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Use of Gain-of-Function Study to Delineate the Roles of *crumbs* in *Drosophila* Eye Development

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Key Words
crumbs · Cell polarity · Adherens junction · Differentiation, photoreceptor · Rhabdomere

Abstract
The cell polarity gene, *crumbs (crb)*, has been shown to participate in the development and degeneration of the *Drosophila* retina. Mutations in *CRB1*, the human homologue of *Drosophila crb*, also result in retinitis pigmentosa and Leber congential amaurosis. In this study, we used the gain-of-function approach to delineate the roles of *crb* in developing *Drosophila* eye. In the third-instar larval stage, eye development is initiated with photoreceptor differentiation and positioning of photoreceptor nuclei in the apical cellular compartment of retinal epithelium. In the pupal stage, differentiated photoreceptors begin to form the photosensitive structures, the rhabdomeres, at their apical surface. Using *GMR-Gal4* to drive overexpression of the Crb protein at the third-instar eye disc, we found that differentiation of photoreceptors was disrupted and the nuclei of differentiated photoreceptors failed to occupy the apical compartment. Using *hs-Gal4* to drive Crb overexpression in pupal eyes resulted in interference with extension of the adherens junctions and construction of the rhabdomeres, and these defects were stage-dependent. This gain-of-function study has enabled us to delineate the roles of Crb at selective stages of eye development in *Drosophila*.

Introduction

The *Drosophila* eye develops from a layer of polarized epithelium. Eye development is a progressive process that involves differentiation of photoreceptors, nuclear migration, pattern formation and rhabdomere morphogenesis [30]. Many studies have focused on the identification of signaling molecules in the regulation of eye development [15, 16, 21, 24]. However, there are limited data on the relationships between cell polarity and eye development.

The cell polarity gene, *crumbs (crb)*, is known to play a significant role in establishing cell polarity in ectoderm-derived epithelia. *crb* encodes a single-pass transmembrane protein which is expressed exclusively in the apical plasma membranes of polarized epithelial cells in *Drosophila* [27]. The Crb protein includes an extracellular domain, which contains 30 EGF-like domains and 4 laminin AG domain-like repeats, and a short cytoplasmic domain [27]. The intracellular C-terminus of Crb contains an EERLI motif that has been shown to interact with Discs Lost [3] and Stardust [1, 9]. The lack of *crb* in *Drosophila* embryos causes severe defects in epithelial development and eventually results in embryonic lethality [26, 27]. Overexpression of Crb on a wild-type background disrupts epithelial integrity, resulting in a multilayered epidermis [29]. In addition, *crb* is also important in the development of photoreceptor cells. In *crb* mutants, zonulae adherens, a type of adherens junction, fail to extend and assemble properly in pupal eyes. The consequence of

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zonula adherens defects is malformation of the rhabdomere [10, 20]. Mutation of *crb* also results in progressive, light-induced retinal degeneration [11]. Furthermore, mutation of *CRB1*, the human homologue of *Drosophila crb*, causes severe forms of retinitis pigmentosa and Leber congential amaurosis [5, 6]. Together, these studies provide strong evidence that *crb* plays an important role in retinal development and prevents light-dependent retinal degeneration.

Although the function of *crb* in rhabdomere morphogenesis and the prevention of retinal degeneration has been studied, the role of *crb* in distinct stages of eye development remains unclear. To clarify the role of *crb* in different stages of eye development, we used stage-dependent Gal4 lines to drive overexpression of Crb. We then examined how Crb affects distinct stages of eye development. Our results show that overexpression of *crb* using *GMR-Gal4* in the early larval stage disrupted differentiation and nuclear positioning in photoreceptors. Using *heat-shock-Gal4* to overexpress *crb* at selected stages of pupal development (PD) caused different degrees of defects in extension of adherens junctions and rhabdomere morphogenesis. In summary, this study delineated the distinct roles of Crb in *Drosophila* eye development by temporal expression of Crb at selective stages of the developing eye.

Materials and Methods

Drosophila *Stocks and Expression of Transgenes*

Drosophila melanogaster, w^{1118}, was used as the wild type. *UAS-crb*^{wt} transgenic flies, which express full-length Crb, and *UAS-crb*^{intramyc} transgenic flies, which express the cytoplasmic domain of Crb [29], were gifts from Dr. Elisabeth Knust of the Institute of Genetics at the University of Düsseldorf in Germany. *hs-Gal4* and *GMR-Gal4* flies were provided by Dr. Henry Sun (Institute of Molecular Biology at Academia Sinica, Taiwan). Flies were raised on standard corn meal-agar media at 25°C unless stated otherwise.

