ABSTRACT

The Role of Spectraplakin in *Drosophila* Photoreceptor Morphogenesis

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Cell polarity, the correct positioning of membrane proteins in the apical and basolateral domains, is critical for the proper development of retinal photoreceptor cells. The regulation of cell polarity is controlled by cell polarity complexes. Crumbs (Crb), a transmembrane protein belonging to the Crb complex, has a critical role in the regulation of the rhabdomeres and adherens junctions during photoreceptor cell elongation. In turn, the cell polarity complexes are influenced by other regulators. Here, I found that Spectraplakin, an actin-microtubule cross-linking protein, participates in the regulation of the localization of Crb during photoreceptor morphogenesis. The Spectraplakin genes are highly conserved throughout evolution, which makes the *Drosophila* eye an excellent model. The Spectraplakin gene in *Drosophila* is known as *short stop*, *shot*. Shot localizes at the rhabdomere terminal web, located at the Crb domain, and serves as a transition zone for the constant delivery of proteins needed for the photoreceptor. Genetic analysis data indicates that Shot and Crb require each other reciprocally for correct targeting to the final sites within photoreceptor cells. Mutations in *shot* affect photoreceptor morphogenesis by causing cell polarity defects.
THE ROLE OF SPECTRAPLAKIN IN DROSOPHILA PHOTORECEPTOR MORPHOGENESIS

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DEDICATION

I would like to dedicate this thesis to my parents for their continuous encouragement and guidance in my academic pursuits, to my sister for her support and advice throughout my life, and to Dr. Sang-Chul Nam for his supervision and faith in me during this honors thesis project and his support in my future career.
CHAPTER ONE

Introduction

The importance of epithelial cell polarity in photoreceptor development and its implications in retinal diseases

The terminal differentiation of a cell into a specific cell type with specialized functions depends on which genes are expressed within the cell. The expressed genes serve as a blueprint for the production of proteins, which are the workhorses of the cell. The developmental course of a cell depends on the type of proteins found within it and where the proteins are located. One method by which a cell differentiates into its final form is through the establishment of polarity within the cell. Cell polarity results from the asymmetric distribution of organelles and proteins in the cytoplasm. This apical-basal cell polarity is important for epithelial morphogenesis and cell fate specification, which determine the final shape of a particular cell or organ.

A structure in which cell polarity exhibits its importance is the retinal photoreceptors of the eye. In humans, a loss of polarity in the photoreceptor cells as a result of genetic mutations can lead to several retinal diseases, such as retinitis pigmentosa (den Hollander et al., 1999) and Leber Congenital Amaurosis (den Hollander et al., 2001). Retinitis pigmentosa initially causes night blindness followed by tunnel vision and progressive visual loss due to the degeneration of photoreceptor cells in the retina (den Hollander et al., 1999). Leber Congenital Amaurosis is characterized by sluggish pupillary response and severe vision loss due to abnormal development of photoreceptor cells (den Hollander et al., 2001). There is currently no treatment for
retinitis pigmentosa but some forms of Leber Congenital Amaurosis can be treated by
gene replacement therapy (den Hollander et al., 2001).

Mutations in the CRB1 gene are present in 4% of retinitis pigmentosa cases
(Bernal et al., 2003; den Hollander et al., 2004) and 10-15% of Leber Congenital
Amaurosis cases (den Hollander et al., 2001; Lotery et al., 2001). CRB1, a cell polarity
gene, localizes to the inner segment (analogous to the rhabdomere stalk) between the
outer segment (analogous to the rhabdomere) and the adherens junction of
photoreceptors. It is required for the formation of the apical domain and adherens
junctions during photoreceptor morphogenesis (reviewed in Richard et al., 2006). The
CRB genes are highly conserved across multiple organisms, and most mammalian cell
polarity genes that contribute to photoreceptor morphogenesis have orthologs in other
organisms (reviewed in Richard et al., 2006). Thus, we can relate the mechanisms
governing the development of polarity in photoreceptor cells in a genetic tractable model
organism to those in humans by studying gene homologs and orthologs. In this study, the
Drosophila eye is used as a model to study how Spectraplakin regulates the apical Crb
domain and adherens junction and affects the development of photoreceptor cells.

