical layer of the photoreceptor (Pellikka et al., 2002). Spectrin has been shown to be colocalized with and bound to the submicrovillar ER subdomain in honeybee photoreceptors (Baumann 1998), showing that it may be important in maintaining functional ER subregions in the insect photoreceptor. All the previous research points to spectrin as being essential to *Drosophila* photoreceptor development, including specific functions for α-spectrin, β-spectrin, and β_H-spectrin in the developing photoreceptor, specifically as it relates to the establishment and maintenance of apical-basal cell polarity.

Known Interactions of Spectrin and Crumbs

As mentioned earlier, it has been shown that β_H-Spectrin colocalizes at the rhabdomere stalk with Crumbs, and β_H-Spectrin interacts with Crumbs, genetically and biochemically (Medina et al., 2002, Pellikka et al.,2002). The eye phenotype of β_H-Spectrin was enhanced by the presence of one copy of *crumbs* mutant, indicating that the *β_H-spectrin* and *crumbs* genetically interact in rhabdomere formation.

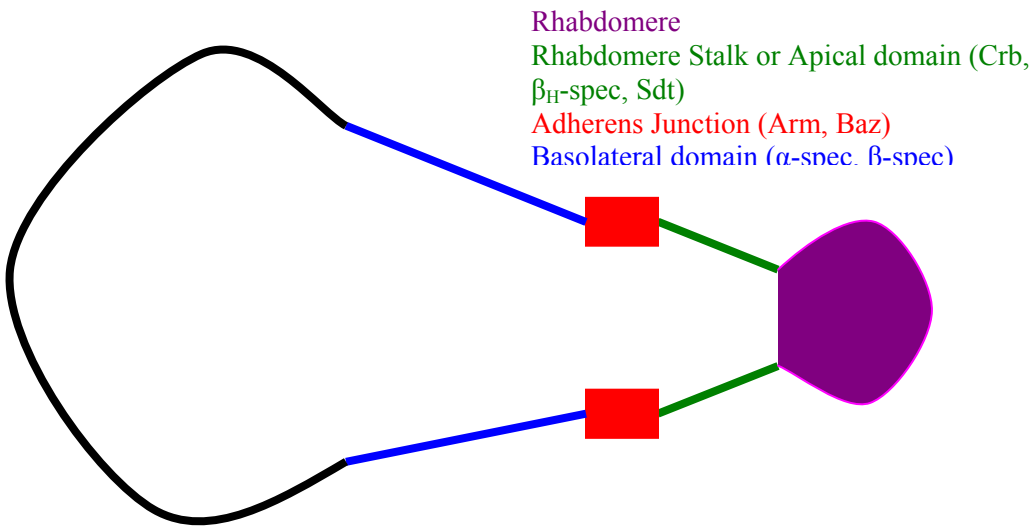

Figure 4. Polarized photoreceptor cell. The rhabdomere is the most apical domain but it does not contain Crumbs or spectrin. α-spectrin is found throughout the cell, β-spectrin is found only in the basolateral membrane, and β_H-spectrin is found only in the apical membrane.

The rhabdomere stalk localization of β_H-Spectrin depends on Crumbs, but the apical membrane targeting of β_H-Spectrin was not noticeably affected.

Therefore, we think that Crumbs, by organizing spectrin at the membrane, and as a part of the cytoskeletal network that controls photoreceptor pattern formation, acts to coordinate photoreceptor polarity and morphogenesis. Furthermore, the fact that human mutations in CRB1 lead to pathologies such as retinitis pigmentosa (RP12) (den Hollander et al., 1999) emphasizes the importance of deciphering the molecular networks associated with Crumbs in *Drosophila*. The human orthologue of β_H, β_V-spectrin, is strongly expressed in photoreceptor cells (Stabach and Morrow, 2000). This raises the possibility that a similar interaction between CRB1 and β_V-spectrin exists in human photoreceptor cells.

Mosaic Genetics

One limitation of using *Drosophila* as a model organism is that very often homozygous mutants for spectrin are embryonically-early instar larval lethal (Lee et al, 1997; Conder et al., 2007; Zarnescu and Thomas, 1999). Because of this lethality, the late-development function of spectrin mutants can not be studied easily. However, this limitation can be overcome because the FLP/FRT system can generate mosaic tissue clones, in which both the wild type and the mutant tissue are expressed within the same eye.

Genetic mosaics can be extraordinarily useful in the study of biological systems, and can be created intentionally in many model organisms in a variety of ways. They often allow for the study of genes that are important for very early events in development, making it otherwise difficult to obtain adult organisms in which later effects would be apparent. Furthermore they can be used to determine the tissue or cell type in which a given gene is required and to determine whether a gene is cell autonomous. That is, whether or not the gene acts solely within the cell of that genotype, or if it affects neighboring cells which do not themselves contain that genotype, but take on that phenotype due to environmental differentiation. Genetic mosaics are a particularly powerful tool when used in the commonly studied fruit fly, where they are created through mitotic recombination. Mosaics were originally created by irradiating flies heterozygous for a particular allele with X-rays, inducing double-strand DNA breaks which, when repaired, could result in a cell homozygous for one of the two alleles. After further rounds of replication, this cell would result in a patch, or "clone" of cells mutant for the allele being studied.

