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# Interaction of Par-6 and Crumbs complexes is essential for photoreceptor morphogenesis in *Drosophila*

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## SUMMARY

**Apicobasal cell polarity is crucial for morphogenesis of photoreceptor rhabdomeres and adherens junctions (AJs) in the *Drosophila* eye. Crumbs (Crb) is specifically localized to the apical membrane of photoreceptors, providing a positional cue for the organization of rhabdomeres and AJs. We show that the Crb complex consisting of Crb, Stardust (Sdt) and Discs-lost (Dlt) colocalizes with another protein complex containing Par-6 and atypical protein kinase C (aPKC) in the rhabdomere stalk of photoreceptors. Loss of each component of the Crb complex causes age-dependent mislocalization of Par-6 complex proteins, and ectopic expression of Crb intracellular domain is sufficient to recruit the Par-6 complex. We also show that the absence of Par-6 complex proteins results in severe mislocalization and loss of Crb complex. We further demonstrate that Dlt directly binds to Par-6, providing a molecular basis for the mutual dependence of the two complexes. These results suggest that the interaction of Crb and Par-6 complexes is required for the organization and maintenance of apical membranes and AJs of photoreceptors.**

Key words: *Drosophila*, Crumbs, Discs-lost, Par-6, Photoreceptor, Rhabdomere

## INTRODUCTION

The establishment and maintenance of cell polarity is an essential feature of all eukaryotic cells and is crucial for the integrity of the organism. Recent studies have begun to reveal the molecular basis of apicobasal cell polarity by identifying important proteins involved in cell polarity determination and junction formation (Bilder, 2001; Ohno, 2001). Accumulating evidence suggests that important cues for the establishment of cell polarity are provided by the function of at least two evolutionarily conserved protein complexes. One of these complexes consists of Crb, Sdt, and Dlt (hereafter 'Crb complex') and the other contains Par-6, aPKC and Bazooka (Baz) (hereafter 'Par-6 complex').

The *crb* and *sdt* genes were identified genetically as essential components for organizing apicobasal polarity and AJs in embryonic epithelia (Bachmann et al., 2001; Bhat et al., 1999; Hong et al., 2001; Tepass et al., 1990). Genetic interaction studies suggested that *sdt* acts downstream of *crb* in the same pathway (Grawe et al., 1996; Tepass and Knust, 1993). Molecular analysis of Crb and Sdt has shown that they are directly associated in the apical plasma membranes of epithelial cells (Bachmann et al., 2001; Hong et al., 2001). Crb is a transmembrane protein with a long extracellular domain and a short C-terminal cytoplasmic tail. The Crb-Sdt interaction is mediated by the single PDZ domain of a MAGUK (a membrane-associated guanylate kinase) family protein Sdt and the C-terminal PDZ domain binding motif (PBM) of the cytoplasmic domain of Crb (Bachmann et al., 2001; Hong et al., 2001). Crb complex proteins are evolutionarily conserved. Studies in mammalian cell culture systems indicate that Pals1 (mammalian Sdt homolog) links PATJ (Dlt homolog) and CRB1 (Crb homolog) to form a Crb complex (Roh et al., 2002). Pals1 (or Sdt) binds to the Crb-PBM motif ($Crb^{PBM}$) via its single PDZ domain. The L27 (Lin-2 and Lin-7 homology) domain of Pals1 interacts with a novel protein-binding motif located at the N terminus of PATJ (or Dlt) termed MAGUK recruitment domain (MRE) (Roh et al., 2002). However, in vivo function of vertebrate Sdt homologs is largely unknown except that a zebrafish homolog Nagie oko localizes to the apical cell junctions of the retinal neuroepithelium and may function as a putative scaffolding factor (Wei and Malicki, 2002).

