Abstract

Spectrins, Spastin, and *Centrosomin* as Partners of Cell Polarity Genes in *Drosophila* Photoreceptor Morphogenesis

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Cell polarity is a common feature of most eukaryotic cells. Recent studies revealed that two heterotrimeric protein complexes, Crumbs (Crb)- Stardust (Sdt)-Patj (Crb complex) and Par-6-aPKC-Bazooka (Baz, Par-3) (Par complex), play essential roles in the establishment and maintenance of apical-basal cell polarity. Using *Drosophila* photoreceptor as a model system, this dissertation identified three potential partners of these cell polarity proteins, which are Spectrins, Spastin and Centrosomin, and examined their roles in the photoreceptor morphogenesis.

Spectrins are major proteins in the cytoskeletal network. Although Spectrins are dispensable for retinal differentiation in eye imaginal discs during larval stage, photoreceptors deficient in α- and β-Spectrins display dramatic apical membrane expansions during pupal eye development. β_{Heavy}-Spectrin (Karst) localizes apically, while β-Spectrin is preferentially distributed in the basolateral region. Overexpression of β-Spectrin causes a strong shrinkage of apical membrane domains, and loss of β-Spectrin causes an expansion of apical domains, implying an

antagonistic relationship between β-Spectrin and Karst. These results indicate that Spectrins are required for controlling photoreceptor morphogenesis through the modulations of cell membrane domains.

By using of anti-acetylated-tubulin antibody, I found that there are stable microtubules in the developing photoreceptors of *Drosophila*. Spastin is a microtubule-severing ATPase involved in constructing neuronal and non-centrosomal microtubule arrays. The *spastin* mutation causes mild defects at the distal section, but the apical domain was dramatically reduced at the proximal section of the developing pupal eye. Spastin overexpression caused the expansion of the apical membrane domain from apical to basolateral in the developing photoreceptor. These results strongly suggest that *spastin* is essential for apical domain biogenesis during rhabdomere elongation in *Drosophila* photoreceptor morphogenesis.

Centrosomin (Cnn) is a core protein for centrosome. I found that Cnn is dispensable for retinal differentiation in eye imaginal discs during the larval stage. However, photoreceptors deficient in Cnn display dramatic morphogenesis defects including the mislocalization of Crb and Baz during pupal eye development. Cnn overexpression caused the expansion of the apical Crb membrane domain, Baz and adherens junctions. These results strongly suggest that the interaction of Baz and Cnn is essential for apical domain and adherens junctions modulation during photoreceptor morphogenesis.

Spectrins, *Spastin*, and *Centrosomin* as Partners of Cell Polarity Genes in *Drosophila* Photoreceptor Morphogenesis

by

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To all my family members

for their constant support and unconditional love.

I love you all dearly.

CHAPTER ONE

Introduction

Cell Polarity in Eukaryotic Cells

Cell polarity is a common feature of most eukaryotic cells, which relies on the spatial differentiation and organization of diverse cellular components, such as proteins, cell cytoskeleton and organelles (Mellman and Nelson, 2008). From simple unicellular organism to complicated multicellular organism, all the cells exhibit sort of cell polarity. For example, the budding yeast has budding site, the epithelial cell has apical and basolateral domain, the neuron has axon and dendrite, and the embryo cell has anterior and posterior areas. In unicellular organisms, cell polarity builds a multifunctional cell which can properly react to external environment signaling and reproduce itself. For the higher multicellular organism, polarized cells functionally assemble together to form a variety of organs and tissues. The establishment and maintenance of cell polarity is important for many processes, such as signaling transduction, cell to cell communication, cell differentiation, proliferation, and cell migration. Failing to establish correct cell polarity causes diverse diseases, including many nervous and retinal diseases.

The best studied polarized cells are epithelial cells. Evolutionally, epithelial polarity is the origin of almost all the types of cell polarity and ~60% mammalian cells are derived from epithelia (Albert, 2008). Each epithelial cell has distinct plasma membrane domains, apical membrane, basolateral membrane, and AJs which

can help it to connect to other epithelial cells. Each membrane domain has different protein components, providing them distinct functions and properties.

Polarized cell domain differentiation requires spatial cues to direct intracellular protein trafficking and distribution. Three coordinated mechanisms are proposed as followings. Firstly, recognization of intrinsic protein-sorting codes regulate protein trafficking and distribution. Secondly, intracellular signalling and cell skeleton define the plasma membrane domains to which proteins are delivered. Third, cell–cell and cell–substrate adhesion that are involved in neighbouring cells and the extracellular matrix (ECM) provide cues that orientate cells in three-dimensional space. Integration and coordination of three mechanisms control and maintain normal cell domains (Mellman and Nelson, 2008).

Drosophila*, an Ideal System*

Drosophila melanogaster, or the common fruit fly, is a widely studied model organism for developmental studies. Compared to other animal model organism like zebra fish and mouse, *Drosophila* has relatively small genome, which consists of 4 pairs of chromosomes. Three pairs of them are autosomal chromosomes and one pair is sex chromosomes. The *Drosophila* genome is fully sequenced, providing us the ability to analyze and manage most of the genes. Additionally, about 75% of known human disease genes have a recognizable match in the genome of *Drosophila* and 50% of fly protein sequences have mammalian homologies, thus the research on *Drosophila* will definitely accelerate the understanding of the pathogenesis of human genetic diseases (Reiter et al, 2001).

Drosophila photoreceptors, the light signaling transduction cells in the eyes, emerge from epithelial cells of eye discs during third instar larvel stage and mature at pupal stage. The reasons why I choose *Drosophila* photoreceptor as the material to study epithelial polarity are as followings.

First, the adult compound eye of *Drosophila* consists of an array of approximately eight hundred hexagonal ommatidia. Each ommatidium contains eight photoreceptors and support cells, pigment cells and a cornea. So totally there are over sixty hundred photoreceptors in one eye, providing a large amount of experimental materials.

Secondly, *Drosophila* has relatively short life span and strong reproduction ability. Each female fly can lay approximately five hundred eggs at one time and from eggs to adult flies it only takes eleven days at room temperature (20-22℃). These physiological features provide an easy way to get designed genotype and abundant materials in a short time.

Lastly and importantly, *Drosophila* photoreceptor and mammalian photoreceptor have highly conserved cell structures and domains (Figure 1). The rhabdomere of *Drosophila* photoreceptor, which is equal to outer segment of mammalian photoreceptor, is made up of stacks of cell membranes that occupy approximately 90% of the whole cell membrane (Izaddoost et al., 2002; Pellikka et al., 2002). The biological pigment molecules are on the membranes that absorb light and then transfer it to chemical signal. The rhabdomere stalk, similar to inner segment of mammalian photoreceptor, is very rich in mitochondria that provide energy for signaling

transduction. Cell domains followed by rhabdomere stalk are the AJs, connecting the cytoskeleton of neighboring cells. The structural and functional resemblance between *Drosophila* photoreceptor and mammalian photoreceptor makes the studies on *Drosophila* photoreceptor can facilitate the human eye research and help to find therapies for human retinal diseases (Izaddoost et al., 2002; Nam and Choi, 2003; Nam and Choi, 2006; Nam et al., 2007; Pellikka et al., 2002).

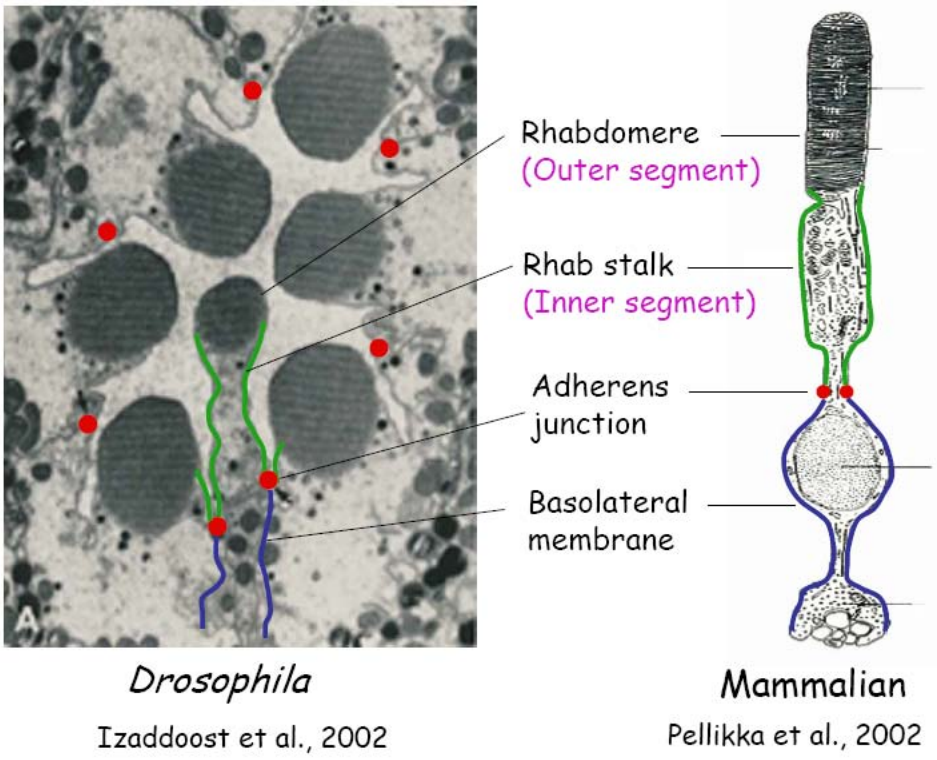

Figure 1: The photoreceptors structure of *Drosophila* and Mammalian. It shows the highly conserved apical and sub-apical domains in photoreceptors Ltd: [NATURE] (Izaddoost et al., 2002; Pellikka et al., 2002), copyright (2002).

Protein Complexes involved in Drosophila *Photoreceptor Morphogenesis*

Drosophila photoreceptors evolve from epithelial cells. During the third instar larval stage, the clusters of 8 photoreceptor cells are generated in the eye disc

epithelium, but morphogenesis of photoreceptor cells takes place mainly during the following pupal stage (Nam and Choi, 2003; Nam and Choi, 2006; Nam et al., 2007). By 37% pupal development (pd), the apical region of each photoreceptor cell is involuted 90°, which reorients the apical side toward the center of the cluster (Kumar, 1995; Longley, 1995). At 55% pd, rhabdomeres begin to develop from the apical surface of photoreceptor cells (Longley, 1995). During this time, developing rhabdomeres undergo dramatic vertical extension from the distal region of photoreceptor cells to the proximal base of the retina (Figure 2).

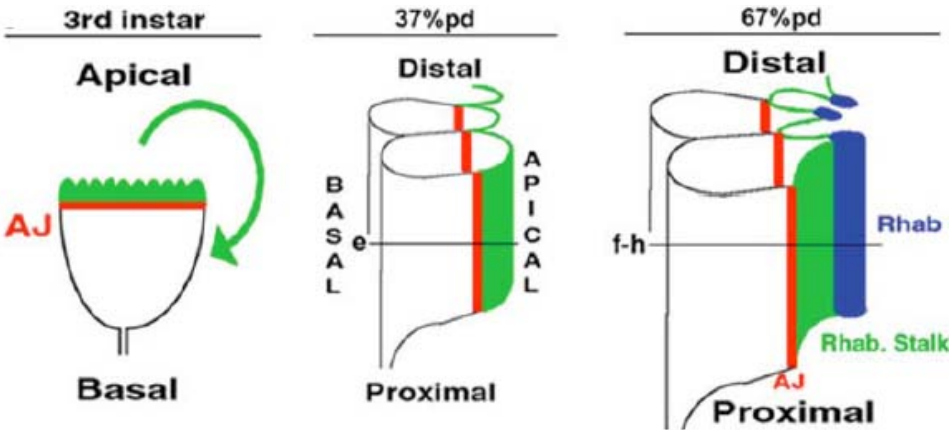

Figure 2: Photoreceptors morphogenesis in *Drosophila*. During putal development, photoreceptor cells undergo the distal to proximal elongation and the apical membrane domain localizes at the center of the photoreceptor clusters surrounded by the AJ and basolateral domains. Ltd: [NATURE] (Izaddoost et al., 2002), copyright (2002).

Although the underlying mechanisms for photoreceptors morphogenesis remains unclear, previous studies on *Drosophila* revealed that a small number of evolutionarily conserved proteins play important roles in epithelial cell polarization. These polarity proteins form two major heterotrimeric cassettes consisting of Crb-

Sdt-Patj (Crb complex) and Par-6-aPKC- Baz(Par-3) (Par complex) in the apical cell membrane, which function in generation and maintenance of photoreceptor polarity.

Crb, together with Sdt and Patj (Bachmann et al., 2001; Hong et al., 2001; Nam and Choi, 2003; Nam and Choi, 2006; Richard et al., 2006) is required for extension of rhabdomeres and formation of AJ along the distal-proximal axis of the photoreceptor cell, although it is not essential for establishing apical basal cell polarity (Izaddoost et al., 2002; Pellikka et al., 2002). Mutations in mammalian homolog of Crb, CRB1, cause retinal diseases including retinitis pigmentosa 12 and Leber Congenital Amaurosis (LCA) in humans (den Hollander et al., 2001a; den Hollander et al., 1999).

In addition to Crb, Par-6 complex is also required for proper organization and maintenance of apical photoreceptor membranes in the eye (Hong et al., 2003; Izaddoost et al., 2002; Nam and Choi, 2003; Nam and Choi, 2006; Nam et al., 2007; Pellikka et al., 2002). Genetic evidence suggests that Par-6 complex is required for localization of the Crb complex to the apical membrane whereas the Crb complex may be necessary for maintenance of Par-6 complex proteins in the apical region (Hong et al., 2003; Nam and Choi, 200).