More recently the use of a transgene incorporated into the *Drosophila* genome has made the system far more flexible. The Flip Recombinase (or FLP) is a gene from the commonly studied yeast *Saccharomyces cerevisiae* which recognizes "Flip Recombinase Target" sites, which are short sequences of DNA, and induces recombination between them. FRT sites have been inserted transgenically near the centromere of each chromosome arm of *Drosophila melanogaster* (Xu and Rubin, 1993). The FLP gene can then be induced selectively, commonly using either the heat shock promoter or the GAL4/UAS system. The resulting clones can be identified either negatively or positively.

In negatively marked clones the fly is transheterozygous for a gene encoding a visible marker (commonly the green fluorescent protein, GFP) and an allele of a gene to be studied (both on chromosomes bearing FRT sites). After induction of FLP expression, cells that undergo recombination generate progeny that are homozygous for either the marker or the allele being studied. Therefore the cells that do not carry the marker (which are dark) can be identified as carrying a mutation. It is sometimes inconvenient to use negatively marked clones, especially when generating very small patches of cells, where it is more difficult to see a dark spot on a bright background than a bright spot on a dark background. It is possible to create positively marked clones using the so called MARCM (pronounced mark-em) system, which stands for "Mosaic Analysis with a Repressible Cell Marker" and was developed by Liqun Luo, a professor at Stanford University. In this system the GAL4/UAS system is used to globally express GFP. However the gene GAL80 is used to repress the action of GAL4, preventing the expression of GFP. Instead of using GFP to mark the wild type chromosome as above,

GAL80 serves this purpose, so that when it is removed, GAL4 is allowed to function, and GFP turns on. This results in the cells of interest being marked brightly in a dark background (Lee and Luo, 1999).

Rationale

Spectrins are plasma membrane-associated cytoskeletal proteins implicated in several aspects of cell morphogenesis, development and function, including cell polarity and photoreceptor development. To test the potential functional roles of spectrins for photoreceptor morphogenesis, this study characterized *Drosophila α-spectrin* mutation in the eye. The *Drosophila* genome contains only one *α-spectrin* gene (Dubreuil et al., 1998), making it an ideal system to genetically manipulate spectrin levels. More specifically, this experiment studied the spectrin mutations using *Drosophila* eyes. First, *Drosophila* photoreceptors are accessible *in vivo* by a variety of genetic and cell biological techniques, including mosaic analysis, overexpression studies, immunohistochemistry, and confocal microscopy. Second, the development, morphology and function of the *Drosophila* photoreceptors are well described and relatively invariant from animal to animal. Third, since the eye is not an essential tissue for an animal's viability, this is a great system to analyze both the essential developmental genes. Furthermore, the FLP/FRT system can generate mosaic tissue composed of both wild-type and mutant cells in the same tissue. These features make the photoreceptor an excellent structure for the detection and quantification of any changes resulting from *spectrin* disruption.

Specific Aims

Epithelial cells are polarized cells which are directed by two protein complexes, the Crumbs complex and the Par-6 complex. Cell polarity is essential for epithelial morphogenesis and cell division. Photoreceptor cells are specialized epithelial cells that have light-sensing subcellular structures. Spectrin is a plasma-membrane associated cytoskeletal protein which has been shown to cause developmental defects in epithelial cells. This study examined the relationship between the cytoskeletal protein spectrin and the cell polarity proteins, such as Crumbs, to identify and explain the specific functions of spectrin, with special emphasis on α-spectrin, in the developing *Drosophila melanogaster* photoreceptor. The following specific aims about *Drosophila* photoreceptor development can be addressed:

Specific Aim 1: Find and analyze the specific functions of α-spectrin in the development of the *Drosophila* photoreceptor.

Specific Aim 2: Find and analyze the specific functions of β-spectrin in the development of the *Drosophila* photoreceptor.

Specific Aim 3: Find and analyze the interactions between α-spectrin and β-spectrin in the development of the *Drosophila* photoreceptor.

CHAPTER TWO

Materials and Methods

Fly Stocks and Genetics

All *Drosophila* strains were grown and maintained at room temperature. The wild-type strain is CS. The analysis of the function of the *α-spectrin* gene in eye development has been hindered by the presence of another gene called *roughoid (rhomboid-3)* that causes the "rough eye" phenotype (Wasserman et al., 2000), localized (61F8) near the *α-spectrin* gene (62B4). All previously available α-*spectrin* mutants also had the *roughoid* mutation (not shown). This study cleaned up the two null alleles of α-*spectrin* mutants, α-*spectrin* ^{lm102} and α-*spectrin*^{rg41}(Lee et al., 1993) using the conventional homologous recombination. The cleaned-up *α-spectrin* mutants were completely rescued to adulthood from early larval lethality without the "rough eye" phenotype using the ubiquitin-promoter based expression of α-spectrin cDNA construct (*ubi-spec*) (Lee et al., 1993). This study further confirmed the absence of the roughoid phenotype with cone cell analysis in the α-spectrin mutant cells. No cone cell or pigment cell defects (not shown) which are affected by the presence of *roughoid* mutation (Wasserman et al., 200) were found. Therefore, the newly clean null α-*spectrin* mutant lines are ready for further mutational analysis for photoreceptor eye morphogenesis.