To induce the expression of transgenes in the eye discs, the *UAS-crb*^{wt} and *UAS-crb*^{intramyc} flies were crossed with *GMR-Gal4* flies to create *GMR>crb*^{wt} and *GMR>crb*^{intramyc} transgenic flies. To induce the expression of *crb* in pupal eyes, the *UAS-crb*^{wt} flies were crossed with *hs-Gal4* flies to make *hs>crb*^{wt} flies. The selective stages of *hs>crb*^{wt} pupae (35, 40, 45 and 50% of PD) were heat-treated at 37°C for 45 min using a thermocycler. After heat shock, the flies were raised at 20°C to 55% PD for immunocytochemical study.

Confocal Laser Scanning Microscopy

For immunocytochemistry, selective stages of developmental eyes were dissected in Ringer's solution (55 m*M* NaCl, 15 m*M* $MgSO_4$, 40 m*M* KCl, 4.5 m*M* $CaCl_2$, 20 m*M* glucose, 50 m*M* sucrose, 10 m*M* PIPES, pH 6.95) and then fixed in 4% paraformaldehyde in PBS for 20 min. After three washes with PBS plus 0.2% Triton X-100 (PBST), the eyes were incubated in primary antibody and phalloidin (2 μg/ml; Sigma, St. Louis, Mo., USA) in PBST at 4°C overnight. The primary antibodies used in this study included rabbit anti-Bar (1:100) [8], mouse anti-Armadillo (Arm; 1:50) [22], mouse anti-Crb (cq4; 1:50) [27] and rat anti-Elav (1:50) [23]. The monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa, USA. The stained eyes were washed three times with PBST, and then incubated with secondary antibodies. The secondary antibodies were either anti-mouse IgG or anti-rabbit IgG conjugated with FITC or Cy5 (Jackson ImmunoResearch Lab, West Grove, Pa., USA). The stained eyes were mounted in mounting medium and examined under a ZEISS LSM510 confocal laser scanning microscope. For three-dimensional reconstruction, the eyes were examined using confocal Z sections (with 0.3 μm between sections). The series sections were then reconstructed with three-dimensional software supplied with the ZEISS confocal module. The images were processed using Adobe Photoshop 6.0.

Results

Expression of Crb in Drosophila *Eye Development*

To examine the role of *crb* in *Drosophila* eye development, we stained the developing eyes in each selected stage with anti-Crb antibody and anti-Arm antibodies. Arm is a marker for adherens junctions, which separate the polarized epithelial cell into apical and basolateral domains [22]. In the third-instar eye disc (fig. 1A), Crb staining was colocalized with Arm staining at the apical surface of photoreceptors. At 35% PD (fig. 1B), expression of Crb and Arm remained at the apical surface of photoreceptors. At 45% PD (fig. 1C), Crb staining began to separate from Arm staining and concentrated at the subapical region, which later develops into the stalk domain. At 55% PD (fig. 1D), Crb staining clearly separated from Arm staining and concentrated at the stalk domain. The immunocytochemical results indicate that Crb is located at the apical surface of the photoreceptors in the early stages of development and then gradually concentrates at the prospective stalk domain of the photoreceptors as development moves to 55% PD.

Overexpression of Crb Disrupted the Differentiation of Photoreceptors

To study whether *crb* plays a role in differentiation of photoreceptors, we overexpressed full-length Crb *(UAS-crb*^{wt}*)* and the cytoplasmic domain of Crb *(UAS-crb*^{intramyc}*)* behind the morphogenetic furrow under the control of *GMR-Gal4* flies. We then used anti-Elav antibodies, which labeled the nuclei of differentiated photoreceptors, and anti-Bar antibodies, which labeled the nuclei of photoreceptors R1 and R6, to examine whether differ-

entiation of photoreceptors was affected by Crb overexpression. In the control wild-type flies (fig. 2A) and heterozygous *GMR-Gal4* flies (data not shown), phalloidin and anti-Elav double staining revealed a regular pattern of ommatidial clusters and nuclei of differentiated photoreceptors in the third-instar eye disc. In *GMR>crb*^{wt} flies (fig. 2B), this regular array of ommatidial lattices became irregular and the number of Elav-positive cells was significantly reduced. In *GMR>crb*^{intramyc} flies (fig. 2C), the regular array of ommatidial lattices was also affected. However, the number of Elav-positive cells was slightly reduced compared to *GMR>crb*^{wt} flies.