Interestingly, although Par-6 and aPKC, like the Crb complex, are localized to the apical membrane of photoreceptors, Baz is targeted to the AJ domain (Choi et al., 2007; Nam and Choi, 2003; Nam and Choi, 2006; Nam et al., 2007). The differential localization of Baz and Par-6/aPKC raises the question of how Baz localization is regulated and whether Baz functions independently from Par-6 and

aPKC. Recently, it has been found that Baz is a nodal component for recruiting and apical targeting of Par-6 and aPKC in *Drosophila* photoreceptors (Nam and Choi, 2006), despite of their final localization is different. However, it is unknown how the initial polarity by the Baz is established, and how the polarity proteins can be targeted to the apical location.

Previous Studies on Spectrins

Spectrin, best known for its role in maintaining red blood cells shape, is a cytoskeletal protein that lines the intracellular side of the plasma membrane of many cell types. There are two types of spectrins, α-Spectrin and β-Spectrin. Commonly two α-Spectrins and two β-Spectrins form tetramers that connect actins by the help of other proteins like Protein 4.1(Figure 3A). The spectrins-actins network is important for the maintenance of plasma membrane integrity and cytoskeletal structure (Bennett and Baines, 2001). Although in most cases α-Spectrin and β-Spectrin interact with each other to play roles in the cell, they may also have other independent functions. For example, functional independence of β-Spectrin from α-spectrin was found in the axonal patterning of the embryonic nervous system (Garbe et al., 2007; Hulsmeier et al., 2007) and Na1K1-ATPase localization in mid-gut (Dubreuil et al., 2000).

In invertebrates, there are one *α-Spectrin* gene and two *β-Spectrin* genes, which encode for α-Spectrin, β-Spectrin, and β_{Heavy}-Spectrin (Karst). In *Drosophila* epithelial cells, $(\alpha–\beta)_2$ tetramers are restricted to the basolateral membrane, whereas $(\alpha–\beta_H)_2$ tetramers localize to the apical membrane and the AJ (Figure 3B) (Dubreuil et al., 1997; Lee et al., 1997; Thomas and Williams, 1999; Thomas et al., 1998Longley,

1995). Genetic studies with mutants indicate that all three spectrin subunits are essential for animal viability, but their functions in developing organisms vary depending on the cell or tissue type (Thomas, 2001).

Recently, it has been shown that Karst colocalizes with Crb at the rhabdomere stalk, the region between the rhabdomere and AJ of a photoreceptor cell, and interacts with Crb (Medina et al., 2002; Pellikka et al., 2002). The rhabdomere stalk length is reduced in photoreceptors of karst mutant adult eyes. This phenotype was enhanced by the presence of one copy of crb mutation, indicating that karst and crb genetically cooperate for rhabdomere stalk maintenance. The rhabdomere stalk localization of Karst depends on Crb (Pellikka et al., 2002). Thus, to identify the specific roles of Spectrins in photoreceptor development is of considerable interest (Chen et al., 2008).

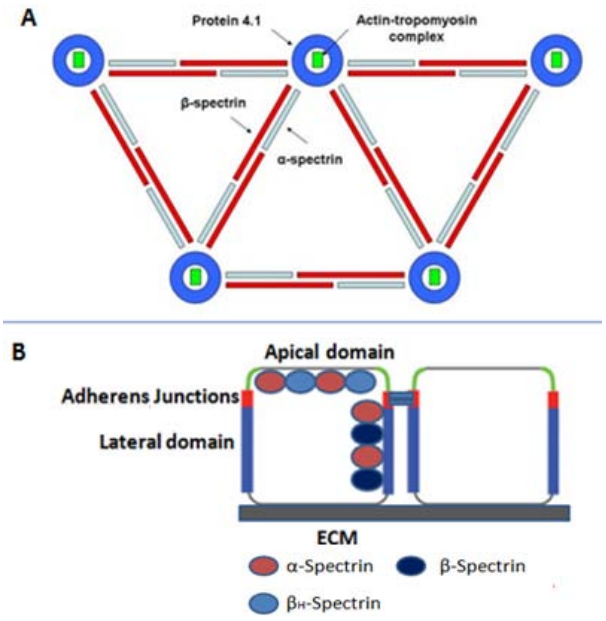

Figure 3: Spectrins and Spectrins localization at *Drosophila* epithelial cells. (A) Two α-Spectrins and two β-Spectrins form tetramers that connect actin-based cytoskeleton. (B) In *Drosophila* epithelial cells, $(\alpha\text{-}\beta)_2$ tetramers localize to the lateral membrane, whereas $(\alpha\text{-}\beta_H)_2$ tetramers are at the apical membrane and the AJ.

Previous Studies on Spastin

Spastin is a microtubule-severing AAA ATPase that involved in microtubules network remodeling (Hazan et al., 1999; Salina et al., 2005; Evans et al., 2005). The mechanism by which spastin breaks apart microtubules remains unclear. But previous studies proposed a model that six spastin proteins may form a hexameric ring and bind to microtubules by radial arms (Figure 4). After the active hexamer binding to tubulin, it may slurp it to the central pole and weaken the interaction between tubulins and thus destabilize the microtubule lattices (Roll-Mecak and Vale, 2008).

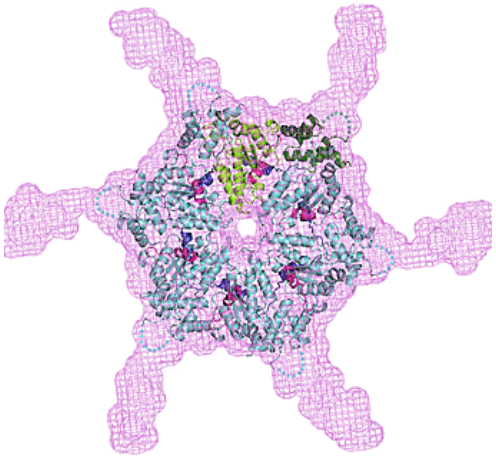

Figure 4: A structural model for the spastin hexameric ring. The radial arms bind to the microtubule, while the central ring uses ATP energy to ratchet a piece of the tubulin protein into the central pore (Roll-Mecak and Vale, 2008).

In human, mutations of spastin are related to hereditary spastic paraplegia (HSP), a group of neurological disorders that characterized by progressive weakness and stiffness in the legs that can lead to complete loss of function. The disease, which affects about 20,000 people in the United States, can also cause mental retardation

and abnormalities of the retina and skin. Despite the fact that spastin plays a role in HSP, however, the biology of the protein and the cellular pathological of the disease is still unknown (Roll-Mecak and Vale, 2006).

Drosophila genome has a highly conserved *spastin* homolog. Recently, functions of *Drosophila* spastin were analyzed in developing neuromuscular junctions using genetic mutational studies, which showed that spastin strongly modulates both synaptic architecture and neurotransmission strength in neuromuscular junctions (Trotta et al., 2004; Sherwood et al., 2004). Besides, motor axon outgrowth defects were observed in *spastin* mutant zebra fish embryo (Wood et al., 2006; Chen et al, 2010).

Previous Studies on Cnn

Centrosome, major microtubule organizing centers (MTOCs) in animal cells, composed of a pair of centrioles that recruit and organize a large number of proteins to form the pericentriolar matrix (PCM). Cnn, a large coiled-coil protein, firstly identified in *Drosophila* that encoded by a homeotic target gene (Heuer et al., 1995). Further analysis revealed that it is the core component in the PCM that can recruit most PCM components (Lucas and Raff, 2007; Megraw et al., 1999; Vaizel-Ohayon et al., 1999). In humans, two Cnn-related proteins were found, which named by CDK5RAP2 and Myomegalin. Human CDK5RAP2 have been linked to brain size during development. Mutations in CDK5RAP2 can result in autosomal-recessive primary microcephaly (Bond et al., 2005), a disorder closely related to defects in centrosome function (Bond and Wood, 2006).

In most animal cells, polarized arrays of microtubules are nucleated from the centrosome. Centrosomes organize symmetric microtubule arrays of uniform polarity, where microtubule-minus ends are embedded in the centrosome while the highly dynamic-plus ends extend toward the cell periphery. Within the pericentriolar matrix, many proteins including Cnn assemble into a scaffold that docks the γ-tubulin ring complex, which nucleates and controls microtubule growth (Oemega et al,. 1999). The γ-tubulin ring complex is composed of γ-tubulin and other accessory factors (Oemega et al,. 1999). Cnn localizes to the pericentriolar matrix and from there other centrosomal proteins load onto the centrosome, includingγ-tubulin (Chen et al., 2011). Centrosomes are non-functional without the addition of Cnn (Figure 5).

Although centrosome is the principal and most important MTOC in the cell, non-centrosomal arrays of microtubules were also found in many cell types (Keating and Borisy, 1999). Non-centrosomal MTOCs are frequently generated in differentiated cells and are likely to expand the functional repertoire of the microtubule cytoskeleton. This is particularly true during the differentiation of specialized cell types in multicellular organisms (Brotolini and Gundersen, 2006). A number of microtubule-organizing structures have been identified in interphase cells. Among these are the nuclear envelope (Bugnard et al., 2005), plasma membrane (Malikov et al., 2004), and Golgi (Chabin-Brion et al., 2001; Efimov et al., 2007; Miller et al., 2009). Sometimes, the relocation of microtubule-anchoring proteins to non-centrosomal sites, such as the apical cell surface, occurs during development (Brodu et al., 2010). As γ-tubulin ring complex is responsible for organizing all the

microtubules arrays, and Cnn is closely related to γ-tubulin, it is reasonable to assume that Cnn can also regulate non centrosomal MTOCs (Chen et al., 2011).

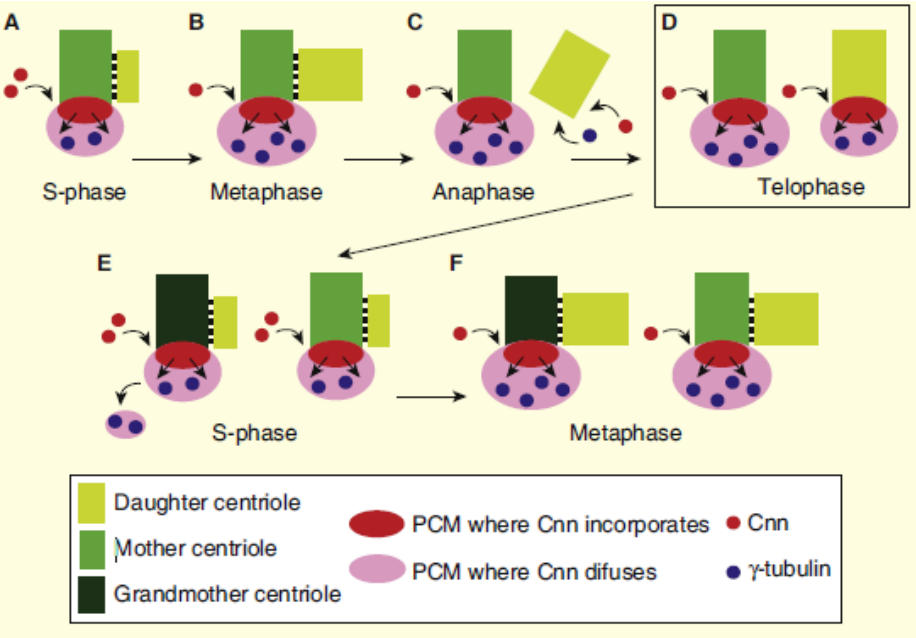

Figure 5: Spatiotemporal dynamics of Cnn at centrosomes in *Drosophila*. (A,B) Cnn (red dots) is incorporated in the centrosome in the vicinity of the centriole (red area) before diffusing into the surrounding PCM (pink), where it regulates the recruitment ofγ-tubulin and potentially other protein complexes (blue dots). During S phase, the levels of Cnn continuously increase, which correlates with higherγ-tubulin levels, and both reach a maximum in M phase. (C) In late mitosis, when the daughter centriole disengages, it begins recruiting its own domain of Cnn andγ-tubulin. (D) By telophase, the mother centriole has been recruiting Cnn for a longer period, reflected by the larger associated PCM domain compared with daughter centrioles. This generates a transient asymmetry in centrosome size (boxed highlighting). (E) During S phase of the next cell cycle, both centrosomes re-establish equivalent levels of Cnn andγ-tubulin. They both incorporate Cnn but the mother centrosome releases PCM 'flares'. (F) In metaphase, both centrosomes have similar maximum levels of Cnn and γ-tubulin. Ltd: [Current Biology] (Gomez-Ferreria and Pelletier, 2010), copy right (2010)

Drosophila *Genetic Techniques*

The common and powerful techniques in *Drosophila* studies are enhancer and suppressor screens to identify genes involved in particular pathways, Flp/FRT system

to conduct loss of function analysis and *GAL4*-UAS system to conduct gain of function analysis.

Enhancer and suppressor screens in *Drosophila* are widely used to identify genes involved in a developmental pathway. One problem in studying gene function is that loss-of-function mutations in almost all genes are recessive, which means that one copy of wild type genes can provide enough proteins to maintain normal developmental pathways. However, *Drosophila* scientists develop a method to identify these genes by building a sensitized genetic background. In this background a known gene mutant partially disrupts a particular pathway and resulted in a specific phenotype. And then recessive mutations in a second gene were expressed on this background. Genes are related to this pathway can be identified as the enhanced or suppressed phenotypes can be observed (St Johnston, 2002).