Morphogenesis of Drosophila photoreceptor

The compound eye of Drosophila is composed of approximately 770 ommatidia
with each ommatidium consisting of eight photoreceptor cells (R1-R8), which are thin
elongated cells formed in the eye imaginal disc during the third instar larval stage. The
outer photoreceptors (R1-R6) are radially arranged around the inner R7 and R8
photoreceptors, which are stacked on top of each other in the center. The plasma
membrane of each photoreceptor cell is divided into a photosensitive region (the
rhabdomere) and a photo-insensitive region (the basal membrane) (Leung et al., 2010). The rhabdomere localizes to the apical region and contains numerous rhodopsins and microvilli that capture photons and transduce them into visual responses, similar to the function of the vertebrate rods and cones. Surrounding the rhabdomere is the apical domain and adherens junction, and the basolateral domain is the outermost layer (Figure 1). The protein E-cadherin (E-cad) is concentrated at the adherens junction and is important for cell adhesion. The acetylated tubulin (stabilized microtubule) localizes between the adherens junction and the basolateral membrane and functions in maintaining cell shape. Both of these markers are essential in maintaining cell polarity.

![Diagram](image)

**Figure 1: Morphogenesis of Drosophila pupal photoreceptor**

(A) Side view of a photoreceptor at mid-stage pupal eyes. The photoreceptor elongates from distal to proximal in the direction of the arrow. (B) Cross-section of mid-stage pupal eyes photoreceptor. The rhabdomere (light blue) localizes in the center. The apical domain (green) localizes basal to the rhabdomere. The E-cad localizes at the adherens junction (red), which is basal to the apical domain. The basolateral domain (black) is located basally to the adherens junction. The acetylated tubulin (blue) localizes outside of the adherens junction.
Figure 2: Developmental Stages of Drosophila photoreceptor

(A) In the third instar larval stage, the apical domains face the retinal surface. (B) At 37% pd, the photoreceptor cells rotate 90° causing the apical domains to face each other in the center. The cells begin to elongate from distal to proximal. (C) At 67% pd, the rhabdomeres develop from the apical domains and elongate proximally until they reach the retinal floor. (adapted from Izaddoost et al. 2002)

During the third instar larval stage, the apical domains of the photoreceptors face the retinal surface (Figure 2A). At 37% pupal development (pd), the photoreceptor cells rotate by 90°, which reorient the apical domains toward the center and the basolateral domains on the outside of the ommatidium. During the involution, the adherens junctions located between the photoreceptor cells are maintained. The photoreceptor cells then begin to elongate from distal to proximal until the adherens junctions reach the proximal base of the retina and anchor the cells in place (Figure 2B). At 67% pupal development (pd), the apical domains give rise to the rhabdomeres and the supporting stalks, both of which then undergo elongation in the direction parallel to the adherens junctions until they reach the retinal floor. Later in the development of the pupal photoreceptor, the rhabdomeres take on a more elliptical shape and the microvilli elongate and vary in
length. The outer rhabdomeres withdraw from the center of the cluster, resulting in the
closer resemblance of the photoreceptors to their adult form (Figure 2C; Longley and
Ready, 1995).

**Three protein complexes that contribute to Drosophila photoreceptor cell polarity**

Among the many protein complexes that contribute to the formation of cell
polarity in *Drosophila* photoreceptors, three particularly important complexes have been
identified. These are the Crumbs (Crb) complex of Crb, Stardust (Sdt), and Patj (protein
associated with tight junctions), the Par complex of Bazooka (Baz), Par-6, and an atypical
protein kinase C (aPKC), and the Scrib complex of Scribble (Scrib), Discs-large (Dlgs),
and Lethal giant larvae (Lgl).