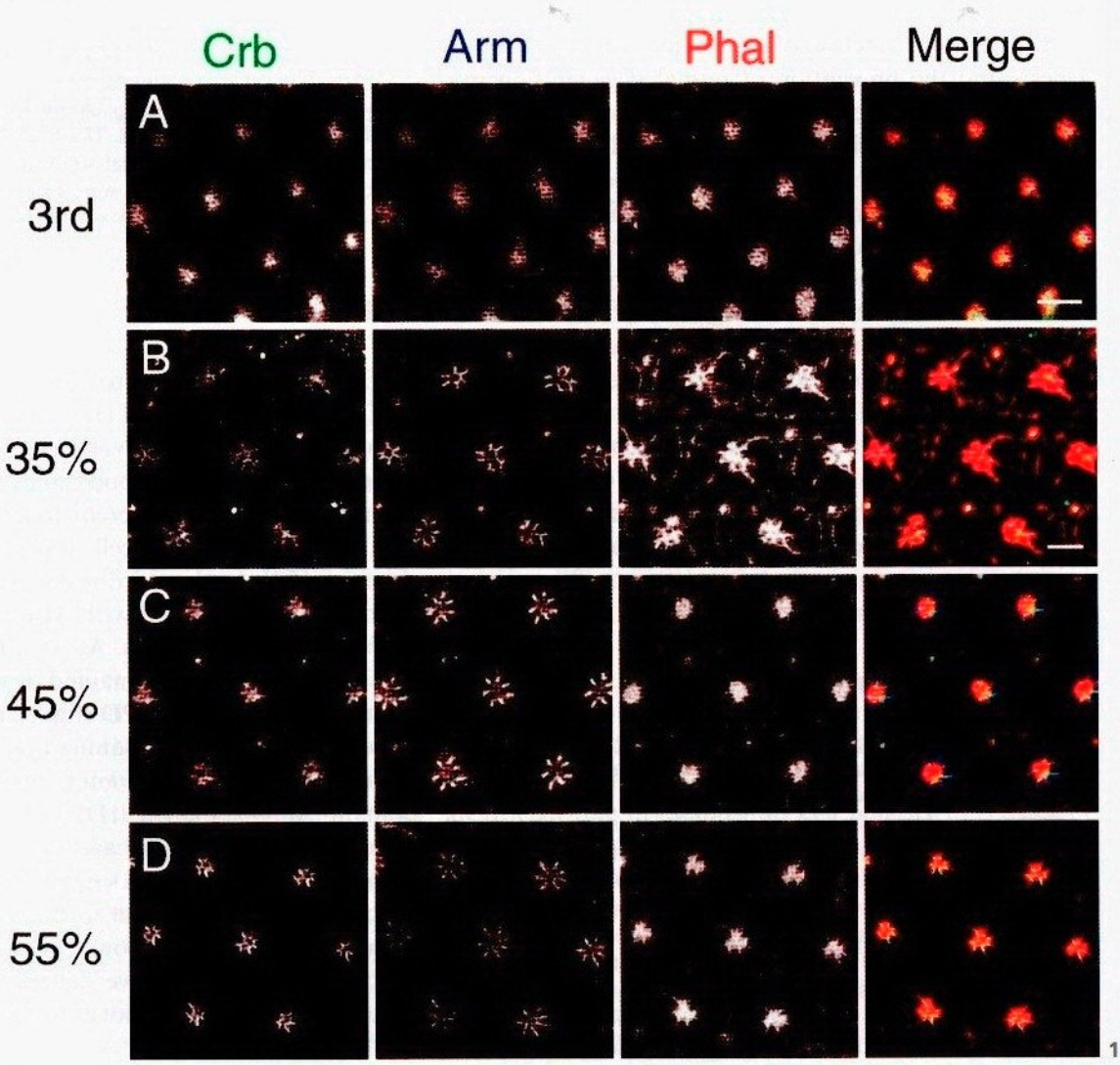
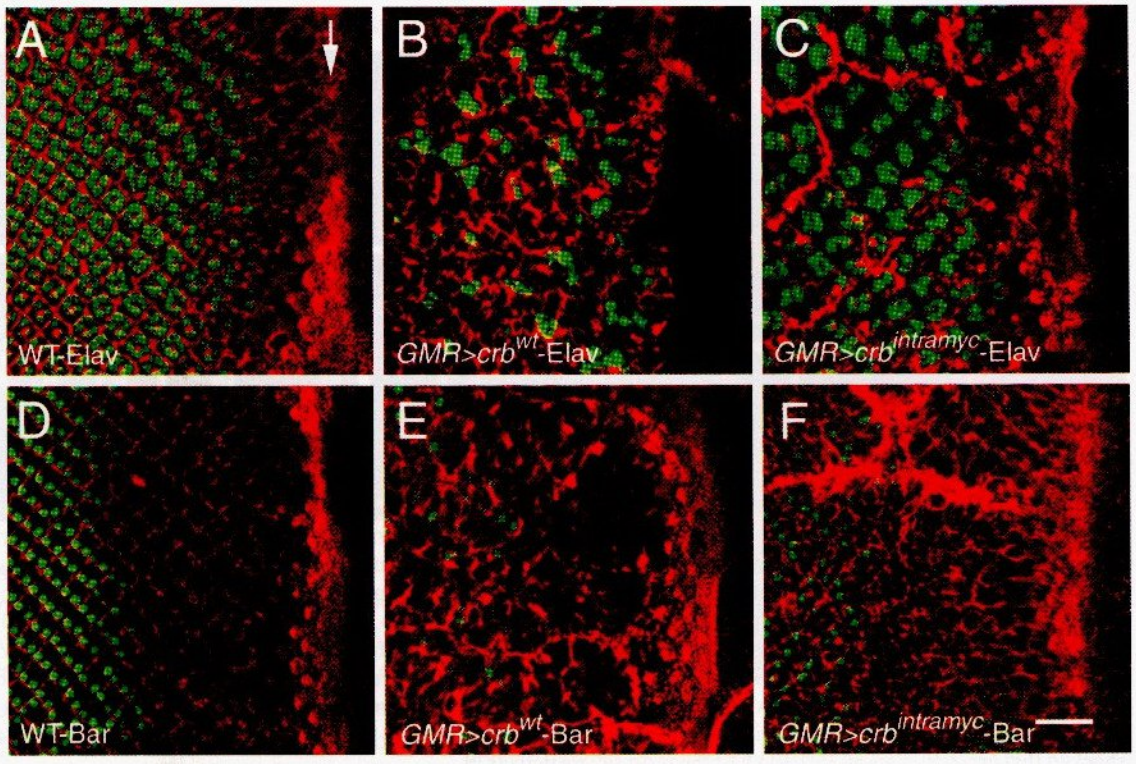