In addition to the Crb complex, apicobasal cell polarity is also regulated by the Par-6 complex. Par-6 (partition-defective-6), initially found in *C. elegans*, is essential for the establishment of asymmetry of early worm embryo and for asymmetric localization of Par-3 (the homolog of *Drosophila* Baz). Par-6 interacts with aPKC and Baz to form a conserved ternary protein complex and affect aPKC activity. In contrast to Crb and Sdt, Par-6 complex proteins are not only crucial for epithelial cell polarity but also important for asymmetric division of various cell types in different organisms (Rose and Kemphues, 1988; Suzuki et al., 2001; Wodarz et al., 2000). In

*Drosophila*, homologs of these proteins (Par-6, aPKC and Baz) colocalize at the apical side of epithelial cells and neuroblasts in the embryo, and are essential for the establishment of apicobasal asymmetry in both of these cell types (Kuchinke et al., 1998; Petronczki and Knoblich, 2001; Wodarz et al., 2000). Mammalian homologs (PAR-6, aPKC and Par-3 or ASIP) form a similar ternary complex that localizes to the tight junctions (Ohno, 2001). Studies in cultured epithelial cells have suggested that this complex is important for the formation of tight junctions and the specification of apical and basolateral membrane domains (Izumi et al., 1998; Joberty et al., 2000; Lin et al., 2000; Suzuki et al., 2001). Thus, the Par-6 complex appears to act as a conserved functional cassette for the generation of apicobasal asymmetries in diverse cell types (Ohno, 2001). Recent studies on embryonic epithelia in *Drosophila* have provided genetic evidence for interaction of Crb and Par-6 complex proteins (Bilder et al., 2003; Tanentzapf and Tepass, 2003). Although a physical interaction between Sdt and Par-6 has been found in mammalian cell cultures (Hurd et al., 2003), its role in epithelial tissues in developing animals has not been demonstrated.

In this study, we focus on the analysis of mechanisms how the apical domains of photoreceptor cells are organized. The apicobasal polarity is prominent in the photoreceptors owing to the photosensitive organ, rhabdomere, formed on the apical surface of the cell. During pupal eye development, the apical domain of differentiating photoreceptors undergoes dynamic reorganization of the cell shape and size, resulting in the formation of rhabdomeres and new AJs (Kumar and Ready, 1995; Longley and Ready, 1995). Recent studies have shown that Crb plays important roles in morphogenesis of the photoreceptor rhabdomere, providing evidence that at least some proteins involved in the apicobasal polarity of embryonic epithelia are essential for the organization of photoreceptors (Izaddoost et al., 2002; Pellikka et al., 2002). Crb is specifically localized to the rhabdomere stalk, a membrane domain that is juxtaposed apically to the emerging rhabdomere and basally to the AJ. Crb is required for positioning and growth of rhabdomere and AJ during the crucial period of photoreceptor extension along the proximodistal axis of the retina. Further analysis of Crb function has shown that the intracellular domain is necessary for the recruitment of AJ as well as localization of Dlt (Izaddoost et al., 2002). Importantly, the mammalian homolog of Crb localizes to the region corresponding to the rhabdomere stalk membrane, that is, the inner segment between the outer segment (analogous to the rhabdomere) and the AJ of rod photoreceptors (Pellikka et al., 2002). Furthermore, mutations in *CRB1*, one of Crb homologs in human, cause severe retinal dystrophies such as retinitis pigmentosa type 12 (RP12) and Leber congenital amaurosis (LCA) (den Hollander et al., 1999; den Hollander et al., 2001a). These studies suggest that Crb and other molecular components involved in the specification of apical membrane of photoreceptors might be evolutionarily conserved.

To understand further the molecular events required for apical morphogenesis of photoreceptors, we examined the localization, function and interaction of components of Crb and Par-6 complexes. We show that all proteins except Baz in the Crb and Par-6 ternary complexes colocalize to the rhabdomere stalk and are mutually required for their localizations. Baz localizes to the AJ but is essential for apical targeting of Crb and Par-6 complex proteins. Furthermore, these two protein complexes are linked by direct binding of Dlt and Par-6, providing the molecular basis for the interdependence of these two protein complexes.