![Figure 3: Localization of cell polarity protein complexes in photoreceptors](image)

*Schematic view of longitudinal (left) and tangential (right) sections of a photoreceptor
cluster during 40-45% pd. The Crb complex, together with Par-6 and aPKC, localize at
the apical domain (green) while Baz of the Par complex localizes at the adherens junction
(red). Due to antagonism with the Par complex, the Scrib complex localizes at the
basolateral domain (blue). These protein complexes contribute to the formation and
maintenance of apical and basolateral identity and cell polarity.*

The Crumbs (Crb) complex along with the Par complex, with the exception of
Baz, colocalize to the apical membrane of the photoreceptor cell, specifically the
rhabdomere stalk (Figure 3). The interaction between the two complexes is crucial for epithelial cell polarity. Crb is an apical transmembrane protein that provides a positional cue for the formation and growth of the rhabdomeres and adherens junctions during the elongation of the photoreceptor along the proximodistal axis of the retina. The intracellular domain of Crb interacts with Sdt and Discs-large (Dlt) to ensure the proper assembly of the adherens junction (Izaddoost et al., 2002; Médina et al., 2002). The extracellular domain is also essential for the function of photoreceptors but no known proteins interacting with it have been identified yet (Lemmers et al., 2004). Within the Par complex, only Par-6 and aPKC localize to the rhabdomere stalk while Baz localizes to the adherens junction (Nam and Choi, 2003). Nevertheless, Baz is essential for targeting the Crb and Par complex proteins to the apical domain. The Scrib complex, on the other hand, antagonizes with the Par complex and is pushed toward the other end of the photoreceptor cell, the basolateral membrane (Bilder et al., 2003). These three protein complexes establish a degree of polarity within the cell but there are other structures, such as microtubule cytoskeletons, that also play crucial roles in determining cell polarity and morphogenesis.

Spectraplakin’s role in the regulations of apical Crb domain in developing Drosophila photoreceptors

Microtubule cytoskeletons are important structures in determining cell shape, cell polarity, and vesicle trafficking. Thus, microtubule reorganization during development is essential for morphogenesis. The presence of stable microtubules in developing Drosophila pupal photoreceptors has been found and they are associated with Crb localization. Spastin, a microtubule-severing ATPase involved in assembling microtubule
arrays, has been shown to help regulate the apical localization of Crb (Chen et al., 2010). It has also been found that Kinesin-1 and Kinesin-2, the microtubule-based motors, participate in Crb localization in developing photoreceptors (Mukhopadhyay et al., 2010). Consequently, Spectraplakin, an actin-microtubule cross-linker, might be involved in the regulation of stable microtubules and apical Crb domain during photoreceptor morphogenesis.

Spectraplakins are a family of giant cytoskeletal cross-linking proteins that have been highly conserved throughout animal evolution. The *spectraplakin* genes in mammals are *BPAG1* and *MACF1* and in *Drosophila* is *short stop* (*shot*). Spectraplakin proteins share characteristics of both the spectrin and plakin superfamilies. Proteins of the spectrin superfamilies contain an N-terminal actin-binding domain and thus are able to cross-link actin filaments. Members of the plakin superfamilies contain a plakin domain at the N-terminus that contains sites for binding to adhesion receptors and intermediate filaments. Proteins of the plakin superfamilies can also cross-link other cytoskeletal components, including microtubules. Spectraplakins can bind both actin and microtubules because of their dual nature (Katja et al., 2002).

Several main domains exist within the *spectraplakin* gene from N- to C-terminus: one or two calponin-homology domains, a plakin domain, a plectin-repeat domain, a spectrin-repeat domain, and a GAS2 domain. The calponin-homology domains at the amino-terminal, when present in two tandem copies, are able to bind actin filaments whereas the GAS2 domain at the carboxy-terminal binds microtubules (Katja et al., 2002). This organization suggests that spectraplakin acts as an actin-microtubule cross-linker.
The possibility of microtubule-binding activity in spectraplakins is elucidated by the colocalization of shot and microtubules. Furthermore, microtubule bundles are detached from the plasma membrane in shot mutant embryos (Gregory and Brown, 1998) and deletion of the GAS2 domain results in loss of microtubule binding (Katja et al., 2002).