Whole eye mutant flies were obtained with the following crosses: y *w α-spec*^{rg41}*, FRT-80B/*TM6B *Tb* crossed with *eyless-flp*; *ubi-GFP, FRT-80B* to generate *eyless-flp/+; α-spec*^{rg41}, *FRT-80B*/*Ubi-GFP*, *FRT-80B* which are *α-spec* mutants. These *α*-spec mutant

eyes are mosaic clones, which contain both wild type tissue and mutant tissue. The *eyless-flp* is the eye-specific promoter coupled with the Flip Recombinase, the *ubi-GFP* is an ubiquitin promoter coupled to GFP, the Tm6B *Tb* is the balancer chromosome containing *Tb*, which is the marker gene. The FRT 80B is the Flip Recombinase Target site, where the homologous recombination occurs.

To generate *β-spec* mutants, *β-spec^{em12} FRT-19A* were crossed with *eyless-flp, Ubi-GFP/FRT-19A* to generate *β-spec FRT19A/Ubi-GFP FRT19A; eyless-flp/+* progeny. For β_H*-spec* mutants, *FRT 80B karst/TB6B Tb* was crossed with *eyless-flp; FRT 80B, Ubi-GFP* to generate the *eyless-flp/+; FRT80B karst/ FRT80B, Ubi-GFP* progeny. This study also used the *lm102* strain of *α-spectrin* and the *em6* strain of *β-spectrin* in our analysis as well. When using the FLP/FRT genetic system, a balancer chromosome containing a marker gene is necessary to help identify which flies have undergone mitotic recombination.

Drosophila Food Preparation

The food is prepared in a 5 gallon roaster oven. First, add 10 L water. Wait for the water to boil, typically about 1 hour. Then add 62 g of agar. Next, add 600 mL of water and 600 mL of molasses. Then add 280 g of yeast and 580 g of corn meal. Cook for 1 hour. Make sure to stir during the cooking process to ensure homogeneity of the solution. After cooking, add 100 mL of 10% Tegosept solution and 50 mL of propionic acid. These are used to prevent fungus growth and bacterial growth. Then, dispense 8 mL of solution into each vial and 30 mL into each flask and cover with cheese cloth for at least 24 hours. Then, plug each vial with a cotton ball after 24 hours and each flask after 24-48 hours. This makes about 11 L of fly food.

Pupal Eye Dissection and Immunostaining

This study used pupae 48 h after pupation from the vial, using pupae that are slightly darker but still have not fully developed their eyes. These pupae are in the mid-pupal stage (40-50% pupal developmental stage). Place the pupae into a 9 well dissection plate with 1X PBS. There should be two capsular membranes on each pupa, a yellowish-brown outer membrane and a whitish inner membrane. Then, poke a hole in the yellowish-brown outer membrane right in the middle, and remove the top half of the yellowish outer membrane. Then, poke a hole near the top of the white inner membrane and squeeze the inner tissue out from the hole. The eye tissue should look like a dog bone, with the brain connecting the two bulbous imaginal eye discs. If the eyes have already shown pigmentation, then they are too old for analysis. Move the eyes into a clean 9 well dissection plate containing 1X PBS. The eyes then need to be prepared for analysis.

Place all the pupal eyes in the same well. First, remove the PBS and shake with 4% Paraformaldehyde in PBS for 15 minutes. Then shake with Blocking Solution, which consists of 50mM Tris buffer at pH 6.8, 150 mM NaCl, 0.5% NP-40, and 5 mg/ml BSA, for 15 minutes. Next, wash with Wash Buffer, which is 80 mM Tris-Cl buffer at PH 6.8, 150 mM NaCl. 0.5% NP-40, and 1mg/ml BSA. Concurrently, put 1 microliter of each primary antibody into a mini-centrifuge tube. Then add 47 microliters of Wash Buffer for a total volume of 50 microliters. Stain with primary antibodies for at least 4 hours. After staining, shake with Wash Buffer 3 times for 15 minutes. To make secondary antibodies, put 1 microliter of each secondary antibody into a centrifuge tube. Secondary antibodies should correspond to the animal and the color that you want for each of the

primary antibodies. Then, stain with secondary antibodies for at least 4 hours. After staining with secondary antibodies, shake 3 times with wash buffer for 15 minutes. Then shake for 15 minutes with 4% Paraformaldehyde in PBS. Next, wash with PBS. Then, mount on slide with less than a drop of Vectashield mounting solution then add cover slip. Put nail polish on the corners, then the sides of the cover slip to prevent moving the cover slip. Allow the slide to dry, then place in freezer for storage until analysis.

The following primary antibodies were used: Mouse anti-α-Spectrin (3A9, DSHB) (Byers et al., 1987), rabbit anti-β-Spectrin (Dr. Goldstein, UCSD) (Dubreuil and Grushko, 1998; Dubreuil et al., 2000; Lee et al., 1997; Lee et al., 1993), and rabbit anti-β_H-Spectrin (Dr. Thomas, Penn State Univ) (Thomas and Williams, 1999; Thomas et al., 1998). The secondary antibodies used were Cy3-, Cy5-, and FITC-conjugated antibodies that bound to their respective primary antibodies.

Fluorescent and Confocal Microscopy

Fluorescent imaging was carried out on a Zeiss Axioskop 40 fluorescent microscope with a 100x oil immersion objective. Confocal imaging was carried out on an Olympus confocal microscope with an 80X oil objective. All images were processed with PHOTOSHOP (Adobe Systems, Mountain View, CA).