## MATERIALS AND METHODS

### Genetics

Mitotic recombinations were induced by using FLP/FRT method for clonal analysis (Xu and Rubin, 1993). $crb^{11A22}$ (Tepass and Knust, 1993), $sdt^{XP96}$ (Hong et al., 2001; Wodarz et al., 1999) and $dlt^{MY10}$ (Bhat et al., 1999) mutant clones were produced by eye-specific expression of FLP in *y w ey-Flp/+; FRT82B* $crb^{11A22}$*/FRT82B Ubi-GFP, y w ey-Flp/+; p[cdc37*$^{+}$*]/+;* $dlt^{MY10}$ *FRT80B/FRT80B Ubi-GFP* and *y w* $sdt^{XP96}$ $FRT^{9\text{-}2}$*/y w Ubi-GFP* $FRT^{9\text{-}2}$*; ey-Flp/+*, respectively. *par-*$6^{\Delta 226}$ (Petronczki and Knoblich, 2001), $aPKC^{K06304}$ (Wodarz et al., 2000) and $baz^{Xi106}$ (Wodarz et al., 1999) eye clones were produced using *y w par-*$6^{\Delta 226}$ $FRT^{9\text{-}2}$*/y w Ubi-GFP* $FRT^{9\text{-}2}$*; ey-Flp/+*, *y w ey-Flp/+; FRT42D* $aPKC^{K06304}$*/FRT42D Ubi-GFP* and *y w* $baz^{Xi106}$ $FRT^{9\text{-}2}$*/y w Ubi-GFP* $FRT^{9\text{-}2}$*; ey-Flp/+*, respectively. Overexpression of $Crb^{intra}$ was induced by crossing *UAS-*$crb^{Myc\text{-}intra}$, *UAS-*$crb^{Myc\text{-}JM}$ or *UAS-*$crb^{Myc\text{-}PBM}$ (Klebes and Knust, 2000) with the *GMR-GAL4* at 18°C (*UAS-*$crb^{Myc\text{-}intra}$) or room temperature (*UAS-*$crb^{Myc\text{-}PBM}$ or *UAS-*$crb^{Myc\text{-}JM}$).

### Immunohistochemistry

For immunohistochemistry, pupal eyes at 40-50% pupal development (pd) were fixed and stained with combinations of antibodies as previously described (Izaddoost et al., 2002). Primary antibodies used were rabbit anti-Dlt (1:500), mouse anti-Dlt (1:500), rat anti-Crb (1:400) (Bhat et al., 1999), rabbit anti-Sdt (1:100) (Bachmann et al., 2001), mouse anti-Arm (1:200; Hybridoma Bank), rat anti-DE-cad (1:50) (Takeichi, 1988), rabbit anti-Baz (1:500) (Wodarz et al., 1999), rabbit anti-Par-6 (1:500) (Petronczki and Knoblich, 2001) and rabbit anti-PKCζ C20 (1:500; Santa Cruz Biotechnology). TRITC-conjugated phalloidin was from Sigma, whereas fluorescent secondary antibodies were from Jackson Immunochemicals. Images were scanned using a Zeiss LSM laser-scanning confocal microscope.

### DNA constructs

GST-Dlt and its deletion constructs were described previously (Bhat et al., 1999). GST-Dlt-PDZ3 was constructed by deletion from the internal *Bam*HI site in the PDZ4 to the C terminus of Dlt. GST-Dlt-PDZ4 was made by deletion from the N terminus of GST-PDZ34 to the internal *Sca*I site in the PDZ3. GST- or MBP-fusion plasmids of Par-6 and aPKC were obtained by inserting fragments amplified from an embryonic cDNA library (provided from K. Zinn) into pGEX (Pharmacia) or pMal (NEB) in-frame. GST-Baz plasmid was obtained by inserting *Nru*I-*Sal*I fragment containing three PDZ domains (amino acids 110-947) from LD13977 (BDGP; the ORF is identical to that available under GenBank Accession Number AJ130871) into pGEX. MBP-Par-6ΔC was constructed using an internal *Bam*HI site to delete the C-terminal 107 amino acids. GST-Par-6ΔPDZ was generated by PCR-based deletion of an internal domain (amino acids 171-247). GST-Par-6ΔN was made by deletion of N-terminal 136 amino acids using an internal *Eco*RV site.