Spectraplakin exhibits at least four known functions (Figure 4). The first function is crosslinking cytoskeletal components, such as actin and microtubules. This is not a common function among cytoskeletal proteins and is thus a key feature of spectraplakin. The second function is linking the cytoskeleton to plasma membrane proteins. Most researches have been focused on these two functions, but spectraplakin has two other potential functions that are associated with maintaining the integrity of a cell. The last two functions are organizing the interaction between the cytoskeleton and plasma membrane proteins to produce membrane subdomains and acting as scaffold proteins that recruit signaling proteins to sites of cytoskeletal activity (Katja et al., 2002). This thesis focuses on the role of spectraplakin as a cytoskeletal cross-linker in Drosophila photoreceptor development and the genetic interaction between spectraplakin/shot and crb.

Shot interacts with microtubules via two protein domains. In the cell interior, the carboxy-terminal of shot binds the growing microtubule plus end through an interaction with EB1, a protein that localizes to the plus end of the microtubule. In the actin-rich cell periphery, shot interacts with the microtubule lattice by means of its GAS2 domain. This interaction also requires the actin-binding calponin-homology domains, suggesting that shot cross-links actin and microtubules (Applewhite et al., 2010). The coordinated
interactions between actin and microtubules are involved in a variety of cellular processes, including cell migration, mitosis, wound healing, cortical flow, and tissue morphogenesis (Rodriguez et al., 2003). Stabilized microtubules are linked to Crb localization and since spectraplakin can bind both microtubule and actin, it may have a role in regulating Crb localization in developing Drosophila photoreceptors.

![Figure 4: Functions of spectraplakins](image)

There are at least four known functions of spectraplakins: (1) crosslinking cytoskeletal elements, (2) joining the cytoskeleton to plasma membrane proteins, (3) organizing the interaction between the cytoskeleton and plasma membrane proteins to create membrane subdomains, and (4) acting as scaffold proteins that recruit signaling proteins to sites of cytoskeletal activity (Katja et al., 2002).
Hypothesis:

The purpose of my research is to determine the localization of Shot, its genetic interaction with *crb*, and its role in *Drosophila* pupal photoreceptor morphogenesis. Actin-microtubule connections contribute to the proper functioning of cell adhesion, which is the role of the adherens junction. Therefore, I hypothesize that Shot localizes to the adherens junction where it crosslinks actin and microtubules. Stable microtubules have been shown to affect the localization of Crb. Since Shot is able to bind microtubules, it is highly possible that *shot* and *crb* act together to control the formation of apicobasal polarity, which is essential for the normal development of photoreceptor cells. Mutations in *shot* should produce dramatic mislocalization of Crb and stable microtubules. Since Crb is necessary for the proper positioning of the apical membrane domain and adherens junction during photoreceptor morphogenesis, the mislocalization of Crb should cause them to mislocalize as well and result in deformed eyes. If *shot* is determined to be able to regulate Crb localization, then the reverse case of whether *crb* is involved in Shot localization will also be examined.
CHAPTER TWO
Materials and Methods

Genetics

_Drosophila_ strains were grown at room temperature via mitotic recombination, which was induced by using the FLP/FRT method for clonal analysis (Xu and Rubin, 1993). _shot^3_ is a null allele of _shot_ that lacks detectable Shot protein (Lee et al., 2000) and has been completely rescued by the UAS-ShotA transgene (Lee and Kolodziej, 2002). Mutant clones of _shot^3_ were produced by eye-specific expression of ey-Flp (Newsome et al., 2000) or GMR-flp (Lee et al., 2001) in _y w ey-Flp_ or _GMR-flp/+; FRT42D shot^3 / FRT42D Ubi-GFP_, or _y w ey-Flp/+; FRTG13 shot^3 / FRTG13 Ubi-GFP_. _crb^{1/A22}_ is a null allele of _crb_ (Xu and Rubin, 1993). Mutant clones of _crb^{1/A22}_ were produced in _y w ey-Flp/+; FRT82D crb^{1/A22} / FRT82D Ubi-GFP_. Overexpression of _shot_ was induced by crossing UAS-ShotA-GFP or UAS-ShotC-GFP (Lee and Kolodziej, 2002) with GMR-GAL4 (Freeman 1996) at room temperature.