CHAPTER THREE

Results

Drosophila α-spectrin Localization

The localizations of α-, β- and βH-Spectrins have not been fully examined in developing photoreceptors. Thus, prior to mutational analysis of spectrin genes, this study examined the normal pattern of localization of Spectrins in developing larval and pupal photoreceptors of wild-types. This study investigated whether Spectrins are asymmetrically localized with a similar spatial relationship with cell polarity proteins in photoreceptors. Mouse anti-α-Spectrin (3A9, DSHB) (Byers at al., 1987), rabbit anti-β-Spectrin (Dr. Goldstein, UCSD) (Dubreuil and Grushko, 1998; Dubreuil et al., 2000; Lee et al., 1997; Lee et al., 1993), and rabbit anti-βH-Spectrin (Dr. Thomas, Penn State Univ) (Thomas and Williams, 1999; Thomas et al., 1998) antibodies were used to examine their localizations in the developing eyes. The specific subcellular-level localizations of Spectrins were determined by double- or triple-staining with various markers for cell polarity proteins and cell junction proteins (Table 1).

This method has been previously used to determine the specific localizations of unknown proteins in the developing larval or pupal eyes (Nam and Choi, 2003; Nam and Choi, 2006; Nam et al., 2007). The information of the precise localization of Spectrins in the developing photoreceptors is essential to analyze the functional phenotypes derived from mutational analysis.

Table 1. Several markers that distinguish each cell membrane domain and cell junctions

Domains	Markers
apical domain	Crumbs, Stardust, Dpatj, Par-6, aPKC
adherens junction	Arm, E-Cadherin, Bazooka
basolateral domain	Coracle, Dlg, Na-K ATPase, Scribble
rhabdomere	Phalloidin
rhabdomere base	Moesin

Larval Eye Localization of α-spectrin

This experiment examined the localization of α-spectrin at the larval and pupal stages of developing eyes, using a monoclonal antibody of *Drosophila* α-Spectrin, 3A9 (Byers et al., 1987), which is available from Developmental Studies Hybridoma Bank (DSHB).

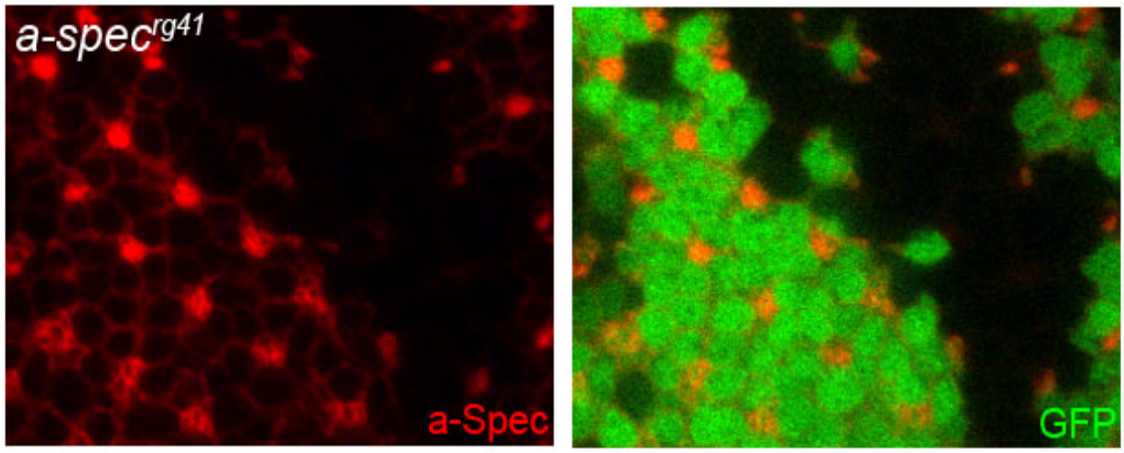

Figure 5. The α-Spectrin proteins are completely absent in α-spectrin mutant clones (absence of green, GFP) in an eye disc of a third-instar larva.

Third-instar larval eye discs of *α-spectrin* mutant clones are completely lacking α-spectrin protein, as shown by the absence of the green GFP, which is linked to the α-spectrin protein. This allows us to verify the specificity of the antibody staining using a protein-null *α-spectrin* mutant (Fig. 5). The α-spectrin is highly concentrated at the cell membranes of the photoreceptor precursor cells during third-instar larval eye discs (Fig. 5). This pattern of high concentration at the cell membrane domains is also shown later in the pupal stages of photoreceptor development. This supports our hypothesis that α-spectrin is localized throughout the cells, but is concentrated at the cell membrane domains. This supports the previous research that showed that α-spectrin was localized throughout the cell.

Wild Type α-spectrin Localization

Later in development, α-spectrin is highly enriched at the basolateral membrane domains and at low concentrations along the apical junctions in pupal eye photoreceptors (Fig. 6). This also supports our hypothesis that α-spectrin is localized throughout the photoreceptor cells during development, but is highly concentrated in the apical and basolateral cell membrane domains. These results imply that α-spectrin is important in photoreceptor development, because it is localized throughout the developing cell. However, it shows very strong enrichment at the basolateral domains, which implies a strong interaction with β-spectrin, which localizes only to the basolateral domain of the developing photoreceptor. The enrichment at the apical domain implies an interaction with B_H-spectrin, which only localizes to the apical membrane.