### In vitro pull-down binding assay using purified proteins

GST-, or MBP-tagged proteins were expressed in *E. coli* strain *BL21-CodonPlus* (Stratagene), and purified by glutathione-agarose (Sigma) or amylose resin (NEB), respectively. Equal amount of purified GST- or MBP- fusion proteins immobilized on glutathione-agarose or amylose in PBS containing 0.1% β-mercaptoethanol, 0.1% BSA, and 0.05% Tween-20 were mixed and incubated with equal amount of purified GST- or MBP-fusion proteins for overnight

at 4°C. After extensive washing with PBS containing 0.1% β-mercaptoethanol and 0.05% Tween-20 or 0.1% NP-40, the bound proteins were eluted with 10 mM glutathione or 10 mM maltose and subjected to SDS-PAGE. Eluted GST- or MBP-fusion proteins were detected by immunoblotting using anti-GST (Sigma) or anti-MBP (NEB) antibody.

## RESULTS

### Crb is required for localization of Sdt at rhabdomere stalk

As the first step to understand the function of Sdt in the retinal epithelium, we examined whether Sdt colocalizes with Crb and Dlt in developing photoreceptors. In third instar eye disc, Crb and Dlt localize to the apical domain of undifferentiated cells and developing photoreceptor clusters. During the pupal stage, the apical domains of each photoreceptor cluster involute 90° in perpendicular to the surface of the retina and extend basally toward the floor of the retina. Therefore, the apical domains of photoreceptors face to each other at the center of each cluster, whereas the other ends of AJs and the apical domain anchor to the retinal floor (Fig. 1A). In pupal retina, Crb and Dlt colocalize specifically to the rhabdomere stalk between the rhabdomeres emerging from the apical surface and the AJs (Izaddoost et al., 2002; Pellikka et al., 2002). As shown in Fig. 1B, an AJ marker Armadillo (Arm) localizes immediately basal to the apical Dlt domain. Sdt co-localizes with Dlt in the eye disc throughout larval development (data not shown). Sdt also shows colocalization with Dlt specifically to the rhabdomere stalk region of the photoreceptor in pupal retina (Fig. 1C-E).

To examine the role of Crb for the localization of Dlt and Sdt in rhabdomere stalk, we generated clones of *crb* null mutation (*crb*$^{11A22}$, hereafter '*crb*$^{-}$') in the eye using the *ey-FLP/FRT* system (Xu and Rubin, 1993). In the absence of Crb, both Dlt and Sdt become mislocalized all together (Fig. 1F-N). Mislocalization of Dlt in *crb*$^{-}$ mutant cells is age dependent: it is relatively mild in the earlier pupal eyes (Fig. 1F-H; ~25% pd), but becomes more severe in the later pupal eyes (Fig. 1I-K; ~35%), and completely diffused to basolateral membrane (Fig. 1L-N; ~50%). The presence of mislocalized Dlt at the membrane in the absence of Crb suggests that there is another positional cue for membrane localization of Dlt.

The intracellular domain of Crb consists of the juxtamembrane domain (JM) and the PDZ domain-binding motif (PBM) (Klebes and Knust, 2000). It has been shown that the JM and PBM are important for AJ and Dlt localization, respectively (Izaddoost et al., 2002). To examine the Crb-dependent localization of Sdt further, Crb$^{PBM}$ was overexpressed in the eye using *GMR-GAL4* (Izaddoost et al., 2002). Crb$^{PBM}$ is the Crb intracellular domain in which the PBM is intact but the JM is mutated (Klebes and Knust, 2000). Overexpression of Crb$^{PBM}$ resulted in mislocalization of Dlt throughout the cell membrane (Fig. 2D). In this case, Sdt was also mislocalized together with Dlt (Fig. 2E,F). The co-mislocalization of Sdt and Dlt was also observed when the Crb$^{intra}$ or Crb$^{Myc\text{-}intra}$ was overexpressed using *GMR-GAL4* (data not shown). These results indicate that the localization of Sdt in apical membrane is strictly dependent on Crb and Crb$^{PBM}$ is sufficient to recruit Sdt.