Reagents and Equipment

PBS (1X)

Fix Solution (4% PF/PBS fixative)

Blocking Solution (50 mM Tris pH 6.8; 150 mM NaCl; 0.5% NP40; 5 mg/ml BSA)

Wash Solution (50 mM Tris pH 6.8; 150 mM NaCl; 0.5% NP40; 1 mg/ml BSA)

Mounting Medium
Dissecting Tweezers
Nine-depression glass spot plates
Cover slips
Glass slides
Clear nail polish
Shaking Platform
Dissecting microscope
Confocal Microscope (Olympus FV1000)

*Primary antibodies*

Mouse anti-acetylated tubulin (Sigma), 1:1000
Rabbit anti-α tubulin (Abcam), 1:200
Rat anti-E-cadherin (Dcad2, DSHB), 1:20
Mouse anti-Shot (Rod1, DSHB), 1:20
Rat anti-Crb 1:400
Sheep anti-GFP (Biogenesis), 1:100
Rabbit anti-aPKCζ (Santa Cruz), 1:500

*Secondary antibodies*

Donkey anti-mouse Cy3 (Jackson ImmunoResearch), 1:50
Donkey anti-sheep FITC (Jackson ImmunoResearch), 1:50
Donkey anti-rabbit FITC (Jackson ImmunoResearch), 1:50
Donkey anti-rat Cy5 (Jackson ImmunoResearch), 1:50
**Dissecting procedure**

*Drosophila* pupae were dissected in 1X PBS solution in a nine-depression glass spot plate by using one tweezer to anchor the pupa in place and the other tweezer to remove the head portion of the pupal case. After removing the anterior part of the pupal case, the anterior inner membrane was perforated. The organs of the fly were gently squeezed out through the hole with a tweezer. The retinas, which were clear and attached to the brains, were collected and transferred to another nine-depression glass spot plate for fixation and staining.

**Immunostaining:**

Pupal retinas were fixed for 15 minutes in 200-250 μL of 4% PF/PBS fix solution on a shaking platform at room temperature. The fixative was then removed and 200-250 μL of a block solution was added and shaken for 5 minutes. Next, the block solution was replaced by a mixture of the primary antibodies at the desired concentrations diluted with a wash solution. The retinas were then incubated for either 4 hours at room temperature or overnight at 4° C. Following the first incubation period, the primary antibodies were removed and the retinas were washed three times with a wash solution and gently shaken at room temperature for 5 minutes each time. Afterward, the retinas were incubated again with the fluorescent secondary antibodies diluted with a wash solution. Following the second incubation period, the retinas were again washed three times with a wash solution while shaking for 5 minutes each time. Subsequently, they were fixed for 15 minutes in 200-250 μL of 4% PF/PBS fix solution while shaking. The fixative was replaced by 1X PBS.
The retinas were transferred onto a glass slide for mounting in 1X PBS solution. Any 1X PBS solutions transferred onto the slide along with the retinas were absorbed using a paper towel. One drop of the mounting solution was added to the retinas on the glass slide, which were then secured with a glass cover slip. To prevent movement of the cover slip, clear nail polish was applied to the sides of the cover slip. Slides were stored at 4°C.

**Imaging:**

Immunofluorescent images of the fly retinas were obtained using the Olympus FV1000 confocal microscope equipped with a 60x oil-immersion objective lens (Plan-Aprochromat, NA 1.42, WD 0.15 mm). The images were analyzed and processed using Image J and Adobe® Photoshop®, respectively.
CHAPTER THREE

Results

Genetic interaction between crb and spectraplakin/shot in Drosophila photoreceptors

The conserved Crb intracellular domain (Crbi\textsuperscript{intra}) (Klebes and Knust, 2000) is overexpressed using GMR-Gal4 (Freeman 1996), which causes the roughening of the eye’s external morphology (Figure 5C). A genetic screen is performed to identify other genes that function with Crb to regulate photoreceptor morphogenesis. When the level of shot protein in the shot\textsuperscript{3}/+ heterozygous background is reduced, the rough eye phenotype of GMR>Crbi\textsuperscript{intra} is dominantly enhanced (Figure 5D) and consistent with 100% penetrance (n>100). This data indicates a strong genetic interaction between crb and shot. Therefore, Shot might act as an additional positional cue for Crb-dependent photoreceptor development.