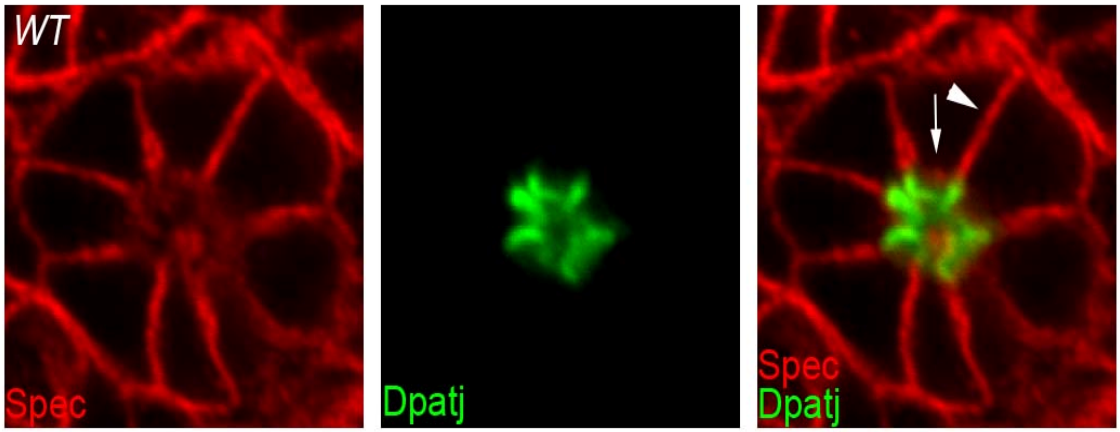

Figure 6. Localization of α-Spectrin in a wild-type pupal eye at 45% pupal developmental stage. The α-Spec (red) localize at all membrane domains with high enrichment at the basolateral domains (arrow-head), as well as at the center (apical domain) of the photoreceptors (Dpatj, green, apical marker).

α-spectrin Mutant Clones Affect Photoreceptor Morphogenesis and Development

Drosophila protein null mutants (*rg41* and *lm102*) for *α-spectrin* are late embryonic or early first-instar larval lethal, with defects in the structure and function of epithelial cells (Dubreuil and Grushko, 1998; Dubreuil et al., 2000; Lee et al., 1997; Lee et al., 1993). To study its effects on late-stage eye development, this study generated mosaic eyes using the FLP/FRT technique (Xu and Rubin, 1993). α-*spectrin* mutant photoreceptors display severely disrupted morphogenesis with mislocalized cell polarity proteins including the Crumbs (Fig. 7) which are essential for photoreceptor morphogenesis, suggesting that the *α-spectrin* is essential for the apical targeting or localization of Crumbs in photoreceptor morphogenesis. Adult eyes of α-spectrin show clear dramatic morphological defects of rhabdomere formation and/or maintenance, as well as retinal floor defects (Fig. 8).

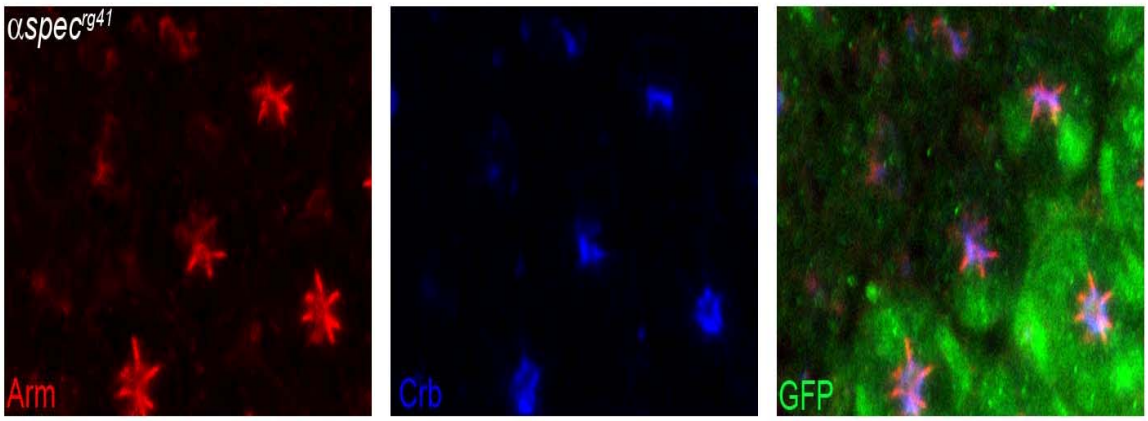

Figure 7. Arm (AJ marker, red) and Crb (apical marker, blue) were absent or mislocalized in the absence of α-Spectrin (marked by the absence of GFP, green) in developing pupal eyes (40% pd). The null mutant, *rg41*, of *α-spectrin* was utilized to generate mutant clones.