Fig. 1. Expression of Crb in *Drosophila* eye development. To determine the expression of Crb, selected stages of developing eyes were stained with anti-Crb, anti-Arm and rhodamine-phalloidin. **A** In the third-instar eye disc, Crb colocalized with Arm at the apical surface of photoreceptors. **B** At 35% PD, Crb staining remained at the apical surface of photoreceptors and was colocalized with Arm staining. **C** As development continued to 45% PD, Crb staining gradually separated from Arm staining and began to concentrate at the stalk membrane. **D** At 55% PD, Crb staining was clearly separated from Arm staining and restricted to the stalk domain of photoreceptors. Scale bar = 5 μm.

Fig. 2. Overexpression of Crb disrupted ommatidial lattices and differentiation of photoreceptors. To visualize ommatidial lattices and differentiation of photoreceptors, the eyes were double-stained for rhodamine-phalloidin and either anti-Elav (**A–C**) or anti-Bar antibodies (**D–F**). **A**, **D** In wild-type (WT) flies, phalloidin staining revealed a regular array of ommatidial clusters (red). Anti-Elav staining (**A**, green) revealed that the nuclei of differentiated photoreceptors formed in regular clusters. Anti-Bar staining (**D**, green) revealed the nuclei of R1 and R6 as regular doublets. **B**, **E** In *GMR>crb*^{wt} flies, the regular array of ommatidial clusters was missing, and random and fewer clusters were found in the eye disc (red). Anti-Elav (**B**,