Figure 5: Genetic interaction between crb and shot in Drosophila photoreceptors
(A) Adult eye phenotype of a wild-type fly. (B) Abnormal eye phenotype of a shot\textsuperscript{3} mutant clone. (C) Rough eye phenotype of a wild-type fly in which Crbi\textsuperscript{intra} is overexpressed. (D) Enhanced rough eye phenotype of GMR>Crbi\textsuperscript{intra} due to reduced shot expression in the shot\textsuperscript{3}/+ heterozygote.
Figure 6: Localization of Shot in Drosophila pupal photoreceptors
(A and B) Shot (red) localizes between E-cad (blue), at the basal side of the aPKC (apical marker, green), and at the apical side of the microtubules (B, green). (C) Shot localizes at the basal side of phalloidin (rhabdomere marker). (D-F) Schematic representation of mid-pupal photoreceptor. Shot (orange) localizes between adherens junction (red), at the basal side of the apical Crb domain (green), at the apical side of the stable microtubule (blue), and at the basal side of the rhabdomere (light blue).

Localization of Spectraplakin/Shot in Drosophila pupal photoreceptors

After finding the genetic interaction between crb and shot, the next step is to determine the localization of Shot in developing wild type photoreceptors. The anti-Shot monoclonal antibody, mAb Rod1, is used to study the localization of Shot in mid-stage developing pupal eyes. Shot has been reported to localize at the adherens junctions in
embryonic and follicular epithelial cells (Roper and Brown, 2003) or with microtubules in oocytes (Roper and Brown, 2004). In this study, the localization of Shot is compared to other subcellular markers of aPKC (apical membrane domain) and E-cadherin (adherens junction) (Nam and Choi, 2003; Nam and Choi 2006; Nam et al., 2007), and cytoskeletal markers of acetylated tubulin (Chen et al., 2009; Chen et al., 2010) and phalloidin (F-actin, rhabdomere) (Cagan and Ready, 1989). In developing pupal photoreceptors, Shot is found to localize between the adherens junctions, at the basal side of the apical Crb domain, and at the apical side of the stabilized microtubules (Figure 6A and 6B). This data suggests that Shot is concentrated at the rhabdomere terminal web (RTW) in developing photoreceptors (Karagiosis and Ready, 2004; Satoh et al., 2005). Based on the localization of Shot, an actin-microtubule cross-linker, the RTW is possibly the region where F-actin and stabilized microtubules meet (Figure 6D-6F). Due to its location and genetic interaction with crb, shot might have a potential role in the localization of Crb and adherens junctions in photoreceptor cells (Figure 6D-6F).

Loss-of-function analysis of spectraplakin/shot in Drosophila pupal photoreceptors

To determine whether Shot is required for photoreceptor morphogenesis in mid-stage pupal, a null mutation of shot, shot\(^3\), is generated using a genetic mosaic technique of FLP/FRT. shot\(^3\) is a null allele that lacks Shot protein and has been completely rescued by the UAS-ShotA transgene (Lee and Kolodziej, 2002). The absence of Shot protein in shot\(^3\) mutants leads to a reduction of the apical Crb domain and the mislocalization from the apical center toward the basolateral areas of the adherens junctions (Figure 7A). In addition, stabilized microtubules are also disrupted (Figure 7B). The loss-of-function analysis of shot strongly suggests that Shot is essential for the maintenance of the apical
Crb domain, adherens junctions, and stabilized microtubules during mid-stage pupal photoreceptor development. In the absence of Shot, photoreceptor cells develop incorrectly leading to abnormal eye phenotypes, such as the rough eye phenotype.