Loss of function analysis in *Drosophila* is always conducted by Flp/FRT system. This system was firstly used in yeast and then was greatly developed in *Drosophila* to produce mitotic clone in specific tissues. Flp is a recombinase that can induce site specific mitotic recombination between its target sites, which called FRT sites. How the Flp/FRT works is showed in Figure 6. The original parent cells before mitosis are heterozygous, in which one chromosome carries a mutant gene while its homologous chromosome carries a wild type gene with a marker which is always GFP. The replication of chromosomes during mitosis produces four sister chromatins. Flp then induce recombination between FRT sites and after recombination each chromosome has a copy of mutant gene and a copy of wild type gene. Then the

sister chromatins separate from each other and then come in to the progeny cells which will have three possible genotypes, the cells have two copies of wild type genes, the cells have one copy of wild type gene and one copy of mutant gene and the cells have two copies of mutant genes. The wild type genes are marked by GFP so the wild type cells have green fluorescence while the mutant cells do not have. If *Flp* gene is drove by eye specific promoter *eyeless*, the expression of FLP can be restricted to eye-antennal imaginal disc and the mitosis recombination will only occurs in the eyes. In this way, the mutant cells and wt cells are generated in the same eye so there is no need to set up control group additionally (Golic and Lindquist, 1989; St Johnston, 2002).

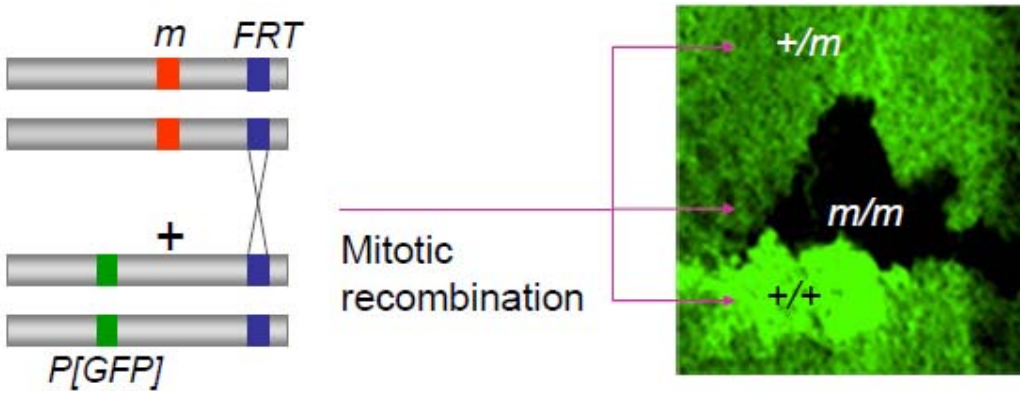

Figure 6: Flp/FRT induced mitotic recombination. Flp recombinase induces recombination between FRT sites. After sister chromatins separating from each other, progeny cells will have three different genotypes. Wild type cells can be identified by its green fluorescence while mutant cells without it.

Gain of function analysis is always conducted by *GAL4*-UAS system which is also from yeast. Gal4 is a yeast transcriptional activator that can bind to upstream activating sequence (UAS) and trigger the expression of gene of interest drove by

UPS (St Johnston, 2008). *Gal4* gene can link to a variety of tissue-specific genomic enhancers and in this way genes expression can be restricted to specific tissues. For example, enhancer *eyeless* induces the Gal4 expression in the eyes while with no influence on other parts of the body.

Rationale

Establishment of correct apical-basal cell polarity (epithelial polarity) is very important to maintain cell function, control cell differentiation and localize cell to proper place. The dysfunction of epithelial polarity is related to a variety of brain and nervous system disorders. Previous studies revealed that there are a small number of proteins play essential roles in establishment and maintenance of epithelial polarity and they are conserved in *Drosophila* and mammalian. By using of *Drosophila* photoreceptors, highly polarized epithelial cells, this study can further analyze the function of known cell polarity proteins and identify more players in the developmental pathways.

First, *Drosophila* photoreceptor and mammalian photoreceptor have highly conserved cell structures and domains. The structural and functional resemblance between them makes the studies on *Drosophila* photoreceptor can facilitate the human eye research and help to find therapies for human retinal diseases. Besides, *Drosophila* has the short life span, strong reproduction ability and a large amount of photoreceptors in the eyes, which provide abundant materials in a short time for experiments.

Secondly, a variety of powerful techniques can be used in *Drosophila*, and *Drosophila* genome is fully sequenced. Together it makes an easy way to analyze and manage most of the genes. The commonly used techniques are enhancer and suppressor screens that identifying genes involved in particular pathways, Flp/FRT system that conduct loss of function analysis and GAL4-UAS system that conduct gain of function analysis.

Thirdly, Genetic studies with mutants indicate that Spectrins are essential for animal viability. One of the spectrins, Karst colocalized with Crb at the rhabdomere stalk, and stalk length is reduced in photoreceptors of karst mutant adult eyes. Thus, to identify the specific roles of Spectrins in photoreceptor development is of considerable interest. Besides, *Drosophilia* have only one α-Spectrin gene and two β-Spectrin genes, which makes it a simple system.

Lastly, microtubules-based cytoskeleton system is the most important cellular transport system, which is closely related to the targeting and transportation of cell polarity proteins. Spastin, a microtubules-severing protein and Cnn, a microtubules organizing protein can regulate microtubules stability and remodeling and thus may influence the establishment of cell polarity. Besides, in human mutations of spastin are related to HSP, and mutations of Cnn are linked to autosomal-recessive primary microcephaly. Thus it is reasonable to further study the roles of Spastin and Cnn during photoreceptors development.

Specific Aims

Specific Aim 1: analyze the roles of Spectrins in photoreceptor morphogenesis which include 1) Genetic interaction between Crb and Spectrins; 2) localization of Spectrins; 3) the possible effects of the *spectrins* null alleles on apical domain and AJ localization in developing *Drosophila* photoreceptor; 4) the possible effects of *spectrins* overexpression mutant on apical domain and AJ localization in developing *Drosophila* photoreceptor.

Specific Aim 2: analyze the role of Spastin in photoreceptor morphogenesis which include 1) Localization of Spastin; 2) the possible effects of the *spastin* null alleles on apical domain and AJ localization in developing *Drosophila* photoreceptor; 3) the possible effects of *spastin* overexpression mutant on apical domain and AJ localization and microtubule stability in developing *Drosophila* photoreceptor.

Specific Aim 2: analyze the role of Cnn in photoreceptor morphogenesis which include 1) Genetic interaction between Baz and Cnn; 2) localization of Cnn; 3) the possible effects of the *cnn* null alleles on apical domain and AJ localization in developing *Drosophila* photoreceptor; 4) the possible effects of *cnn* overexpression mutant on apical domain and AJ localization and microtubule stability in developing *Drosophila* photoreceptor.

The purpose of this dissertation is therefore to specify the localization of three possible partners of cell polarity proteins (Spectrins, Spastin and Cnn) and clarify the roles of them during *Drosophila* photoreceptor morphogenesis.

CHAPTER TWO

Materials and Methods

Fly Stocks and Genetics

All *Drosophila* strains were grown and maintained at room temperature in vials. The *α-spectrin* gene is located at 61F. All current available *α-spectrin* mutants have the *roughoid* mutation in an adjacent region (62B4) that causes the ‘‘rough eye’’ phenotype (Wasserman et al., 2000). I removed *roughoid* mutation from the two null alleles of *a-spectrin* mutant chromosomes, *α-spectrin*^{lm102} and*α-spectrin*^{rg41} (Lee et al., 1993), using conventional meiotic recombination. The *roughoid*$^{+}$*α-spectrin* mutants were completely rescued from early larval lethality to adulthood by the ubiquitin-promoter-based expression of *α-spectrin* cDNA construct (*ubi-spec*) (Chen et al., 2009).

Mitotic recombination was induced by using the FLP/ FRT method for clonal analysis (Xu and Rubin, 1993). *α-spec*^{rg41} (Lee et al., 1993) mutant clones were produced by eye-specific expression of FLP in *y w ey-Flp/+; α-spec FRT82B/FRT82B Ubi-GFP.* *karst*^{1} (Thomas et al., 1998) mutant clones were produced by *y w ey-Flp/+; karst*^{1} *FRT80B/Ubi-GFP FRT80B*. *β-Spectrin*^{G113} (Hulsmeier et al., 2007) mutant clones were produced by *y w β-Spectrin*^{G113} *FRT19A/y w Ubi-GFP FRT19A*; *ey-Flp/+*. Overexpression of *β-Spectrin* was induced by crossing *UAS-β-Spectrin* (Hulsmeier et al., 2007) with *GMR-GAL4* (Freeman, 1996). *spastin*$^{5.75}$ mutant (Sherwood et al., 2004) clones were produced by eye-specific expression of FLP in *y*

w ey-Flp/+; spastin$^{5.75}$ *FRT82B/FRT82B Ubi-GFP*. Overexpression of spastin was induced by crossing *UAS-spastin* (Sherwood et al., 2004) with *GMR-GAL4* (Freeman, 1996).

UAS-Baz (Wodarz et al., 1999) and *GMR-Gal4* (on chromosome 3 from Andreas Bergmann, MD Anderson) were recombined on the same chromosome for a genetic modifier screening. Mitotic recombination was induced by using the FLP/FRT method for clonal analysis (Xu and Rubin, 1999). *cnn*^{hk21} is a null allele with a nonsense mutation that truncates the protein at amino acid 106 (Megraw et al,. 1999). *cnn*^{hk21} mutant clones were produced by eye-specific expression of FLP in *y w ey-flp/+; FRT42D cnn*^{hk21}*/ FRT42D Ubi-GFP*. Overexpression of cnn was induced by crossing *UAS-GFP-Cnn* (Megraw, 2002) with *GMR-GAL4* (Freeman, 1996) at 29°C. *cnn*^{hk21} and *UAS-GFP-cnn* were obtained from Bloomington Stock Center at Indiana University.

Drosophila *Food Preparation*

The food is prepared in a 5 gallon roaster oven. First, add 8 L water. Then add 43.3 g of agar. Next, add 500 mL of water and 500 mL of molasses. Then add 220 g of yeast and 450 g of corn meal. Cook for 2 hours. After cooking, add 75 mL of 10% Tegosept 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 25 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 48 hours (From Dr. Nam lab's protocols).

Pupal Eye Dissection

Pupas at mid pupal stage are taken from the vials, which are slightly yellow but still have not fully developed their eyes. Place the pupas into a 9 well dissection tray with approximately 400 µl of 1X PBS in each well. Using one dissecting tweezers, gently hold the pupa at the posterior end and carefully remove the anterior part of the case by another tweezers. Then use tweezers to poke a pole in the white inner membrane and squeeze the tissue from the hole. Then isolate the developing eye with the brain connecting the two bulbous ends and move it into a clean dissection tray (From Dr. Nam lab's protocols).

Antibody Staining and Mounting

Place the pupal eyes in the same well. First, remove PBS and shake with 4% PF in PBS for 15 minutes. Then shake with Block Buffer Solution for 15 minutes. Next, wash with wash buffer. 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. The ones used in these experiments are Cy3, Cy5, and FITC. 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 20 minutes with 4% PF in PBS. Next, wash with PBS. Then,

mount on slide with less than a drop of mounting solution then add cover slip. Apply nail polish on the corners, prevent moving the cover slip.

The following primary antibodies were used: rabbit anti-PKCζ (Santa Cruz), 1:500; mouse anti-α-Spectrin (DSHB), 1:10; mouse anti-Arm (DSHB), 1:10; rat anti-DEcadherin (DSHB), 1:10; rabbit anti-Baz (Wodarz et al., 1999), 1:500; rat anti-Crb (den Hollander et al., 1999), 1:400; sheep anti-GFP (Biogenesis or Serotec), 1:100; mouse and rabbit anti-Dpatj (den Hollander et al., 1999), 1:500; rabbit anti-β-Spectrin (Byers et al., 1989), 1:500; and rabbit anti-Karst (Thomas and Kiehart, 1994), 1:500; mouse anti-Acetylated tubulin (Sigma), 1:1000; mouse anti-α-tubulin (Sigma), 1:500; mouse anti-β-tubulin (Sigma), 1:500; rabbit anti-Dlg (Lee et al., 2003), 1:1,000; sheep anti-GFP (Biogenesis), 1:100; and guinea pigs anti-Spastin (Sherwood., 2004), 1:300; rabbit anti-Sdt, 1:500 (Hong et al., 2001); rabbit anti-γ-tubulin (Sigma or Abcam), 1:1000; guinea pig anti-Cnn, 1:1000 (Dix and Raff, 2007); rat anti-Elav (DSHB) (O'Neill et al., 1994), 1:50; mouse anti-Lamin (DSHB) (Stuurman et al., 1995), 1:50.

Secondary antibodies conjugated with Cy3, Cy5, or FITC were from Jackson Laboratories. Fluorescent immunostaining and confocal analysis of pupal eyes were carried out as reported. Specificity of the antibody staining was verified by clonal analysis using the protein-null mutant (Sherwood., 2004). Fluorescent images were acquired on an Olympus FV1000 confocal microscope. Image analysis and quantification were performed using ImageJ and Adobe (From Dr. Nam lab's protocols).

CHAPTER THREE

Results

This chapter published as: Chen TW, Chen G, Funkhouser LJ, Nam SC (2009) Membrane domain modulation by Spectrins in *Drosophila* photoreceptor morphogenesis. Genesis 47: 744-750.

Chen G, League GP, Nam SC (2010) Role of spastin in apical domain control along the rhabdomere elongation in *Drosophila* photoreceptor. PLoS One 5: e9480.

Chen G, Rogers AK, League GP, Nam SC (2011) Genetic interaction of Centrosomin and Bazooka in apical domain regulation in *Drosophila* photoreceptor. PLoS ONE 6(1): e16127.