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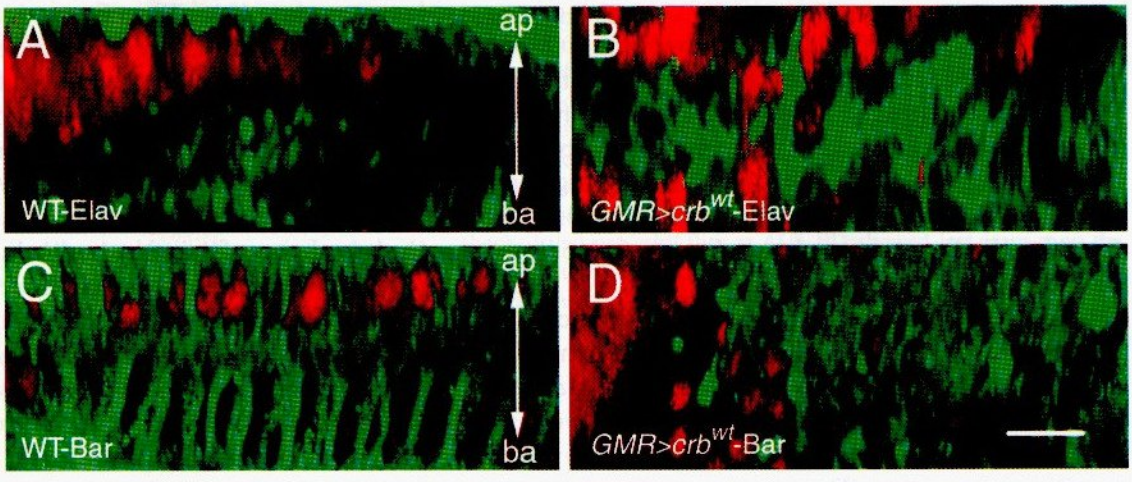

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green) and anti-Bar staining (**E**, green) revealed that the number of differentiated nuclei decreased significantly and regular clusters also disappeared. **C**, **F** In *GMR>crb*^{intramyce} flies, the regular array of ommatidial clusters also disappeared (red), but the defects were not as severe as those seen in the *GMR>crb*^{wt} flies. Anti-Elav (**C**, green) and anti-Bar (**F**, green) staining revealed that the number of differentiated nuclei were fewer than in wild-type flies. The morphogenetic furrow is indicated by the arrow. Anterior is to the right. Scale bar = 10 μm.

Fig. 3. Overexpression of Crb resulted in nuclear mislocalization in differentiated photoreceptors. To visualize the nuclear position, the eye discs were stained with rhodamine-phalloidin (green) and anti-Elav (red in **A**, **B**) or anti-Bar (red in **C**, **D**). Z sections were then taken from selective eye fields using confocal microscopy. The nuclear position was then observed from the lateral view of Z stacks. **A**, **C** In the wild-type (WT) flies, the Elav-positive nuclei (**A**) were located as stereotyped clusters near the apical compartment of the retinal epithelium. Similarly, the nuclei of R1 and R6, which were labeled by anti-Bar antibodies (**C**), also occupied the apical compartment of the retinal epithelium. **B**, **D** In *GMR>crb*^{wt} flies, stereotyped clusters of the Elav-positive nuclei (**B**) disappeared and the nuclei were usually randomly distributed in the retinal epithelium. The nuclei of R1 and R6 were also mislocalized at the retinal epithelium (**D**). ap = Apical surface of the retinal epithelium; ba = basal surface of the retinal epithelium. Scale bar = 5 μm.

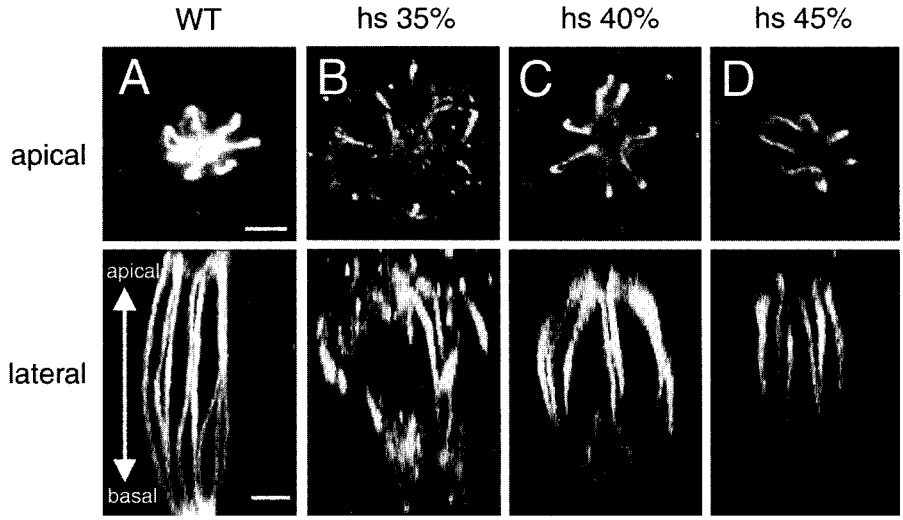

Fig. 4. Overexpression of Crb prevented extension of the adherens junctions. The extension of the adherens junctions was observed from the lateral view derived from the confocal Z stack. **A** In wild-type (WT) eye, the adherens junction was organized as a compact cluster in the center of the ommatidium in the apical view. In the lateral view, the adherens junctions extended smoothly from the apical surface to the basal surface of the retinal epithelium. **B** Heat-shocked (hs) *hs>crb*^{wt} flies at 35% PD had the most severe defects in organization and extension of adherens junctions. In the apical and lateral views, the adherens junctions were completely disorganized. **C**, **D** Heat-shocked (hs) *hs>crb*^{wt} flies at 40% PD (**C**) and 45% PD (**D**) had less severe defects in the organization and extension of adherens junctions, with most defects found at the basal portion of the retinal epithelium. Adherens junctions shown in these figures were stained with anti-Arm antibodies. Scale bar = 2 μm.