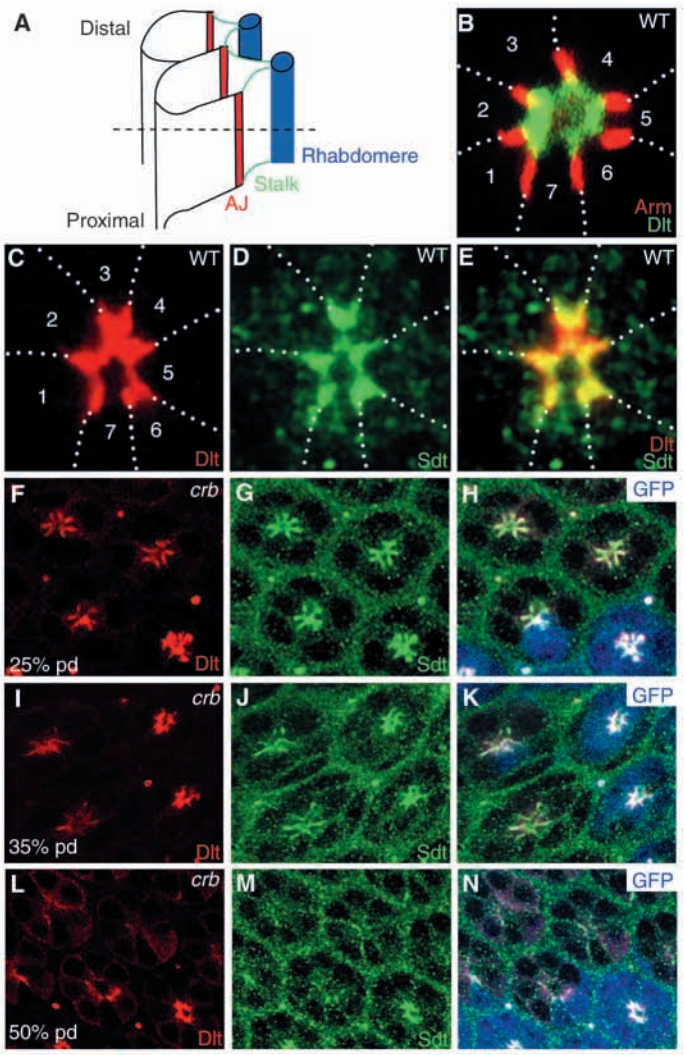



**Fig. 1.** Crb is required for Sdt localization at rhabdomere stalk. (A) A schematic representation of photoreceptor cell in pupal stage. Crb and Dlt colocalize specifically to the rhabdomere stalk between the rhabdomeres emerging from the apical surface and the AJs. Confocal images shown in B-E were sectioned as indicated by the broken line. (B-E) Co-localization of Dlt and Sdt in photoreceptors at ~40% pd. Dlt (green) is localized in apical membrane in the most central region of a photoreceptor cluster whereas AJ (red) is immediately basal to the Dlt domain (B). Later, rhabdomeres form apical to the Dlt domain, which will become the rhabdomere stalk between rhabdomere and AJ. Dlt (C, red) and Sdt (D, green) are colocalized in the rhabdomere stalks. Both proteins show extensive colocalization (E). (F-N) Dlt (F,I,L; red) and Sdt (G,J,M; green) are mislocalized in the *crb*$^{-}$ photoreceptors cell autonomously. In *crb*$^{-}$ mutant cells identified by the absence of GFP staining (H,K,N; blue), Dlt and Sdt are co-mislocalized (H,K,N). Mislocalization of Dlt and Sdt in *crb*$^{-}$ mutant cells is relatively mild in the earlier pupal eyes (F-H; ~25% pd), but becomes more severe in the later pupal eyes (I-K; ~35% pd) and then completely diffused to basolateral membrane (L-N; ~50% pd).