Spectrins in Drosophila *Photoreceptor Morphogenesis*

Genetic Interaction Between Spectrin *and* crb

Previous studies indicated a possible interaction between *karst* and *crb* (Medina et al., 2002; Pellikka et al., 2002), thus I examined the genetic interaction between *crb* and the *spectrin* genes (*α-spectrin, β-Spectrin,* and *karst)* in *Drosophila* photoreceptors. Intracellular domain of Crb (Crb^{intra}) (Izaddoost et al., 2002) was expressed in *spectrin*/+ heterozygous background which has one copy of mutant *spectrin* gene and one copy of wild type gene. The rough eye phenotype caused by Crb^{intra} expression (Grzeschik and Knust, 2005; Izaddoost et al., 2002)was enhanced by the reduced protein expression of any *spectrin* gene (*spectrin*/+) (Figure 7). These results indicate that *crb* may interact with all of the *spectrin* genes.

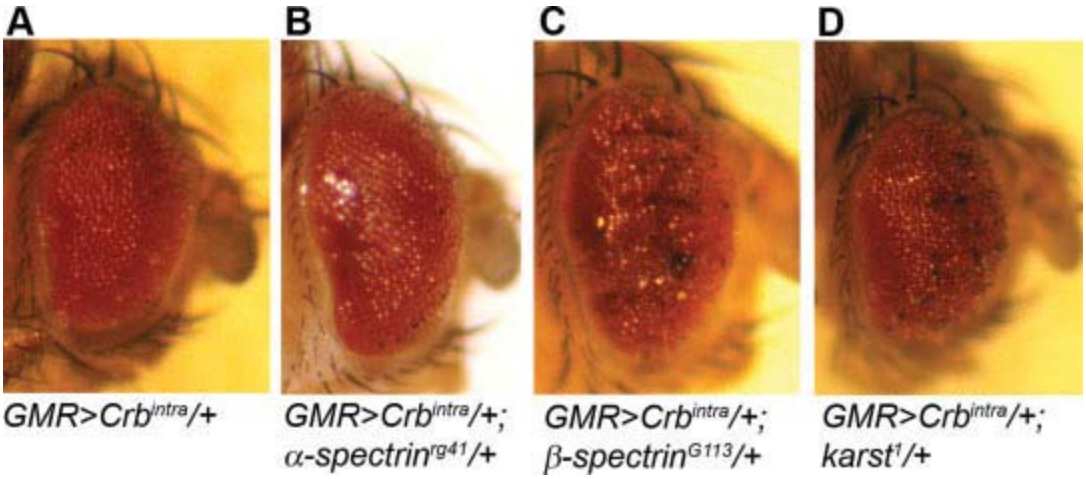

Figure 7: Genetic interaction of *spectrins* and *crb.* A-D: Adult eye phenotypes of *GMR>Crb^{intra}*/+; +/+ (A), *GMR>Crb^{intra}*/+; *α-spectrin*^{rg41}/+ (B), *GMR>Crb^{intra}*/+; *β-Spectrin^{G113}*/+ (C), and *GMR>Crb^{intra}*/+; *karst^{2}*/+ (D).

Localization of Spectrin in the Developing Photoreceptors

In *Drosophila* epithelial cells, $(\alpha–\beta)_2$ tetramers localize to the basolateral membrane, whereas $(\alpha–\beta_H)_2$ tetramers localize to the apical membrane and the AJ (Dubreuil et al., 1997; Lee et al., 1997; Thomas and Williams, 1999; Thomas et al., 1998). I examined the localization of each Spectin at the mid pupal stage of photoreceptors. α-Spectrin distributes to all the cell membrane domains from basal membrane to apical membrane, but has relatively high concentration at the basolateralmembrane domains (Figure 8A). Karst only localizes at the apical domains (Figure 8A', arrowhead) and β-Spectrin primarily localizes at the lateral membrane domains (Figure 8B', arrow). These results display a similar localization pattern as the *Drosophila* epithelial cells.

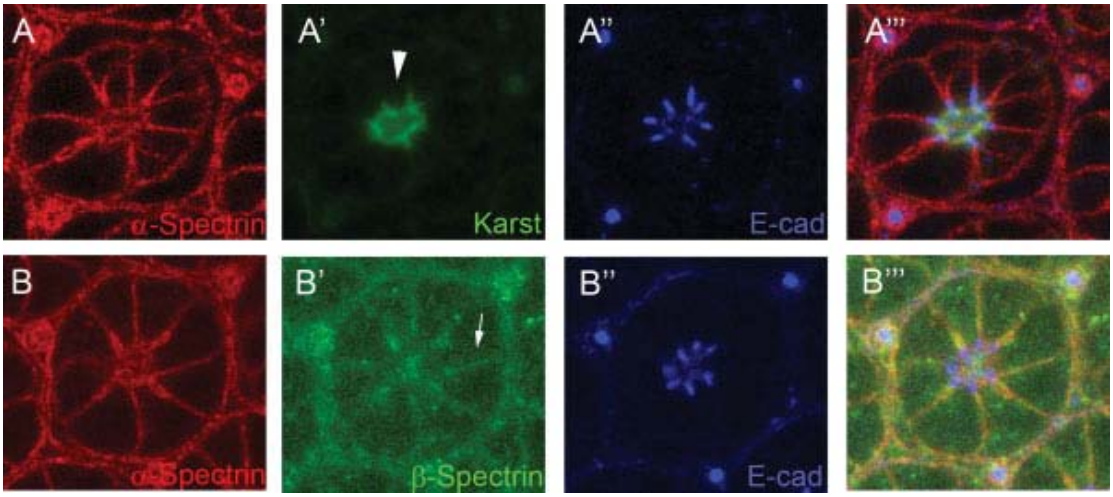

Figure 8: Localization of spectrin in *Drosophila* pupal photoreceptors in developing pupal eyes (40% pd). A, B: The *α*-Spectrin (red) localizes at all membrane domains with high enrichment at the basolateral domains, as well as at the apical domain and AJ (E-cad, blue, A'' and B'') of the photoreceptors. *β*-Spectrin localizes mostly at the lateral membrane domain (B'). Karst specifically localizes at the apical domain of the photoreceptors (A').

Spectrins are Dispensable in Early Eye Pattern Formation

To examine the role of spectrins in photoreceptor development, firstly I checked whether spectrin is required for the normal development and differentiation of photoreceptor precursor cells. I generated mosaic eyes of null or strong hypomorphic mutations of *α-spectrin*^{rg41} (Lee et al., 1993) , *karst*^{1} (Thomas et al., 1998) and *β-Spectrin*^{G113} (Hulsmeier et al., 2007) , using the Flp/ FRT technique (Xu and Rubin, 1993). α-Spectrin is highly concentrated at the cell membranes of photoreceptor precursor cells during third-instar larval eye discs (Figure 9A). In all the loss-of-function mutant clones, *α-spectrin* (Figure 9B), *β-Spectrin*^{G11} (Figure 9C) and *karst*^{1} (Figure 9D), the apical domains and AJs of photoreceptor precursor cells didn't show any obvious defects. These data indicate that spectrins are dispensable for establishment of initial cell polarity and early differentiation in the eye.

Loss-of-Function Analysis of Spectrin Genes during Pupal Eye Development

Although spectrins are dispensable for early eye pattern forming, *α-spectrin* and *β-Spectrin* play an important role in mid-stage pupal eye development (Figure 10). I analyzed *spectrin* mutant clones in mid-stage pupal eyes (Figure 10). In *karst* mutants, mid-stage pupal eyes didn't show any localization defects in Dpatj and Arm (Figure 10A). This result indicated that Karst is dispensable during pupal eye development.

However, in *β-Spectrin* mutants, apical domains (Dpatj) and AJ (Arm) were dramatically expanded from the apical to the basolateral membrane domains (100%, n > 300) in the mid-stage pupal eyes (Figure 10B). And *α-spectrin* mutants displayed severely disrupted morphogenesis with expanded Dpatj and Arm (Figure 10C) in 90%±10% ommatidia examined (n > 400). *α-spectrin* and *β-Spectrin* mutations show similar photoreceptor defects with expansions of apical domain and AJ markers, and the *β-Spectrin* mutation causes more severe phenotypes than that of *α-spectrin* mutation (Figure 10B, C). These data strongly suggest that the α- and β-Spectrins are essential for apical membrane domain maintenance development during pupal eye development (Figure 10B, C). Unlike photoreceptors, the cone cells and pigment cells that surround the photoreceptor cells were not noticeably affected in the *α-spectrin* mutant pupal eyes (Figure 10D). Thus, observed defects in *α-spectrin* mutant eyes are not only specific to the developmental stage but also to the cell type.

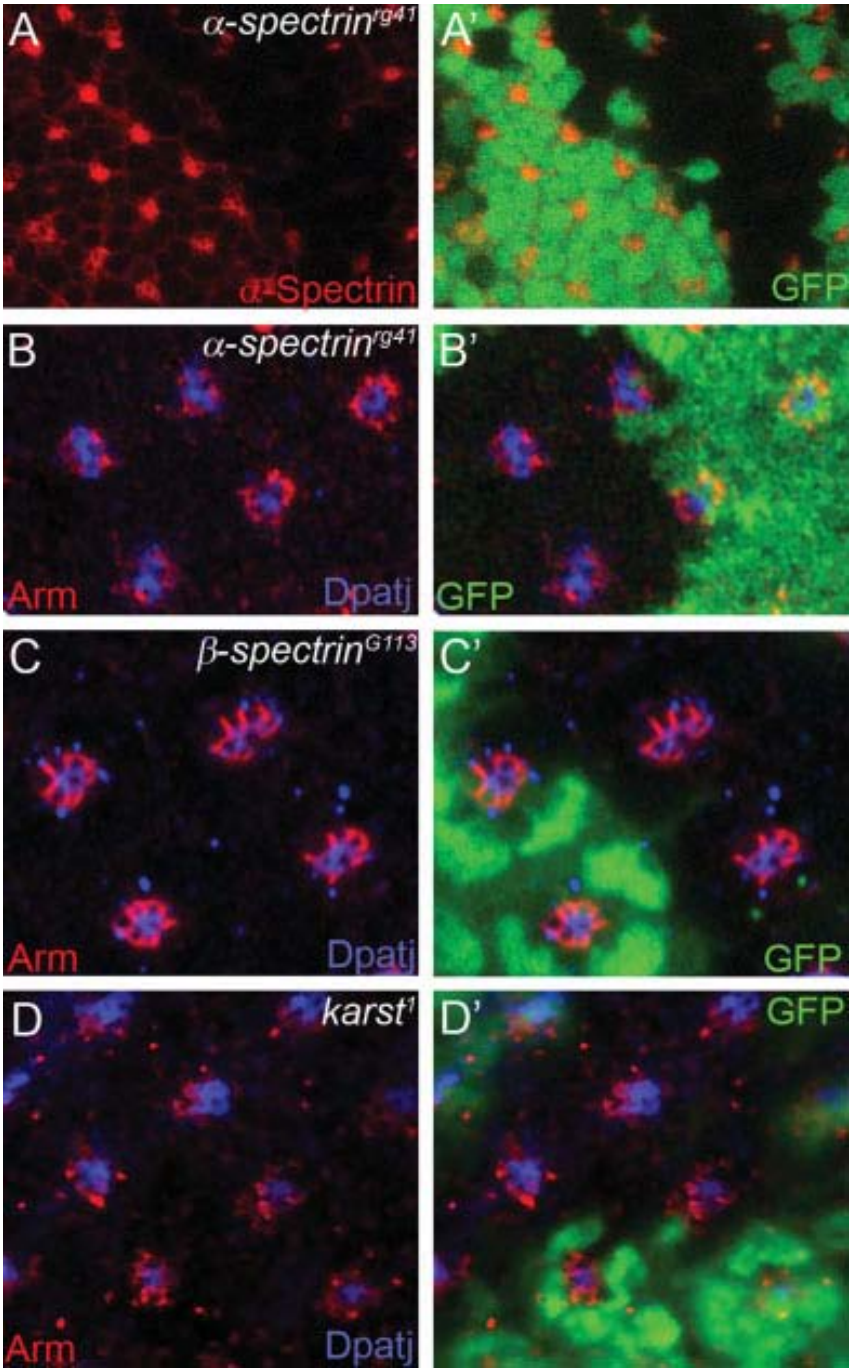

Figure 9: Spectrins are dispensable for early eye pattern formation. A: α-Spectrin (red, A) mainly localizes at the apical domains of developing third-instar larval eye discs. In the mutant clones (absence of green, GFP, A'), α-Spectrin protein is absent. The null mutant, *α-spectrin*^{rg41}, was used to generate mutant clones. B–D: Arm (AJ marker, red) and Dpatj (apical maker, blue) showed little defects in third-instar larval eye discs of *α-spectrin*^{rg41} (B), *β-Spectrin*^{G113} (C), or *karst*^{1} (D) mutant clones. Mutant clones were marked by the absence of GFP (green).