To further investigate whether the differentiation of R1 and R6 was affected in response to overexpression of Crb, we stained the eye disc with anti-Bar antibodies. In the wild type (fig. 2D), Bar staining revealed a regular pattern of R1/6 in the third-instar eye disc. In *GMR>crb*^{wt} flies (fig. 2E), Bar staining almost disappeared; only a few Bar-positive cells were found in the eye disc. In *GMR>crb*^{intramyc} flies (fig. 2F), the Bar-positive cells were also significantly reduced. We believe that the phenotypic difference between *GMR>crb*^{wt} and *GMR>crb*^{intramyc} flies was quantitative, but not qualitative, since *GMR>crb*^{wt} flies had a more severe eye phenotype than *GMR>crb*^{intramyc} flies. Thus, only the eye phenotypes in *GMR>crb*^{wt} flies were shown in the following experiments. In addition, to ensure that the phenotypes were consistent, all experiments were blindly repeated at least five times. Together, the results suggested that overexpression of Crb interrupted differentiation of photoreceptors and regular organization of ommatidial lattices.

Overexpression of Crb Disrupted the Nuclear Position in the Third-Instar Eye Disc

In third-instar larvae, the nuclei of differentiated photoreceptors occupy the apical compartment of the retinal epithelium. To further investigate the effects of Crb overexpression on nuclear position in differentiated photoreceptors, we compared the position of photoreceptor nuclei in wild-type and *GMR>crb*^{wt} flies using anti-Elav and anti-Bar staining. Nuclear position was determined using reconstruction of confocal Z sections and visualized from the lateral view of the retinal epithelium. In wild-type flies (fig. 3A), the Elav-positive nuclei were located in stereotyped clusters near the apical compartment of the retinal epithelium. In *GMR>crb*^{wt} flies (fig. 3B), the Elav-positive nuclei were distributed randomly throughout the retinal epithelium. Consistent with the results of Elav staining, the nuclei of photoreceptors R1 and R6, as revealed by Bar staining, were also located at the apical compartment of the retinal epithelium in wild-type flies (fig. 3C). In *GMR>crb*^{wt} flies (fig. 3D), Bar-positive nuclei were mispositioned in the retinal epithelium. Together, these results suggest that overexpression of Crb affects differentiation of photoreceptors as well as the nuclear position in the retinal epithelium.

Overexpression of Crb Disrupted the Extension of Adherens Junctions in Pupal Eyes

We showed that overexpression of Crb disturbed differentiation of photoreceptors in third-instar eye discs. We then examined whether overexpression of Crb af-

fected eye development in the pupal stage. Previous studies have shown that *crb* is required for rhabdomere morphogenesis due to its role in assembly of the adherens junctions and extending the apical surface of photoreceptors [10, 20]. In these studies, *GMR-Gal4* was used to drive Crb overexpression or *eyeless-FLP* to generate *crb* loss-of-function mosaic clones. The role of *crb* in rhabdomere morphogenesis was then examined. Although these studies provide significant information about the role of *crb* in rhabdomere morphogenesis, the findings could also have been due to the accumulation of defects that occurred in the early stages of eye development. To clarify the roles of *crb* in distinct stages of pupal eye development, we overexpressed Crb at selective stages of PD using *hs-Gal4* lines. This study allowed us to bypass the effect of *crb* at the third-instar eye disc and only examine its role at the pupal stage.