### Sdt is required for correct localization of Crb and Dlt

To determine whether Sdt is essential for the localization of Crb and Dlt at the rhabdomere as seen in embryonic epithelial cells (Bachmann et al., 2001; Hong et al., 2001), *sdt*$^{-}$ mutant

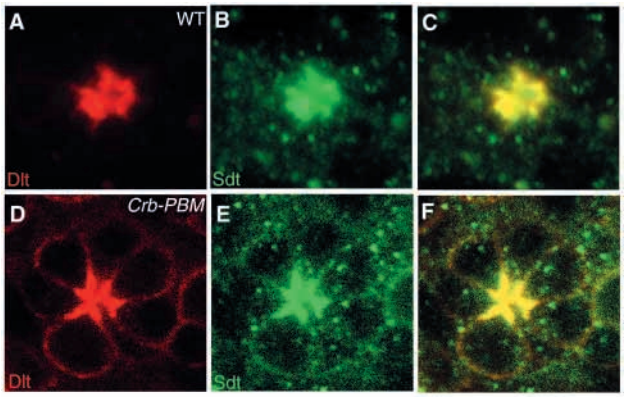



**Fig. 2.** Crb is sufficient to recruit Dlt and Sdt. (A-C) Dlt (A, red) and Sdt (B, green) colocalize in the rhabdomere stalk (C). (D-F) Mislocalization of Dlt (D, red) in the photoreceptor was induced by overexpression of $Crb^{PBM}$ using *GMR-Gal4*. In this situation, Sdt (E, green) was co-mislocalized together with Dlt (F).

clones were generated in the eye using *ey-FLP/FRT*. In $sdt^{XP96}$ (hereafter '$sdt^{-}$') mutant cells, Dlt was greatly reduced to an undetectable level in both early and late pupal development (Fig. 3A-C). This result is in striking contrast to the maintenance of a significant level of Dlt in $crb^{-}$ mutant cells in early pupal stage (Fig. 1F). Hence, Sdt appears to be more crucial than Crb for the localization of Dlt at the rhabdomere stalk. In $sdt^{-}$ mutant cells, Crb is also strongly reduced (Fig. 3D-F).

Loss of Crb from the rhabdomere stalk causes basolateral expansion of AJ distribution (Izaddoost et al., 2002; Pellikka et al., 2002). In $sdt^{+}$ wild-type ommatidia viewed from the top, Arm localizes to the AJ immediately basal to the apical domain of the photoreceptor (Fig. 3G). In $sdt^{-}$ mutant, Arm and *Drosophila* E-cadherin (E-cad) were widely mispositioned (Fig. 3G,H). This phenotype is very similar to that of $crb^{-}$ mutant cells (Izaddoost et al., 2002; Pellikka et al., 2002). The misposition of these AJ markers, Arm and E-cad, was milder in earlier pupal eyes but became more severe in older pupal eyes as shown in $crb^{-}$ clones (data not shown). These results suggest that both Crb and Sdt may be required cell-autonomously for localization and maintenance of AJs.