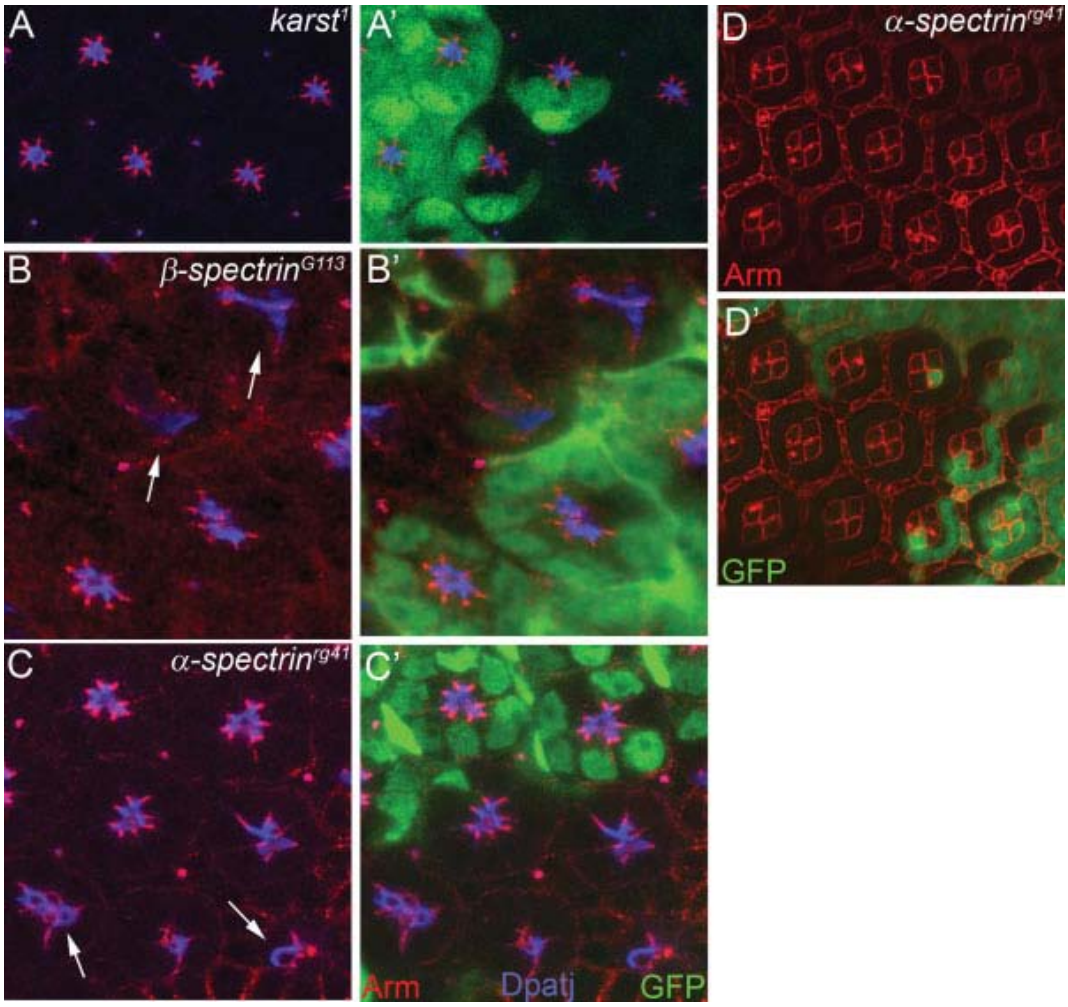

Figure 10: α- and *β*-Spectrins are essential for photoreceptor morphogenesis but Karst is dispensable in the mid-stage developing pupal eyes. Mutant cells were marked by the absence of GFP (green). A: Arm (AJ marker, red) and Dpatj (apical marker, blue) are normal in *karst*^{1} mutants in 40% pd. B: In *β-Spectrin*^{G113} mutant clones of mid-pupal stage, Dpatj (blue) and Arm (red) are expanded from the apical to the basolateral (arrows) in 40% pd. C: In *α-spectrin*^{rg41} mutant clones of mid-pupal stage, Arm is expanded with concurrent Dpatj (blue) expansion (arrow). Often, the expanded Dpatj is displaced basal to Arm (red, arrows), suggesting the severe apical domain expansions (arrows) in 40% pd. D: Eye accessory cells are normal in *α-spectrin* mutants. Cone cells and pigment cells are not affected in *α-spectrin* mutants (marked by the absence of GFP, green, D') in developing pupal eyes (40% pd).

Basolateral Specific Role of β-Spectrin Gene during Pupal Eye Development

In *Drosophila* epithelial cells it was reported that apical membrane protein complexes and basolateral membrane protein complexes antagonized with each other

to maintain the cell domain identity (Bilder et al., 2003; Tanentzapf and Tepass, 2003). As Karst and β-Spectrin localize differently to the apical and basolateral membrane, respectively (Figure 8), I examined the interaction between these two spectrins. I overexpressed wild type β-Spectrin at 25℃ in differentiating retinal cells using GMR-Gal4 and it resulted in dramatic reduction of the apical domain marked by Dpatj (Figure 11B'). In contrast, loss of β-Spectrin causes Karst expansion from apical to basolateral domains (Figure 12A'). This data strongly suggest that β-Spectrin and Karst may have important function in separation and maintenance of distinct cell membrane domains.

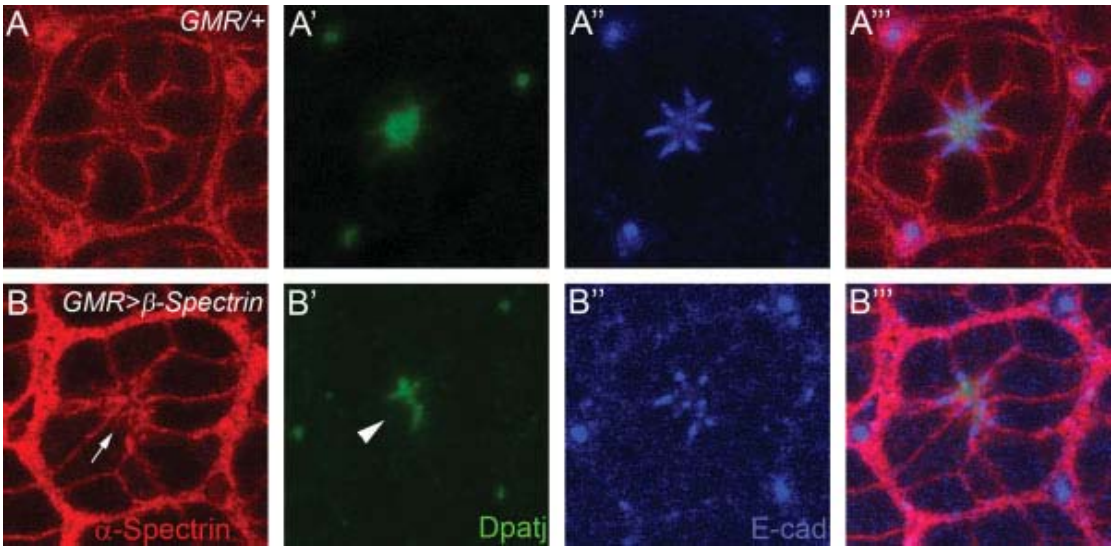

Figure 11: β-Spectrin overexpression causes severe reduction of the apical membrane domain of developing pupal eyes (40% pd). A: Localization of α-Spectrin, Dpatj (apical marker, green, A'), and E-cad (AJ marker, A'') in the *GMR-Gal4*/+ control. B: β-Spectrin overexpression by *GMR-Gal4* causes the shrinkage of the apical domain marked by Dpatj (apical marker, B')

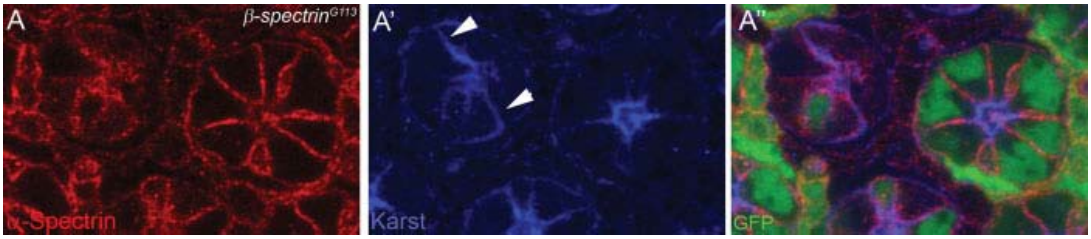

Figure 12: Role of β-Spectrin in localization of α-Spectrin and Karst. (A) α-Spectrin (red, A) and Karst (blue, B) were mislocalized in β-Spectrin mutant clones (marked by the absence of GFP, green, A'') in developing pupal eyes (40% pd). Karst was ectopically expanded from apical to basolateral (arrowheads, A').

Role of Spastin in Drosophila *photoreceptor morphogenensis*

Localization of Stable Microtubules in Drosophila *Photoreceptor*

In animal photoreceptor cells, the surface membrane is enlarged for the storage of opsin and photopigment. Previous studies revealed that insect eyes use an actin-based structure for surface membrane enlargement, but mammalian eyes use microtubule-based structure. I examined whether the *Drosophila* photoreceptor cells have any microtubule-based structures. To identify stable microtubules, I used a monoclonal antibody directed against acetylated-tubulin (Piperno and Fuller, 1985), which specifically labels stabilized microtubules in cilia and axons (Piperno and Fuller, 1985; Wolf et al., 1988). Since the acetylation of microtubules is correlated with the stabilization (Schulze et al., 1987; Schulze and Kirschner, 1987), this antibody is a reliable marker to check the stable microtubules (Piperno and Fuller, 1985). By using the antibody I found that the acetylated-tubulin is highly enriched in the basal to apical domain in the developing pupal eyes (40% pd), (Figure 13A), between and more basal to the adhrens junction (E-cad) (Figure 13B), and in between

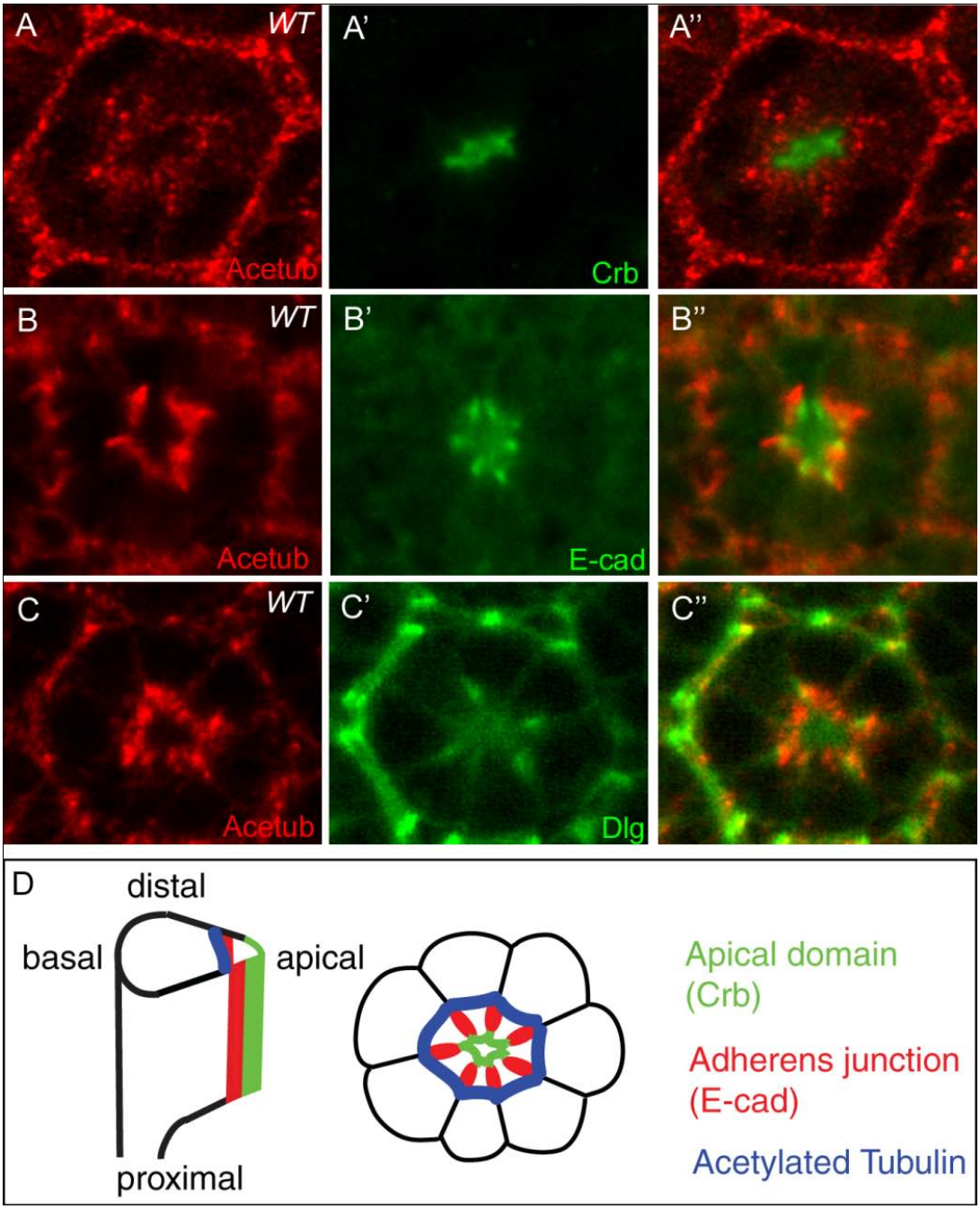

Figure 13: Acetylated microtubules in pupal photoreceptors. Localization of acetylated microtubules in mid-stage (40-45% pd) of pupal eyes were examined. (A) Pupal eyes were stained with acetylated-tubulin (Acetub, red) and Crumbs (Crb, green). The acetylated-tubulin (Acetub, red) localizes more basal to the apical domain (Crb). (B) Acetylated microtubules (red) localizes more basal to the adhrens junction (E-cad, green). (C) Acetylated microtubules localize more apical to the basolateral domain (Dlg, green). (D) Schematic diagram of the localization of stabilized microtubules in pupal photoreceptors. The apical markers (Crb) localize at the apical domain (green). The E-cad localizes at AJ (red) which are more basal to the apical domain. The acetylated-tubulin (blue) localizes at the outside from the AJs (red).

and more apical to the basolateral domain (Discs large, Dlg) (Figure 13C). Based on these results, I propose a model in which the stable microtubules localize on the outside of adhrens junctions in developing pupal eyes (Figure 13D). Given the presence of acetylated microtubules in the *Drosophila* photoreceptors, these stable microtubules may have some potential functions for photoreceptor development.