To examine the role of Crb at distinct stages of pupal eye development, we induced Crb expression at 35, 40 and 45% PD by heat treatment of *hs>crb*^{wt} flies. We then examined how these conditions affected the extension of photoreceptor adherens junctions at 55% PD by antibody staining for Arm, a component of the adherens junction. In 55% PD wild-type flies (fig. 4A) and untreated *hs>crb*^{wt} flies (data not shown), Arm expression indicated that adherens junctions were organized as a belt-like structure at the center of the ommatidium and extended smoothly from the retinal tip to the floor in the retinal epithelium. However, heat-treated *hs>crb*^{wt} flies at 35% PD (fig. 4B) had the most severe phenotype, in which adherens junctions usually terminated prematurely and were randomly distributed over the ommatidium. Heat-shocked *hs>crb*^{wt} flies at 40% PD had incomplete extension of adherens junctions; the adherens junctions near to the retinal tip were quite normal but failed to extend basally (fig. 4C). Heat-treated *hs>crb*^{wt} flies at 45% PD also had defective adherens junctions mainly close to the retinal floor (fig. 4D). Together, these results suggest that overexpression of *crb* disrupted the initiation and extension of adherens junctions in photoreceptors.

Overexpression of Crb Disrupted the Formation of the Rhabdomere Domain

Extension of adherens junctions is the first step of rhabdomere morphogenesis. To further examine how Crb affects the construction of rhabdomeres, we overexpressed Crb at selective stages of PD and then observed their rhabdomere phenotype at 55% PD, a stage when apical extension has finished and rhabdomere precursors begin to be formed. In heat-shocked wild-type (fig. 5A) and untreated *hs>crb*^{wt} flies (data not shown), the actin-enriched rhabdomere precursors, as shown by phalloidin staining, were located among adherens junctions at the apical surface of photoreceptors. In heat-shocked *hs>crb*^{wt} flies at 35% PD, the rhabdomere precursors became irregular with many actin-enriched rhabdomeres mislocalized at the basolateral domain of photoreceptors (fig. 5B, arrows). The number and position of adherens junctions per ommatidium were reduced or increased. In heat-shocked *hs>crb*^{wt} flies at 40% PD, the actin-enriched rhabdomeres were expanded wider than in wild-type flies and some were located at the basolateral membrane of the photoreceptor (fig. 5C, arrows). The array of adherens junctions was also slightly affected. In heat-shocked

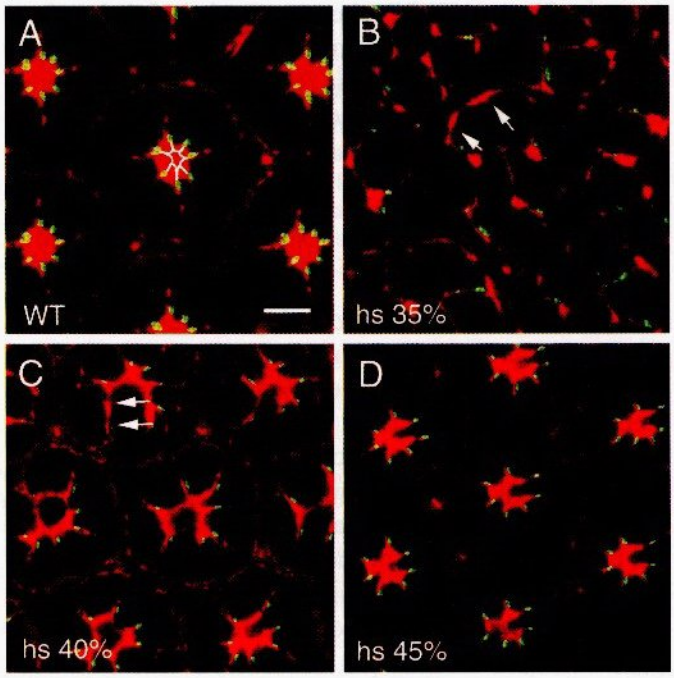

Fig. 5. Overexpression of Crb disrupted the formation of the rhabdomere domain. **A** In the wild type (WT), the rhabdomere domain stained by phalloidin (red, indicated by the white lines) was localized between the adherens junctions stained by anti-Arm antibodies (green) at the apical surface of the photoreceptors. **B** Heat-shocked (hs) *hs>crb*^{wt} flies at 35% PD had a disrupted rhabdomere domain with some of the rhabdomere domains found at the basolateral membrane (arrows). In addition, the array of adherens junctions was also disorganized. **C** Heat-shocked (hs) *hs>crb*^{wt} flies at 40% PD had an expanded rhabdomere domain (arrows). The organization of adherens junctions was expanded wider than in the wild type. **D** Heat-shocked (hs) *hs>crb*^{wt} flies at 45% PD showed less impact on the determination of the rhabdomere domain and the organization of adherens junctions. Scale bar = 5 μm.

hs>crb^{wt} flies at 45% PD, the organization of rhabdomere precursors was nearly normal, as seen in the wild-type flies (fig. 5D). Together, these results showed that overexpression of Crb disturbed the extension of adherens junctions as well as the construction of rhabdomeres.