## Sdt is required for positioning and extension of rhabdomere

In the absence of Crb, rhabdomeres of photoreceptors fail to expand along the growing axis of the cells (Izaddoost

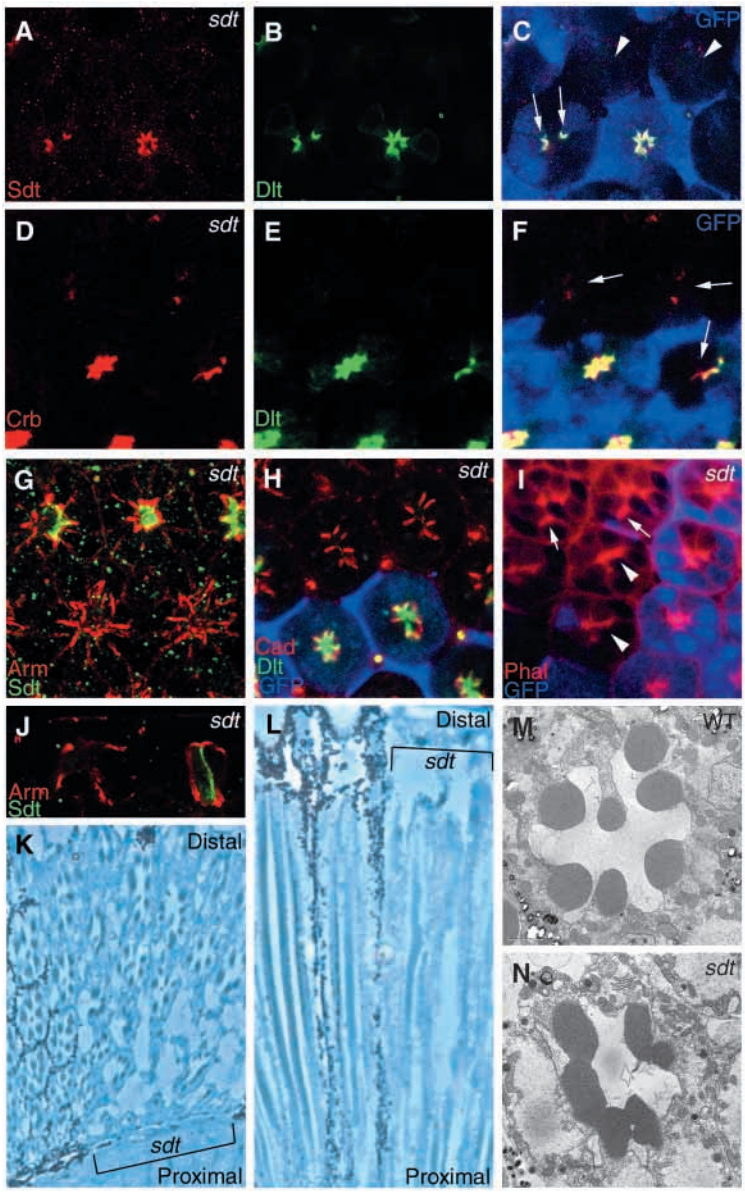



**Fig. 3.** Sdt is required for correct localization of Crb and Dlt. (A-C) Dlt (B, green) is absent (arrowheads) in $sdt^{-}$ clones marked by the absence of GFP (C, blue) or Sdt (A, red) staining. Arrows show the presence of Dlt in $Sdt^{+}$ cells in a mosaic photoreceptor cluster, indicating cell-autonomous function of Sdt. (D-F) Crb (D, red) is absent or strongly reduced in the stalk (arrows in F) of $sdt^{-}$ cells marked by the absence of Dlt (E, green) or GFP (F, blue). (G) Top view of a three-dimensional reconstruction of $sdt^{-}$ clones marked by the absence of Sdt staining (green). Arm staining indicates that AJ was expanded basolaterally and mispositioned in $sdt^{-}$ clones marked by the absence of Sdt (green). (H) DE-cad (red), another marker of AJs is expanded basolaterally. Mutant clones were marked by the absence of GFP staining (blue). (I) F-actins (phalloidin, red) are diffused (arrowheads) and/or displaced from the apical to the lateral position of the photoreceptors (arrows). $sdt^{-}$ mutants are marked by the absence of GFP (blue). (J) Longitudinal section of $sdt^{-}$ clones containing wild-type (right) and $sdt^{-}$ cells (left) marked by the absence of Sdt staining (green). Arm staining is fragmented and widely mispositioned to the lateral positions. (K,L) An oblique (K) and a longitudinal section (L) of $w^{-}$ $sdt^{-}$ adult eye clones marked by the absence of red pigments (labeled with brackets). The oblique section (K) shows arrays of ommatidia from the distal end to the proximal end at the floor of the retina. $sdt^{-}$ ommatidia in the distal region show rhabdomeres, whereas mutant ommatidia in the proximal region show severe disruption. The longitudinal section (L) shows continuous elongated rhabdomeres in $sdt^{+}$ ommatidia and discontinuous disrupted rhabdomeres in $sdt^{-}$ ommatidia. (M,N) Transmission electron micrographs of a wild-type (M) and a $sdt^{-}$ photoreceptor cluster (N) from a mosaic adult eye. These tangential sections were made at the distal region of the eye. The rhabdomeres are bulky and fused in the $sdt^{-}$ photoreceptors (N) compared with the wild type (M).