Orientation of Microtubules in Drosophila *Photoreceptors*

Based on their intrinsic molecular polarity, microtubules have both plus-ends and minus-ends. I checked the microtubule orientation in *Drosophila* photoreceptor using Kin:LacZ (a plus-end marker) and Nod:LacZ (a minus-end marker) (Clark et al., 1997). Each marker was expressed by an eye-specific driver, *GMR-GAL4* (Freeman, 1996), using the GAL4/UAS expression system (Brand and Perrimon, 1993). Although the markers were broadly expressed in the photoreceptor cells, I

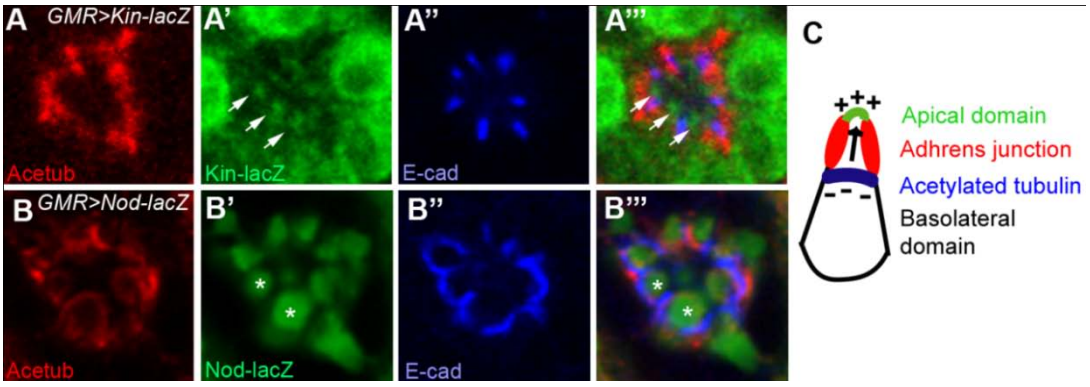

Figure 14: Orientation of microtubules in *Drosophila* pupal photoreceptors. (A, B) The (+) ending-marker (Kin-LacZ (A)) or the (-) ending-marker (Nod-LacZ (B)) were expressed using *GMR-GAL4* at 22°C. Pupal eyes (~45% pd) were analyzed. The (+) ending-markers localize at the apical domains (arrows, A') and (-) ending-markers localize at the rhabdomere base (asterisks, B'). (C) A model of the tubulin orientation in photoreceptor. (+) ending localize at the apical and (-) ending localize at the acetylated microtubule bundles. The minus-to-plus orientation (black arrow) points toward the apical side of the photoreceptor.

identified the apical localization of the (+) endings (Figure 14A*'*, arrows) and the lateral and cell-body localization of the (-) endings (Figure 14B*'*, asterisks). Based on this data, I propose that the microtubules in *Drosophila* eyes are pointed toward the apical domains from the cell bodies (Figure 14C).

Localization of Spastin in Drosophila *Pupal Photoreceptors*

In mammals, Spastin has been shown to modulate the microtubule cytoskeleton (Errico et al., 2002). The *Drosophila* homolog of Spastin appears to affect the abundance and distribution of acetylated microtubules in *Drosophila* neuromuscular junctions (Trotta et al., 2004). To identify one of the potential functions of the stable microtubules in *Drosophila* photoreceptor development, the subcellular localization of Spastin in the mid-stage developing pupal eyes (45% pd) was examined by immunostaining and confocal microscopy. Spastin is highly enriched in the stable microtubules (Fig. 15), as well as in the apical membrane domain (Figure 15, arrow).

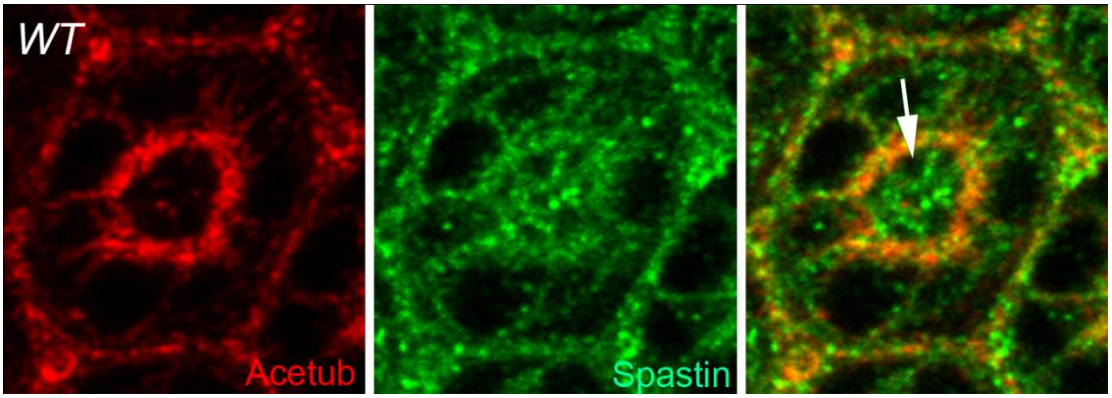

Figure 15: Localization of Spastin in *Drosophila* pupal photoreceptors (45 % pd). Spastin (green) localizes not only at acetylated microtubules (red), but also at the apical membrane domain (arrow) of the mid-stage pupal eyes.

This data strongly indicates Spastin's potential role in the stable microtubules and/or apical domain morphogenesis in *Drosophila* pupal photoreceptors.

Spastin is Required in Apical Domain of Drosophila *Photoreceptor Morphogenesis*

To examine whether Spastin is required for photoreceptor morphogenesis in mid-stage pupal eye development, I generated mosaic eyes of a null mutation of spastin, $spastin^{5.75}$ (Sherwood et al., 2004), using the FLP/FRT-based genetic mosaic technique (Xu and Rubin, 1993). I have found that the *spastin* mutation slightly affected Crumbs localization (50±10%, n=100, Figure 16A) at the distal section, but caused the almost complete loss of the Crumbs at the proximal section of the same pupal eye (100%, n>200, Figure 16B). Since the rhabdomere grows from distal to proximal in developing pupal eyes (Figure 16C, arrow), this mutant phenotype of *spastin* strongly suggests that Spastin is specifically required for the apical membrane domain, including Crumbs, in addition to rhabdomere growth from distal to proximal during photoreceptor morphogenesis. This type of rhabdomere elongation defect was found in the cases of *crumbs* and *par-1* mutations (Izaddoost et al., 2002; Nam et al., 2007).

Similar differential defects along the distal-proximal axis were observed in the case of the stable microtubules. The stable microtubules are relatively normal at the distal section (10±10% reduction, n=100, Figure 16A, red), but the stable microtubules are reduced at the proximal section of spastin mutants (50±10% reduction, n=100, Figure 16B, arrowheads). It was noticed that the complete loss of Crumbs occurred even in the presence of the acetylated microtubules (Figure 16B,

arrowheads), which strongly indicates that the loss of Crumbs was not caused by the absence of the stable microtubules. Instead, this data strongly suggests that the loss of Crumbs at the proximal section of spastin mutants was caused by a direct effect of the spastin mutation, rather than by an indirect influence of the stable microtubules.

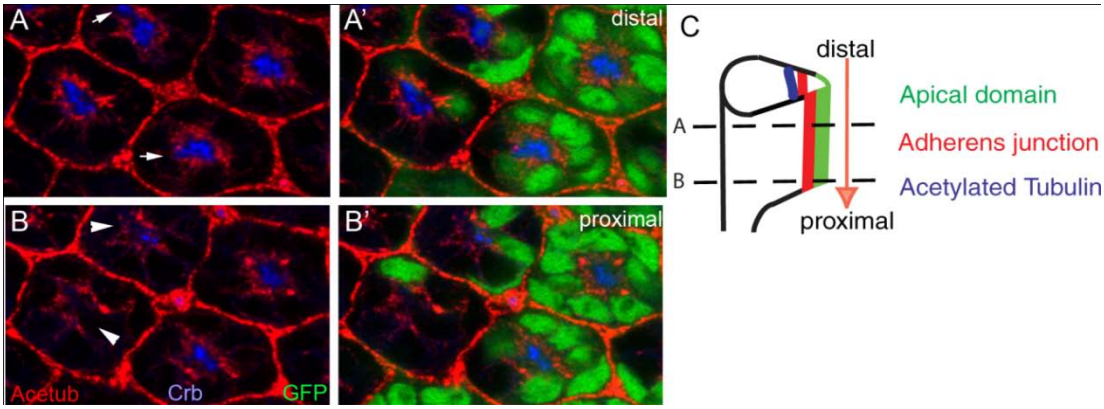

Figure 16: Spastin is essential for photoreceptor morphogenesis in the mid-stage developing pupal eyes. (A, B) Pupal eyes (45% pupal stage) with *spastin*$^{5.75}$ null mutant clones marked by the absence of the GFP (green). Acetylated tubulin (Acetub, red) was decreased or destabilized in the absence of the spastin. Crumbs (Crb, blue) is mislocalized at the distal section (A, arrows) and almost absent at the proximal section (B, arrow-heads) from the same pupal eye. (C) Developing pupal photoreceptors elongate from distal to proximal direction. Distal (A) and proximal (B) sections were marked by dashed-lines.

Overexpression of Spastin Causes Apical Domain Expansion of Drosophila *Photoreceptor*

The loss-of-function analysis of the *spastin* mutation (Figure 16) strongly suggests that *spastin* might affect the apical membrane domain for photoreceptor morphogenesis. Next I analyzed the gain-of-function analysis of *spastin* using eye-specific GAL4 lines, *GMR-GAL4* (Freeman, 1996), to increase the Spastin level in the photoreceptors. I employed the established *UAS-Spastin* (Sherwood et al., 2004) to examine the effects of Spastin overexpression for photoreceptor

morphogenesis. Spastin overexpression in the mid-stage pupal photoreceptors dramatically expanded the apical membrane domains (300±100% expansion, n=100, Figure 17B, green), with concurrent mislocalization/expansion of the adhrens junctions (300±100% expansion, n=100, Figure 17B, blue) from the apical center of the photoreceptor. Although the mislocalization of apical and adhrens junctions was dramatic, there were no defects in cell polarity since the Crumbs (apical marker) still localized more apically compared to the E-cad (adhrens junction marker) (Figure 17B).

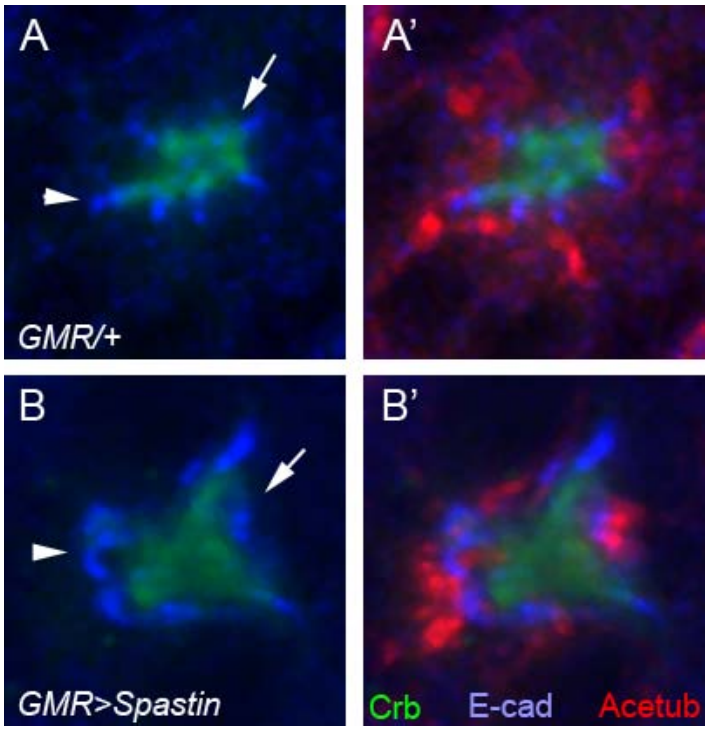

Figure 17: Overexpression of spastin causes the apical domain expansions. Pupal eyes (45% pd) with spastin overexpression driven by GMR-GAL4 at 22°C were examined by Crumbs (Crb, green, apical domain marker), E-cad (blue, AJ marker) and Acetylated- tubulin (Acetub, red). (A) control, *GMR-GAL4/+* (B) *GMR-GAL4/UAS-spastin*. The expanded Crumbs domain (Crb, green, arrow) and E-cad (blue, arrowhead) were caused by the spastin overexpression (B).

My results strongly suggest that Spastin specifically controls the apical membrane domain during pupal eye development based on the loss-of-function (Figure 16) and gain-of-function (Figure 17) phenotypes of *spastin*. Although the apical domains were strongly expanded based on the overexpression of spastin, the stable microtubules were not affected dramatically (Figure 17B), indicating that apical expansion might be caused by the direct effect of spastin overexpression and not by any indirect effects caused by differences in the stable microtubule levels.

Cnn in Drosophila *Photoreceptor Morphogenesis*

Genetic Interaction Between baz and cnn

Previous studies have shown that Baz is essential for localization of Crb and AJ during photoreceptor morphogenesis (Nam and Choi, 2006; Nam et al., 2006). Furthermore, overexpressed Baz in differentiating retinal cells recruits the Crb and AJ to ectopic positions, and causes cell polarity disruptions in photoreceptors (Nam et al., 2006). I overexpressed Baz (Wodarz et al,. 1999) using *GMR-Gal4* (Freeman, 1996), which led to a roughening of the eye's external morphology (Figure 18A). Using this genetically sensitized condition, I performed a genetic screen to identify additional players that function with Baz to regulate photoreceptor morphogenesis. From a pilot screen, I found that the rough eye phenotype of *GMR>Baz* was dominantly enhanced by reducing the level of *cnn* (Figure 19B). This data suggests that there is a strong genetic interaction between *baz* and *cnn*. Overexpression of Baz by *GMR-Gal4* in developing eyes resulted in severe defects in the apical basal

pattern of photoreceptors, with abnormal positioning of Crb and AJ (Nam et al., 2007). In the *cnn/+* heterozygous background to reduce the Cnn level, the rough-eye phenotype of *GMR>Baz* was strongly enhanced. This genetic interaction data strongly suggests that Cnn may provide an additional positional and/or maintenance cue for Crb and AJ positioning, because the Crb/AJ mislocalization caused by Baz overexpression further enhanced by the reduced *cnn* gene dosage (*cnn/+*) (Figure 18).