Discussion

Photoreceptor cells of *Drosophila* derive from a monolayer of polarized epithelium. Beginning at the third-instar eye disc, the photoreceptor precursor cells begin to receive different signals to differentiate as photoreceptors, cone cells and pigment cells [15, 21, 24]. Immunohistochemical studies have shown that the receptor tyrosine kinase, Sevenless, and the transmembrane receptor, Boss, are located or enriched at the apical membrane of undifferentiated cells in controlling the differentiation of the photoreceptor R7 [2]. In addition, during embryonic development, Notch and Arm are located at the adherens junctions in controlling the differentiation of neuroblasts and the determination of segment polarity, respectively [31]. Thus, determination of the apical membrane domains in developing epithelial cells is important to restrict differential signals at specific domains and thus regulates the determination of cell fate. The apical determination in the polarized epithelium is a complex process that has been shown to involve a series of genetic regulations [4, 13, 14, 28]. Overexpression of either full-length Crb or its cytoplasmic domain has been shown to result in expansion of the apical membrane domain in polarized epithelial cells [29]. In this study, we showed that overexpression of full-length Crb or its cytoplasmic domain disrupted photoreceptor differentiation. This result supports the notion that localization of the differential signals at the apical surface is important for cell fate determination in the developing *Drosophila* eye.

During eye development, the nuclei of differentiated photoreceptors form stereotyped clusters and array at the apical compartment of the retinal epithelium. The mechanism to control the apical localization of photoreceptor nuclei is unclear. However, several studies have indicated that cytoskeletal proteins, such as dynactin (Glued) and Klar, participate in nuclear positioning in developing *Drosophila* eyes [7, 19]. In this study, we showed that overexprerssion of Crb disrupted the apical localization of photoreceptor nuclei, suggesting a linkage between the cell polarity gene and cytoskeletal proteins in nuclear localization.

The rhabdomere is a photosensitive structure located on the apical surface of the photoreceptor. During rhabdomere morphogenesis, the photoreceptors turn and then extend their apical surface to face the interrhabdomere space [17]. The identity of the molecules involved in the turning and extension of the apical membrane remains unclear. Previous studies have shown that disruption of *crb* in the third-instar larvae stage disrupted rhabdomere morphogenesis in the pupal stage [10, 20]. However, the eye defects seen in these studies could be due to accumulative defects that occurred in larval and earlier pupal stages. In order to examine the role of Crb in rhabdomere morphogenesis, we used heat shock to drive the expression of Crb, which allowed us to bypass the larval stage and only examine the role of Crb in the pupal stages. Our results showed that overexpression of Crb at the pupal stages produced phenotypes similar to those in *crb* mitotic clones. This finding further strengthens the evidence for a role of *crb* in apical extension during rhabdomere morphogenesis. Furthermore, our results not only showed that overexpression of Crb disrupted the apical extension but also indicated that this effect was stage-dependent (fig. 4). Overexpression of Crb at the time when apical extension had just begun resulted in the most severe defects in the extension of adherens junctions, while overexpression of Crb at lateral stages, when the adherens junctions had already reached the retinal floor, caused less obvious effects.

Malformation of the rhabdomere is associated with the mispositioned adherens junctions in *hs>crb*^{wt} flies, suggesting that formation of the adherens junctions may be required to determine the formation of the rhabdomere domain. Crb has been shown to play an important role in the formation of the zonula adherens in polarized epithelium [25]. A previous study also indicated that the C-terminus of Crb interacts with Disc Lost to mediate the localization and stabilization of E-cadherin at the adherens junction [3, 12]. Thus, in this study, the finding that alteration of Crb expression resulted in mislocalization of the adherens junctions in the photoreceptors is consistent with previous studies. The relationship between adherens junction formation and rhabdomere morphogenesis has also been addressed in a *canoe* mutant [18]. The *canoe* gene encodes a PDZ domain containing a protein that was shown to localize in the adherens junctions in the developing photoreceptors. Mutation of *canoe* also leads to the malformation of the rhabdomere [18]. The mechanism by which adherens junctions regulate rhabdomere morphogenesis remains unclear. It is possible that adherens junctions are required to set the apical domain in the photore-

ceptor. Once the apical domain is determined, the rhabdomeres are then formed specifically at the apical surface. Disruption of the assembly of adherens junctions fails to define the apical domain, resulting in malformation of the rhabdomere. In summary, in this study, we used gain-of-function methods to delineate the roles of *crb* in distinct stages of eye development. Although not physiological, this method provided a phenotypic suggestion of its physiological function.

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