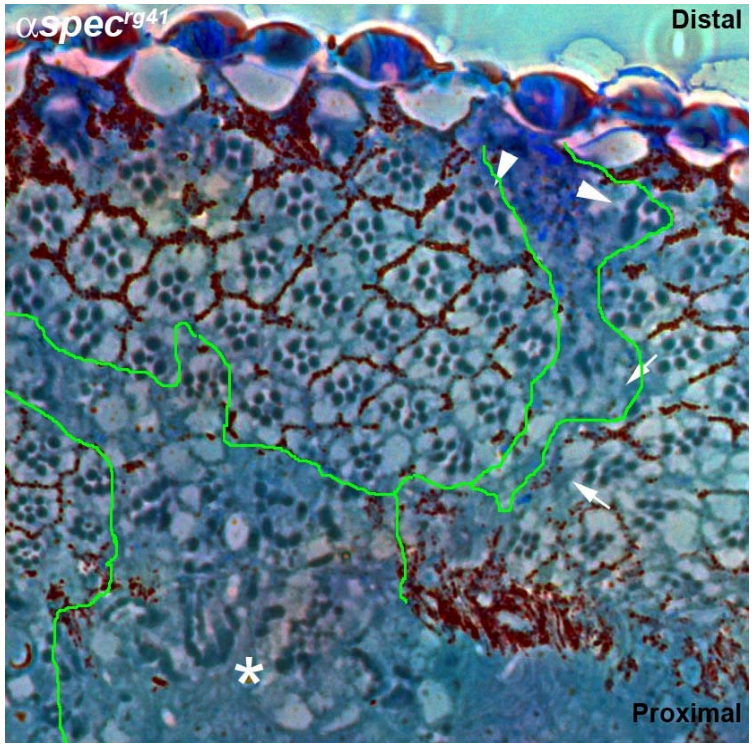

Figure 8. Distal part of the eyes shows the expanded or double-rhabdomeres (arrow-heads) and proximal parts show the loss or absence of the rhabdomeres in adult eye of *α-spectrin* mutants (marked by the absence of red-pigments, green-lines). These phenotypes are similar to those of cell polarity mutants (*crumbs, stardust* or *dpatj)*. Retinal floors were defective in *α-spectrin* mutants (asterisk).

Larval Eye Development of α-spectrin Mutant Clones

Surprisingly, this experiment was unable to detect any abnormalities in the early developing larval eye in α-*spectrin* mutants (Fig. 9), indicating that *α-spectrin* is not essential for the early eye patterning or cell viability. Several other markers including the neuron and glial markers (Elav and Cut) were normal in the α-spectrin mutants of the third-instar larval eye discs (not shown). The photoreceptor defects were only found at the later stage, later than 35% pupal eye development (Fig. 7), suggesting that the *α-spectrin* is only essential for the late photoreceptor morphogenesis like *crumbs*, *stardust*, or *dpatj* (Izaddoost et al., 2002; Nam and Choi, 2003; Nam and Choi, 2006).

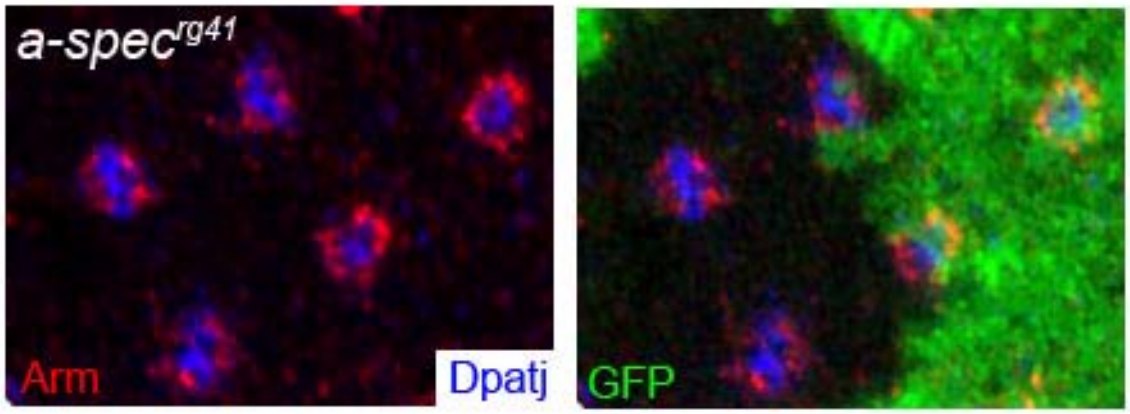

Figure 9. The Arm (AJ, red) and Dpatj (apical, blue) are normal without the *α*-spectrin (absence of green, GFP) in a third-instar larval eye disc.

Pupal Eye Development of β-spectrin Mutant Clones

In order to analyze the β-spectrin mutant's effects on pupal eye development, a strong *β-spectrin [em12]* mutant (Dubreuil et al., 2000) was utilized. The β-spectrin mutation causes almost complete loss of Crumbs and Arm (Fig. 10), which is similar to α-spectrin mutants (Fig. 7). These results show that β-spectrin likely interacts with α-

spectrin to aid in the targeting of cell polarity proteins like Crumbs, an apical membrane protein marker, and Arm, an adherens junction protein marker.