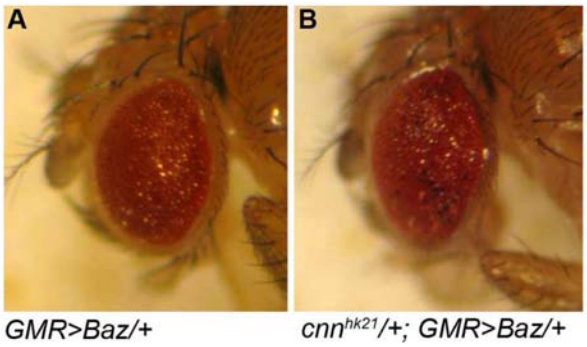

Figure 18: Genetic interaction of *baz* and *cnn* in *Drosophila* eye. (A-B) Adult eye phenotype of +/+; *GMR>Baz*/+ (A) and cnn^{hk21}/+; *GMR>Baz/+* (B).

Localization of Cnn in Drosophila *Pupal Photoreceptors*

Determining the localization of Cnn will provide information on how the acetylated microtubules are originated in developing photoreceptors and how Cnn is linked to the cell polarity genes, including *baz*. Subcellular localization of Cnn was examined at the mid-pupal stages of developing eyes by immunostaining and confocal microscopy. Cnn is highly enriched at the ba and non-centrosomal MTOC. Centrosome- independent MTOC has been reported (Moutinho-Pereira et al., 2009), but the acentriolar MTOC also contains γ-tubulin and requires Cnn for its architecture and function (Moutinho-Pereira et al., 2009). Therefore, I examined the localization

of γ-tubulin and Cnn (Megraw et al., 1999) in the pupal eyes. Cnn also localizes at the same place with γ-Tubulin (Figures 19B' and 19C') which labels all the centrosomal MTOC as well as non-centrosomal MTOC. Based on these confocal image analyses, I identified the relative location of Cnn at the basal, adjacent side of the acetylated-microtubules and at the perinuclear side in the mid-pupal eyes (Figure 19). The localization pattern of Cnn strongly suggests that Cnn may contribute to the organization of microtubule arrays, rather than mitotic cell division, in post-mitotic and fully differentiated cells of the these pupal eyes. In summary, I precisely identified the exact and relative location of Cnn in developing pupal eyes using several markers of the apical domain, AJs, and acetylated-microtubules, as well as specific nuclear markers. Given the presence of Cnn underneath the acetylated microtubules in the *Drosophila* photoreceptors, Cnn may have some potential functions for photoreceptor development as a MTOC or microtubule modulator in the eyes. sal side from the apical (Crb) domain and AJ (E-cad) (Figure 19A'), and also basal and adjacent to the acetylated-microtubules (Figure 19B') (Chen et al., 2010) in the mid-pupal eyes. The localization of Cnn was compared to that of γ-tubulin, a marker for centrosomal

cnn is Required for Localization of Stable Microtubules and Baz

To test whether *cnn* is required for developing photoreceptor architecture, I generated mutant clones of the *cnn* mutation using cnn^{hk21} (Megraw et al., 1999) in photoreceptors using the FLP/FRT-based genetic mosaic technique (Xu and Rubin, 1993) with eyeless-flippase (ey-flp) (Newsome et al., 2000). Since cnn^{hk21} is a null

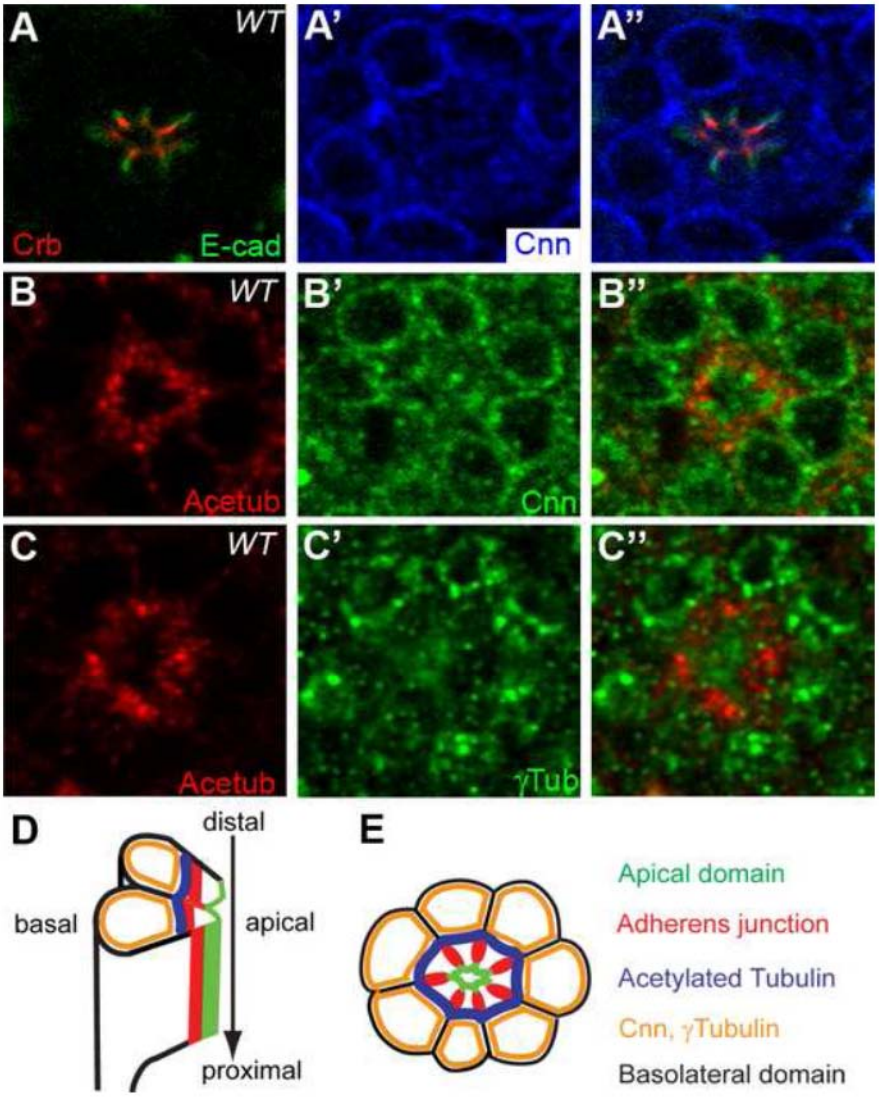

Figure 19: Localization of Cnn in *Drosophila* pupal photoreceptors. Localization of Cnn in mid-stage (50% pd) of pupal eyes were examined. (A) Pupal eyes were stained with Crb (apical marker, red, A), E-cad (green, AJ, A) and Cnn (blue, A'). The Cnn (blue, A') localized more basal to the apical domain of Crb and AJ of E-cad. (B) The Cnn (green, B') localized more basal and adjacent to the acetylated microtubules (Acetub, red, B). (C) Acetylated microtubules (red, C) localized more apical and adjacent to the γ-Tubulin (γ-Tub, green, C').

allele (Eisman et al., 2009), I analyzed cnn^{hk21} mutation to identify the Cnn functions. At the mid-pupal (50%) developmental stage, the *cnn* mutation causes the acetylated microtubules and Baz domains to mislocalize from basal to apical positions (Figure 20A). Based on statistical analysis of Baz membrane domains, about 30±5% (n=30)

of the apical domain size was increased in the *cnn* mutant (ImageJ analysis). The acetylated microtubule bundles, at the basal side of the Baz membrane domains, were also mispositioned (Figure 20A). Interestingly, the ectopic position of acetylated microtubules (Figure 20A, arrowheads) also recruited the ectopically positioned Baz (Figure 20A', arrows) at its apical side. Crb, the apical membrane marker, also mispositioned from apical to basal in the *cnn* mutant (Figure 20B, arrowheads), which is consistent with the Baz/E-cad mispositioning in the *cnn* mutant (Figure 20A' and 20B'). The localization of E-cad, an AJ marker, was co-distributed with Baz (Figure 20C) in the *cnn* mutant, which is consistent with the Baz localization at the AJs in the pupal eyes (Nam and Choi, 2003; Nam et al., 2007). During the extensive morphogenetic rearrangement of the photoreceptors, the absence of Cnn causes the mispositioning and/or defects of the localization of acetylated microtubules. Furthermore, this mispositioned acetylated microtubule recruits the ectopic localization of Baz which causes the mislocalization of Crb and AJ/E-cad. These mutant phenotypes of *cnn* strongly suggest that Cnn is dispensable in early eye pattern formation, but is required for the regular localizations of Crb, Baz/AJ, and acetylated tubulins during photoreceptor morphogenesis in the pupal eyes.

Overexpression of Cnn Causes Apical Domain Expansion in Pupal Photoreceptors

The loss-of-function analysis of the *cnn* mutation (Figure 20) strongly suggests that *cnn* might affect AJ/Baz positioning and the apical membrane domain (Crb) in photoreceptor morphogenesis. Next I conducted a gain-of-function analysis of *cnn* using eye-specific GAL4 lines, *GMR-GAL4* (Freeman, 1996), to increase the Cnn

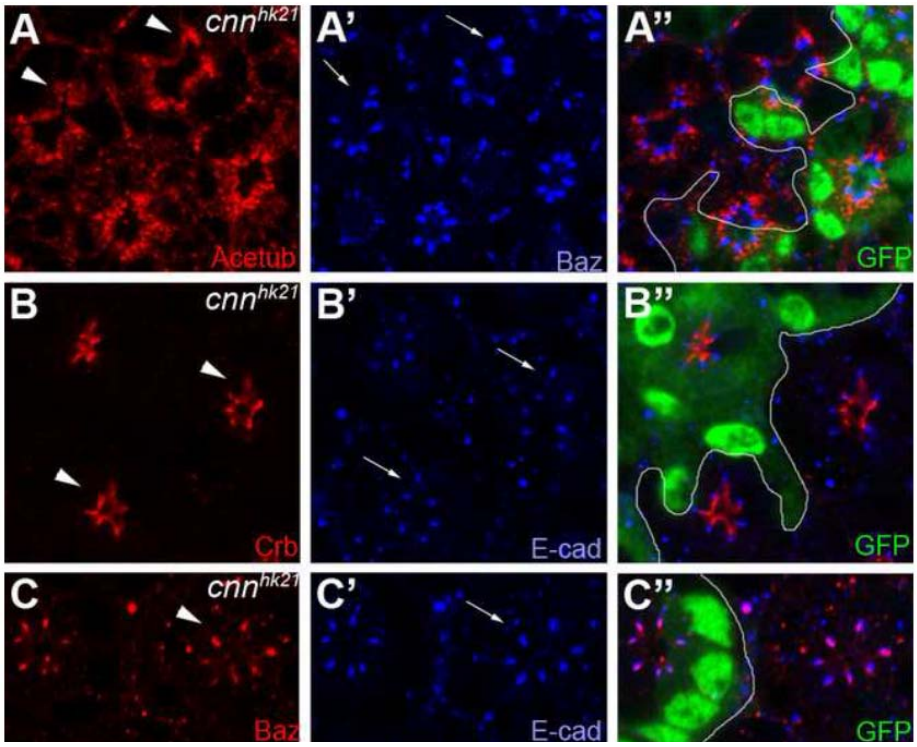

Figure 20: Cnn is essential for photoreceptor morphogenesis in the mid-stage developing pupal eyes. (A, B) Pupal eyes (50% pupal developmental stage) with cnn^{hk21} null mutant clones marked by the absence of the GFP (green). (A) Acetylated tubulin (Acetub, red, A) and Baz (blue, A') were mislocalized from apical to basal in the absence of the Cnn (absence of the GFP, A''). Ectopic mispositioned acetylated tubulins (A) and Baz (A') are indicated by arrowheads (A) and arrows (A'), respectively. (B) Crb (red, arrowheads, B) and E-cad (blue, arrows, B') were mislocalized from apical to basal in the absence of the Cnn (absence of the GFP, B''). (C) Baz (red, arrowhead, C) and E-cad (blue, arrow, C') were co-mislocalized from apical to basal in the absence of the Cnn (absence of the GFP, B').

level in the photoreceptors. I employed the established *UAS-GFP-Cnn* (Megraw et al., 2002) to examine the effects of Cnn overexpression for photoreceptor morphogenesis. Cnn overexpression in the mid-stage (50%) pupal photoreceptors dramatically expanded the apical membrane domains in an apical to basal fashion (300±100% expansion, n=100, Figure 21B), with concurrent mispositionings of Baz (Figure 21B') from the apical center of the photoreceptor. Similarly, the AJ marker E-cad showed the same pattern as Baz (data not shown) in the case of Cnn

overexpression. In rare occasions of Cnn overexpression (<5%), the Crb domain expansion was so dramatic that the apical Crb domains were completely removed from the apical position and reallocated to a much more basal location (Figure 21C). Although the mislocalization of Crb and Baz/E-cad was dramatic, there were no defects in cell polarity since the Crb apical marker still localized more apically compared to Baz/AJ (Figure 21B and 21C). Interestingly, it was noticed that the ectopically positioned Baz always recruits Crb at its apical side (Figures 21B and 21C, arrows).