Figure 7: Shot is required for photoreceptor morphogenesis in mid-stage pupal eyes (A and B) Mosaic retinal tissues showing Shot\(^3\) null mutants mark by the absence of GFP (indicates normal photoreceptor cells) and dotted lines (green, A’ and B’). Crb (red, A, arrow) is dramatically diminished, E-cad (blue, A, arrowhead) is mislocalized from the apical center toward the basolateral areas, and stabilized microtubules (Acetub, red) are mislocalized.

Gain-of-function analysis of spectraplakin/shot in Drosophila pupal photoreceptors

Since the loss-of-function analysis of shot demonstrates the importance of shot in maintaining the apical Crb domain, adherens junctions, and stabilized microtubules, shot
might also have a role in the correct positioning of Crb/E-cad/Acetub in photoreceptors.

A gain-of-function analysis of shot is conducted using an eye-specific Gal4 driver, GMR-Gal4, to test this hypothesis. GMR-Gal4 increases shot expression in the photoreceptors by binding to the eye-specific UAS enhancer located on the shot gene.

![Figure 8: Overexpression of Shot causes the mislocalization of the apical domain, adherens junctions, and stabilized microtubules](image)

Pupal eyes (45% pd) with Shot overexpression using GMR-Gal4 are examined by Crb (apical domain marker, red, A-C), E-cad (adherens junction marker, A-E), and acetylated-tubulin (Acetub, stable microtubules marker, red D-E). (A, D) Control, GMR-Gal4/+; (B, E) GMR-Gal4/UAS-ShotA-GFP causes complete cell polarity defect; (C) GMR-Gal4/UAS-ShotC-GFP does not cause any mislocalization.

Shot exists in several isoforms, which are ShotA, ShotB, and ShotC, depending on the alternative RNA splicing of the shot gene (Roper et al., 2002). The Shot isoforms differ only at the amino-terminus while the central and carboxy-termini remain the same. ShotA has an actin-binding domain but ShotC does not because it lacks one calponin
homology domain and two copies of this domain are needed to bind actin (Leung et al., 1999; Lee and Kolodziej, 2002). The overexpression of ShotA using GMR-Gal4 in mid-stage pupal photoreceptors results in the dramatic mislocalized distribution of ShotA-GFP and the mislocalization of Crb and E-cad (Figure 8B). Shot, Crb, and E-cad become tangled when all three proteins are concurrently mislocalized (Figure 8B”). However, the most dramatic mislocalization occurs in the stabilized microtubules. The stabilized microtubules are completely displaced from the apical areas to the basolateral areas (Figure 8E). Thus, the primary target of Shot may be the stabilized microtubules, which may then affect the localization of Crb and E-cad. On the other hand, the localization of Crb and E-cad are not affected in the overexpression of ShotC (Figure 8C). Both ShotA and ShotC localize to the RTW, indicating that the actin-binding domain may not be required for the localization of Shot. However, the ShotA-induced mislocalization of Crb/E-cad/Acetub requires the actin-binding domain since ShotC, which lacks the actin-binding domain, does not cause the mislocalization. These results indicate that the overexpression of ShotA induces complete cell polarity defect.

Crb is required in Shot localization during photoreceptor elongation

The genetic interaction between crb and shot suggests that Shot may provide an additional positional cue for Crb-dependent photoreceptor development (Figure 2). Furthermore, shot mutational analyses indicate Shot’s role in the localization of Crb in photoreceptors (Figure 7 and 8). The effect of Crb on Shot, however, has not yet been determined. Therefore, it is necessary to examine Crb’s role in the localization of Shot in photoreceptors. The allele crb11A22, a null allele of crb that lacks Crb protein expression (Tepass et al., 1990), is generated using a genetic mosaic technique (Xu and Rubin,
1993). It has been reported by Izaddoost et al. (2002) that E-cad is mislocalized from apical to basal in the *crb* null mutants (Figure 9A’, arrowhead). In this study, it is noted that Shot also is mislocalized (Figure 9A, arrow) as much as E-cad, suggesting that Crb is required for the localization of Shot at the “RTW” in the pupal photoreceptors.