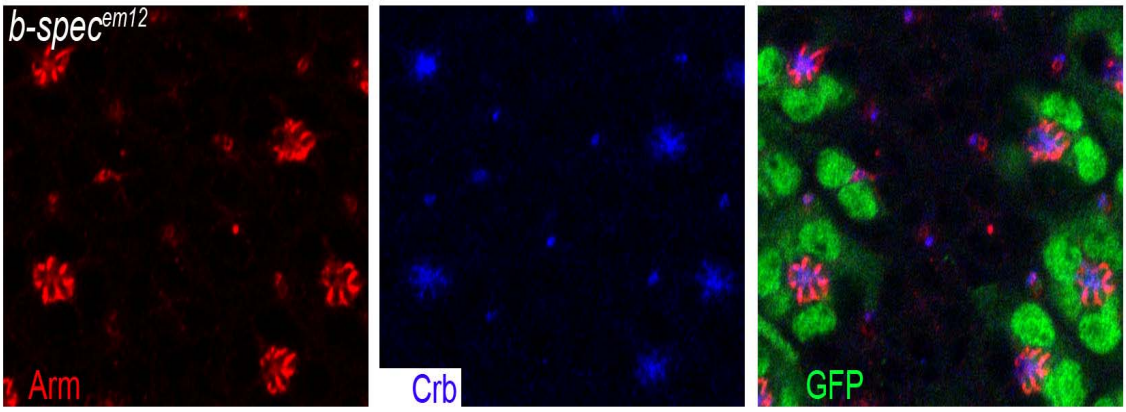

Figure 10. Loss of β-spectrin (marked by the absence of GFP, green) shows the absence or strong mislocalization of Arm (AJ marker, red) and Crb (apical marker, blue) in developing pupal eyes (40% pd). The em12 allele of β-*spectrin* was used as a null mutation.

Relationship Between α-spectrin and β-spectrin

In order to investigate the relationship between *α-spectrin* and *β-spectrin*, this study examined the localization of β-Spectrin in *α-spectrin* mutants (Fig. 11). Without *α-spectrin*, the localization and targeting of β-Spectrin was not changed (Fig. 11), indicating that either *β-spectrin* acts upstream to α-spectrin or it acts independently of α-spectrin for membrane targeting. These results support previous research that β-spectrin, while important when interacting with α-spectrin, also likely has some specific functions that may be independent of α-spectrin. The rabbit anti-β-spectrin antibody was obtained from Dr. Goldstein of UCSD (Dubreuil and Grushko, 1998; Dubreuil et al., 2000; Lee et al., 1997; Lee et al., 1993).

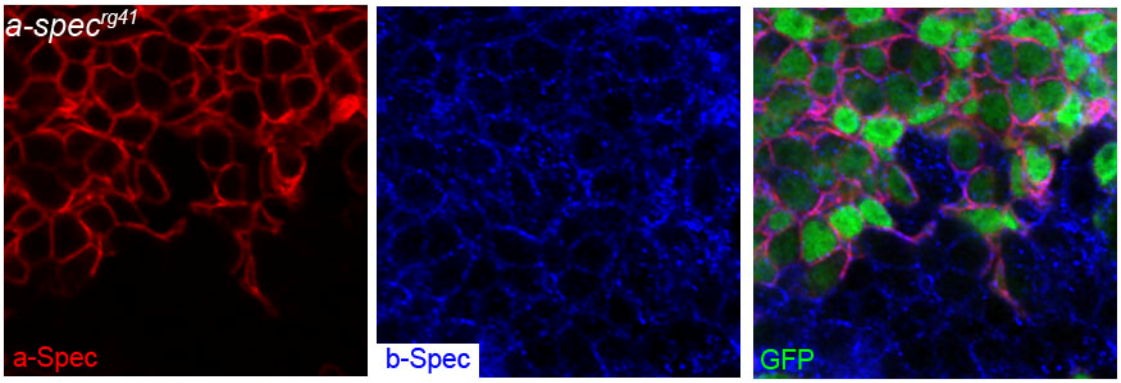

Figure 11. Loss of α-Spectrin (marked by the absence of GFP, green) does not affect the membrane targeting of β-Spectrin (blue).

Based on these results, the α- and β-Spectrins are essential components for Crumbs localization at the photoreceptor cells. Further work could further help to understand the roles of *spectrins* in photoreceptor morphogenesis and their potential interactions with *crumbs*, a gene associated with human retinal degeneration.

CHAPTER FOUR

Discussion and Conclusions

Spectrins are plasma membrane-associated cytoskeletal proteins implicated in several aspects of cell morphogenesis, development and function, including cell polarity and photoreceptor development. Spectrin forms a tetramer which interacts with actin and ankyrin to maintain cell structure and function. Spectrin is conserved throughout the animal kingdom, and *Drosophila* serves as a good model system because it only has one alpha subunit and two beta subunits which do not colocalize. Since spectrin is an essential cytoskeletal protein, it has also been implicated in maintaining cell polarity. To test the potential functional roles of spectrins for photoreceptor morphogenesis, this study characterized *Drosophila α-spectrin* mutation in the eye. The *Drosophila* genome contains only one *α-spectrin* gene (Dubreuil et al., 1998), making it an ideal system to genetically manipulate spectrin levels. More specifically, this study examined the spectrin mutations using *Drosophila* eyes. It is important to study the effects of spectrin in the developing photoreceptor because spectrin causes cell polarity defects. Cell polarity defects in human photoreceptors cause retinal eye diseases such as retinitis pigmentosa. By studying *Drosophila* spectrin, this study may be able to discover similar functions in humans.

The *Drosophila* eye is an ideal model for studying cell polarity in *Drosophila*. In *Drosophila* retinal development, photoreceptor morphogenesis is the final stage of photoreceptor development that occurs during pupal stage. The process of

morphogenesis, occurring at this terminal differentiation stage, is critically dependent upon maintenance of apical-basal polarity in the photoreceptors.