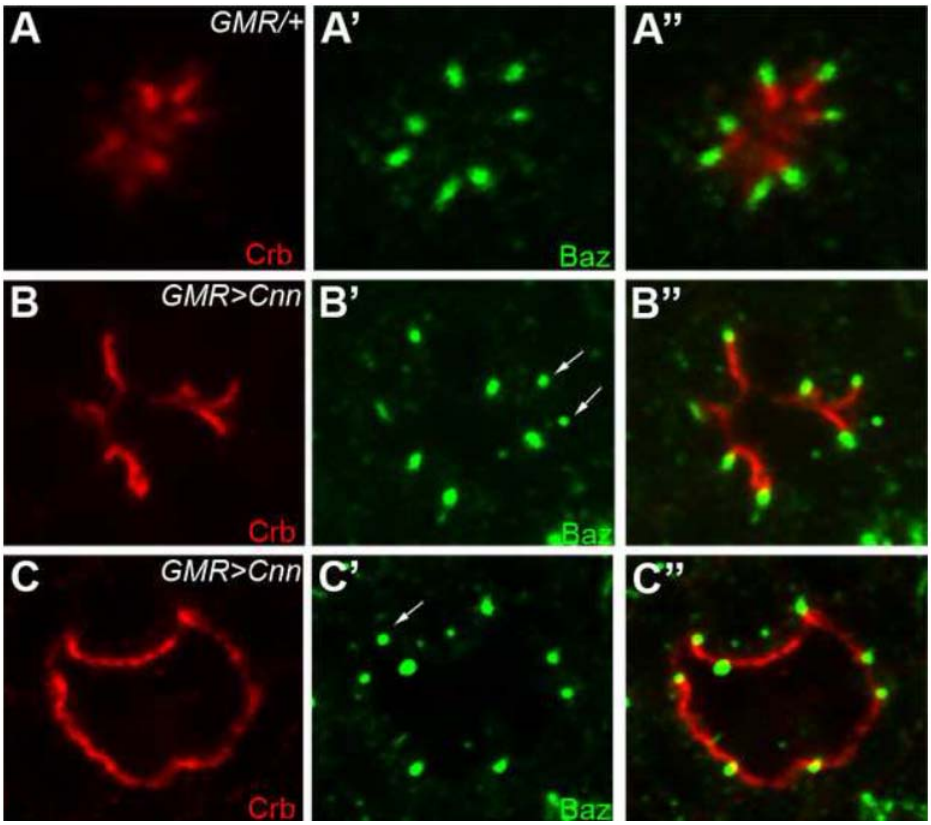

Figure 21: Overexpression of Cnn causes the apical domain expansions. Pupal eyes (50% pupal developmental stage) with Cnn overexpression driven by *GMR-GAL4* at 29˚C were examined by Crb (green, apical domain marker), and Baz (green). (A)control, *GMR-GAL4/+*. (B, C) *GMR-GAL4/UAS-GFP-Cnn*. The expanded Crb domain (red) and Baz (green) were caused by the Cnn overexpression (B). The ectopic localizations of Baz are indicated by arrows (B', C').

CHAPTER FOUR

Conclusions and Discussion

This chapter published as: Chen TW, Chen G, Funkhouser LJ, Nam SC (2009) Membrane domain modulation by Spectrins in *Drosophila* photoreceptor morphogenesis. Genesis 47: 744-750.

Chen G, League GP, Nam SC (2010) Role of spastin in apical domain control along the rhabdomere elongation in *Drosophila* photoreceptor. PLoS One 5: e9480.

Chen G, Rogers AK, League GP, Nam SC (2011) Genetic interaction of Centrosomin and Bazooka in apical domain regulation in *Drosophila* photoreceptor. PLoS ONE 6(1): e16127.

In this dissertation, I examined the role of Spectrins, Spastin ad Cnn in apical basal cell polarity in *Drosophila* photoreceptor morphogenesis. The results strongly indicated that all these three cell skeleton proteins play important functions in regulation of apical cell membranes and AJs of photoreceptors.

1) Spectrins

Spectrins interact with actin to form a supportive network at the intracellular side of the plasma membrane. In *Drosophila* there are one *α-Spectrin* gene and two *β-Spectrin* genes and $(\alpha–\beta)_2$ tetramers are restricted to the basolateral membrane, whereas $(\alpha–\beta_H)_2$ tetramers localize to the apical membrane and the AJ. During the third instar laval stage when the epithelial cells of eye disc initially develops to photoreceptors, loss of Spectrins didn't show any obvious defects. These data showed that Spectrins are dispensable for the establishment of cell polarity and the initial targeting of cell polarity proteins.

However, at the mid pupal stage, loss of α and β-spectin resulted in severe defects in the photoreceptor. Both apical domains and AJs expanded from apical to basal, which indicate $(\alpha–\beta)_2$ complex play an essential role in later maintenance and of proper cell membrane domains. These results are consistent with the phenotype in other polarity mutants. For example, the Crb complex proteins are also dispensable during laval stage but play an essential role in the later photoreceptor morphogenesis (Izaddoost et al., 2002; Nam and Choi, 2003, 2006). Compared to $(\alpha–\beta)_2$ complex, $(\alpha–\beta_H)_2$ complex seems to be dispensable for the photoreceptor development in the pupal stage. Previous studies revealed that the apical Spectrins are involved in rhabdomere stalk length regulation in later stages (Pellikka et al., 2002).

Previous studies suggested that the protein complexes which localize to the apical membrane and basolateral membrane may act antagonistically and thus could be involved in cell domains identity (Bilder et al., 2003; Tanentzapf and Tepass, 2003). I found there is a similar antagonistic relationship between apical Karst and basolateral β-Spectrin (Figures 11 and 12). Reduction of basolateral β-Spectrin caused the expansion of apical Karst (Figure 12) and an increase in β-Spectrin caused the reduction of apical domains (Figure 11). These data suggest that spectrins actively participate to modulate or maintain the distinct membrane domains.

As Spectrins are important components of cytoskeleton backbone, firstly I assumed that the enhanced rough eye phenotype resulted by spectrins mutants in the Crb overexpression background is caused by general damage to the cytoskeleton.

However, further studies of Spectrins seem not to support this explanation. On one hand, mutation of spectrins only affect late-stage pupal development, not early eye patterning. On the other hand, overexpression of β-Spectrins showed severe reduction of the apical domain. These observations suggested that Spectrins may have specific functions in modulation or maintenance of photoreceptor cell domains.

2) Spastin

In animal photoreceptor cells, the surface membrane is enlarged for the storage of opsin photopigment. Insect eyes use an actin-based structure for surface membrane enlargement, but mammalian eyes use microtubule-based structure. I examined whether the *Drosophila* photoreceptor cells may have any microtubule-based structures. There is a distinguished structure which is specifically labeled by anti-acetylated- tubulin antibody in the developing photoreceptors of *Drosophila*. Given the specific localization of stable microtubules in developing pupal *Drosophila* photoreceptors, these subcellular structures might provide a functional role for photoreceptor morphogenesis.

Spastin is an ATPase that binds microtubules and localizes to the spindle pole and distal axon in mammalian cell lines. Furthermore, its *Drosophila* homolog, *Drosophila spastin*, has recently been shown to regulate microtubule stability and synaptic function at the *Drosophila* larval neuromuscular junction (Sherwood et al., 2004; Trotta et al., 2004). My genetic analysis of the spastin mutation strongly indicates that spastin not only modulates the microtubules, but also modulates the apical Crumbs membrane domain during rhabdomere elongation (Figure 16). The

apical membrane modulation activity of spastin was further confirmed by the obversion that spastin overexpression which caused a dramatic expansion of the apical membrane domain.

Based on the highly concentrated localization of Spastin and Kin-lacZ (a plus-ending marker of microtubules) in the apical domains of the photoreceptors (Figure 14 and 15), I propose that the apically localized Spastin might control the apical Crumbs domains. This apical domain-specific function of Spastin is based on the following results; (i) Apical domain localization of Spastin (Figure 15), (ii) Loss of spastin causes apical domain defects (Figure 16), and (iii) Overexpression of spastin causes apical domain expansions (Figure 17).

Spastin has microtubule-severing activities *in vitro*. Therefore, microtubule-severing activity of Spastin may facilitate the apical Crumbs domain, since loss of spastin caused loss of Crumbs, and gain of spastin caused the expansion of the Crumbs domain. Furthermore, this facilitating activity of Spastin for the apical domain could be independent from the main stable microtubule structures which are located far beneath the apical domains (Figure 13D). This possibility is supported by the more direct influence on apical Crumbs, as the stable microtubules were not dramatically changed, relatively speaking, by either spastin mutants or spastin overexpression (Figure 16 and 17).

During the massive growth of the rhabdomeres in the pupal retina, many membrane materials, including Crumbs, will be targeted into the growing apical membranes. Spastin may participate in this material transport to the apical

membrane domain during rhabdomere growth, although the initial targeting is spastin-independent. The outcome of this study will provide useful information for understanding the molecular genetics mechanism of spastic paraplegia. Although the spastin mutation subtly affects the main microtubules, this genetic approach will provide more convincing clues concerning the microtubule-based processes in photoreceptor morphogenesis.

Thus, analysis of the microtubule-modulating Spastin in *Drosophila* photoreceptors may provide important insights into the understanding of the functional basis of the microtubule-based structure and the microtubule-related genes involved the formation and development of photoreceptors. Evolutionary conservation in the structure and function of eye morphogenesis genes makes the *Drosophila* eye an excellent model for studying the genetic and molecular basis of retinal cell organization. My studies suggest there is a microtubule-based structure in insect photoreceptors which was assumed to be entirely composed of an actin-based cytoskeleton. This knowledge may broaden the usefulness of *Drosophila* photoreceptors as a model system for microtubule-related photoreceptor development and disease.

In summary, I examined the role of Spastin in the regulation of the apical Crumbs domain in developing photoreceptors. My data strongly suggests that Spastin plays important functions in the modulation of cell membrane domains including the apical domains of photoreceptors during pupal eye development. Because proper maintenance of the apical Crumbs domain is important for the

massive growth observed in rhabdomeres at the apical region of photoreceptor cells, malfunction of spastin results in severe defects in the formation of functional photoreceptors.

3) Centrosomin

Here I examined Cnn as a potential microtubule organizer and/or modulator in the developing pupal photoreceptors. Based on the results in which (1) Cnn was identified as a genetic interaction partner of Baz, (2) Cnn localized at the basal side of the acetylated microtubules, (3) acetylated microtubules were defective in the *cnn* mutant, and (4) Baz was mislocalized in the absence of Cnn, I propose that Cnn may contribute to the organization of microtubule arrays which might in turn contribute to Baz localization, rather than mitotic cell division, in post-mitotic and fully differentiated photoreceptor cells in the pupal eyes.

I investigated where Cnn localizes compared to other cell polarity markers, including the Crb and Par complexes, in mid-stage pupal photoreceptors. The localization results of Cnn and γ-Tubulin in pupal eyes strongly indicate that Cnn and γ-Tubulin localize at a perinuclear position, as well as adjacent to the basal side of the acetylated microtubules (Figure 18).

Cnn has a microtubule organizing activity. Therefore, the expected results in the loss of *cnn* are the loss of acetylated microtubules in the pupal eyes. However, most of the acetylated microtubules were still present, although they were mislocalized and mispositioned from apical to basal locations in the *cnn* mutants (Figure 19A). One potential possibility is that non-centrosomal and

non-Cnn-dependent-MTOC is not affected in *cnn* mutants. However, my results (Figure 19) strongly suggest that the presence of the Cnn-independent MTOCs are not sufficient to preserve the acetylated microtubules in their correct location and that Cnn is thus essential in the proper Crb/Baz localization in the pupal photoreceptors.

Baz defects might not be caused by the defect of Baz membrane targeting because (1) there were no early eye defects in the larval or early pupal stage, (2) there were no cell polarity defects, and (3) only the Crb/Baz/AJ domains were defected. Therefore the *cnn* mutant defects might be the apical membrane domain controls which may be regulated by the Baz-dependent apical targeting of Par-6/aPKC or Crb/Sdt/Patj. This hypothesis is further supported by the gain-of-function of Cnn which shows a dramatic expansion of Crb (apical domain). Further research is needed to answer these important questions.

Since Cnn functions in many diverse cellular processes, it was assumed to be impossible to examine a specific developmental defect in the photoreceptor. However, analysis of the *cnn* mutation in the photoreceptor showed defects that are quite specific in the pupal photoreceptors based on (1) a lack of defects in early eye pattern formation during the larval stage, and (2) an absence of defects detected in eye accessory cells of pigment cells and cone cells in the pupal stage eyes (data now shown).

Here I identified the structural and functional roles of Cnn in photoreceptor morphogenesis and polarity. I have characterized the centrosome in the developing photoreceptors and examined its roles in *Drosophila* photoreceptor morphogenesis.

Understanding the genetic basis for photoreceptor organ development is important for finding cures for retinal degeneration caused by genetic defects. Because of the conservation of cell polarity genes and Cnn in higher mammals including humans, similar cooperative mechanism between Baz and Cnn could have a role in the development and degeneration of the human photoreceptor.

Future Research

The establishment of cell polarity in eukaryotic cells is a complicated and continual process that involves in many signaling pathways and a variety of cell polarity proteins. To get a clear and exact model of the underlying mechanism by which cell polarity is established, it is still a long way to go. Future work should focus on finding more players in establishment and maintenance of cell polarity and indentify the biochemical and genetic interactions between each other.

Because of the existence of stable microtubules in *Drosophila* photoreceptor and its obvious functional role in material transportation and cell domain maintenance, the most possible player in cell polarity network will be the proteins related to the regulation of microtubules, including microtubules associated proteins (MAPs) and microtubules based motors. Thus the short term studies should examine the roles of these proteins. In the long term studies, the relationship between stable microtubules structures and the known cell polarity proteins, especially these important protein complexes including Crb complex and Par complex, should be further studied to clarity the roles of stable microtubules in photoreceptors, which will facilitate the research on *Drosophila* photoreceptor morphogenesis.

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