*Figure 9: Crb is required for Shot localization in mid-stage pupal eyes* (A) GFP (green, A”) is absent in pupal eyes with *crb*11A22 null mutation. Shot (red, A, arrow) and adherens junction (E-cad, blue, A’, arrowhead) are mislocalized from apical to basal.
The localization of Shot is compared to that of the apical membrane domain, adherens junctions, stabilized microtubules, and rhabdomere in *Drosophila* pupal photoreceptors. Shot is found to localize in between the adherens junctions, at the basal side of the apical domain and rhabdomere, and at the apical side of the stabilized microtubules (Figure 6). This region is located at the end of the rhabdomere and is known as the rhabdomere terminal web (RTW) and may be the interface where the stabilized microtubules and F-actin convene. As an actin-microtubule cross-linker, Shot may join actin and microtubules together at the RTW.

Based on the genetic interaction between *shot* and *crb*, Shot may provide an additional positional cue for Crb in photoreceptor morphogenesis since reduced level of Shot (*shot^3/+*) enhances the rough-eye phenotype caused by the overexpression of *crb* (Figure 5). From comparative genetic analysis, Shot and Crb require each other reciprocally to localize at their target sites of RTW and rhabdomere stalk, respectively. Shot and Crb may be associated in one of the following ways: (i) *shot* acts upstream of *crb*, (ii) *shot* acts downstream of *crb*, or (iii) *shot* and *crb* control the parallel pathway in photoreceptor development.

The mutational analysis of *shot* indicates that Shot alters the localization of the apical membrane domain during rhabdomere elongation. Overexpression of *shot* leads to a dramatic mislocalization of the apical membrane domain (Figure 8). Based on its
capacity to bind both actin and microtubule and its activity as an actin-microtubule cross-
linker, Shot possibly affects actin (rhabdomere) and/or microtubule during photoreceptor
morphogenesis. This postulation is further supported by the absence of Crb
mislocalization in the overexpression of ShotC, which lacks the actin-binding domain
while the overexpression of ShotA, which contains the actin-binding domain, causes Crb
mislocalization.

Although Shot and Crb localize to different positions within the cell, they are able
to communicate with each other in order to regulate photoreceptor development in at
least several possibilities: (i) a potential interaction when they co-localize during the
trafficking before their final targeting, (ii) a potential interaction in previous
developmental time, or (iii) a potential interaction at the interface where the two
subcellular compartments meet. The RTW is where the stabilized microtubules and actin
meet. Consequently, Shot may regulate stabilized microtubules and actin, and thus the
localization of Crb and adherens junctions.

Since Shot participates in the regulation of stabilized microtubules, it is predicted
that the loss of Shot results in defective stabilized microtubules, which is observed in the
loss-of-function analysis of shot mutation (Figure 7B). In addition, the stabilized
microtubules are also defected in the gain-of-function study of ShotA-GFP
overexpression (Figure 8E). Therefore, the stabilized microtubules appear to be the main
target of Shot. The defected microtubules may then affect the activity of Crb and E-cad
through the microtubule-based trafficking (League and Nam, 2011; Mukhopadhyay et al.,
2010) and/or other microtubule-based cell polarity (Siegrist and Doe, 2007). Crb and E-
cad may also be affected through other mechanisms, such as the direct targeting of Shot
toward Crb/E-cad or actin-based cell polarity. *ShotA-GFP* overexpression leads to the mislocalization of Acetub around the cells (Figure 8E), which can be caused directly by the binding of ShotA-GFP to Acetub or indirectly by the mislocalization of the rhabdomere terminal web.

The presented study has shown that Spectraplakin/Shot is essential for the correct targeting of the apical domain, adherens junctions, and stabilized microtubules in *Drosophila* photoreceptors. Mutations in *shot* affect its functions and thus photoreceptor morphogenesis by inducing cell polarity defects. The data strongly indicates that Spectraplakin/Shot plays an important role in the modulation of cell membrane domains, including the apical Crb domains of photoreceptors during pupal eye development. The *Crb and Spectraplakin* genes have a high degree of evolutionary conservation from *Drosophila* to higher mammals, including humans, making them useful for comparative study. Therefore, the development and degeneration of human photoreceptor may be based on similar cooperative mechanism between Crb and Spectraplakin.
REFERENCES