This study wanted to test whether or not the development of the larval eye discs would be affected by the lack of spectrin in the *α-spectrin* mutant clones, as spectrin was believed to be essential for structural integrity. Previous studies seemed to support that idea, as human erythrocytes with spectrin deficiencies had structural defects that led to diseases like hereditary elliptocytosis and hereditary spherocytosis. However, after experimentation this study discovered that the larval development appeared normal in the *α-spectrin* mutant clones, which supports the idea that spectrin is not essential for early eye patterning or cell viability. This was an unexpected result, as it was thought that there could be structural defects in early eye development. On the other hand, this supports our belief that spectrin has a specific role in photoreceptor development and does not cause general cell viability defects.

The α-spectrin mutant photoreceptors displayed severely disrupted morphogenesis with mislocalized cell polarity proteins including Crumbs which is essential for photoreceptor morphogenesis, suggesting that *α-spectrin* is essential for the apical targeting or localization of Crumbs in photoreceptor morphogenesis. Also, adult eyes of α-spectrin mutants showed clear dramatic morphological defects of rhabdomere formation and/or maintenance, as well as retinal floor defects. These results suggest that α-spectrin is essential in the targeting, formation, and maintenance of proper cell polarity in photoreceptors.

β-spectrin mutants also severely disrupted photoreceptor morphogenesis, and showed nearly complete loss of specific cell polarity proteins like Crumbs and Armadillo,

which is a similar phenotype to the α-spectrin mutants. Those results showed that β-spectrin likely interacts with α-spectrin to target cell polarity proteins like Crumbs and Armadillo. This study then investigated the relationship between *α-spectrin* and *β-spectrin*, by examining the localization of β-spectrin in *α-spectrin* mutants. The localization and targeting of β-Spectrin was not affected by the loss of α-spectrin. This implies that either β-spectrin acts upstream of α-spectrin in membrane targeting or they act independently of each other in membrane targeting.

β_H-spectrin has been shown to interact with Crumbs genetically (Medina et al., 2002; Pellikka et al., 2004), and the presence of a *crumbs* mutant augments the phenotype of β_H-spectrin. These results show that *crumbs* and *β_H-spectrin* interact genetically in rhabdomere formation. This study has discovered that Crumbs is necessary for β_H-spectrin to localize to the rhabdomere stalk, but it is not necessary for apical membrane targeting of β_H-spectrin. The human orthologue of β_H, β_V-spectrin, is strongly expressed in photoreceptor cells (Stabach and Morrow, 2000). This raises the possibility that a similar interaction between CRB1 and β_V-spectrin exists in human photoreceptor cells. Based on these results, this study implies that Crumbs, as a part of the cytoskeletal network that controls photoreceptor pattern formation, acts to coordinate photoreceptor polarity and morphogenesis by organizing spectrin at the membrane. Also, because human mutations in CRB1 lead to pathologies such as retinitis pigmentosa (RP12) (den Hollander et al., 1999), it is important to uncover and interpret the molecular networks associated with Crumbs in *Drosophila*. It appears that spectrin is an essential part of one of the molecular networks associated with Crumbs in *Drosophila*, and that spectrin plays

an essential role in development, targeting, and maintenance of cell polarity in the *Drosophila* photoreceptor.

In conclusion, spectrin appears to function in late photoreceptor morphogenesis, playing an essential role in the localization of cell polarity proteins. Spectrin appears essential in apical targeting of Crumbs during photoreceptor morphogenesis, but it is not essential in early eye patterning or cell viability. Each individual subunit of spectrin appears to have a specific function, with α-spectrin functioning in apical membrane targeting, β-spectrin which appears to function independently of α-spectrin in cell membrane targeting, and β_H-spectrin which appears to function in rhabdomere formation and localization of Crumbs to the rhabdomere stalk.

Future Research

There are several future directions to take after this project. One would be to research the specific functions of the other cytoskeletal proteins in the *Drosophila* photoreceptors, such as ankyrin, actin, dystrophin, and pleckstrin. Many of these other proteins interact with spectrin, so after studying spectrin it is possible to study these other proteins to see how they help form the cytoskeleton and how they interact to affect the development of cell polarity. Previous research has already shown that spectrin interacts with both ankyrin and actin, and this study showed that spectrin interacts with cell polarity proteins in cell membrane targeting and cell polarity establishment and maintenance, and by studying the other interacting proteins with spectrin, investigators can possibly gain some insight on what indirect effects these cytoskeletal proteins can have on the establishment of cell polarity through interaction with spectrin.

Another future direction would be to then apply the study of spectrin to human spectrin. Since *Drosophila* spectrin is conserved in mammals, once this study identified the specific functions of *Drosophila* spectrin others can hopefully identify spectrin defects in humans and possibly find what eye diseases are caused by defects in spectrin cytoskeletal formation. The cell polarity proteins that interact with spectrin on the plasma membrane have been identified as the cause of retinal diseases like retinitis pigmentosa. This study shows that spectrin plays a major role in cell polarity, so investigators are now one step closer to finding a solution for retinal diseases caused by mislocalized or defective cell polarity genes.

The end goal of studying spectrin in *Drosophila* photoreceptors is to find the functional roles of the spectrins in photoreceptor morphogenesis and development. Since it has a functional similarity to mammalian eyes, future researchers could possibly identify the same functions in human retinal development and can eventually help prevent and possibly find a solution to certain human retinal eye diseases.

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