et al., 2002; Pellikka et al., 2002). To determine whether Sdt is also required for growth and/or maintenance of rhabdomeres, we examined *sdt*⁻ mutant photoreceptors using phalloidin (Fig. 3I) as an F-actin marker. Phalloidin staining showed fragmented or mispositioned rhabdomeres (Fig. 3I), indicating that rhabdomeres fail to grow normally and/or maintain the structure along the length of photoreceptor cells. In a normal ommatidium, the apical surface of photoreceptors points toward the center of each cluster. In *sdt*⁻ mutant clones, the apical domain of photoreceptors failed to orient to the center, resulting in the formation of rhabdomeres in displaced positions (Fig. 3I, arrows). These defects in AJs and rhabdomeres in *sdt*⁻ mutant clones mimic the phenotypes of *crb*⁻ photoreceptors (Izaddoost et al., 2002; Pellikka et al., 2002). As the JM domain of Crb is important for correct positioning of AJ (Klebes and Knust, 2000; Izaddoost et al., 2002) and the PBM domain of Crb is important for binding the Sdt (Bachmann et al., 2001; Hong et al., 2001), *sdt*⁻ phenotypes are likely to be due to the loss of Crb in *sdt* mutant clones.

AJs in *sdt*⁻ photoreceptors (Fig. 3G) were mispositioned basolaterally. A side view of wild-type ommatidium (Fig. 3J) shows smooth and well-defined AJs spanning the length of the retina from the surface to the floor. But, the *sdt*⁻ cells (Fig. 3J) show discontinuous and mispositioned Arm staining. Furthermore, a longitudinal section of the *sdt*⁻ clones in adult eye shows defects of rhabdomere elongation (Fig. 3L). An oblique section of the same eye (labeled with bracket) shows that the rhabdomeres are missing in proximal region, but are relatively well organized in distal region (Fig. 3K). This phenotype of discontinuous rhabdomeres is similar to that of the *crb*⁻ mutant (Izaddoost et al., 2002; Pellikka et al., 2002). Transmission electron microscopy was used to examine the ultrastructure of *sdt*⁻ rhabdomeres and AJs in adult eyes. Rhabdomeres and AJs of wild-type ommatidia adjacent to *sdt*⁻ mutant clones in the same mosaic eye are uniform in size and properly positioned (Fig. 3M). But, the *sdt*⁻ photoreceptors show bulky and fused rhabdomeres (Fig. 3N). The similar adult phenotype of *sdt*⁻ and *crb*⁻ clones suggests that Crb and Sdt are involved in the same cellular function.

## Par-6 and aPKC colocalize with Crb complex in the rhabdomere stalk

It has been shown that *baz*, encoding one of the components of Par-6 complex, genetically interact with *sdt* (Müller and Wieschaus, 1996). This led us to postulate that Crb and Par-6 complexes might function together for the formation and/or maintenance of rhabdomere and AJ in the photoreceptors. To test this, we examined whether the components of the

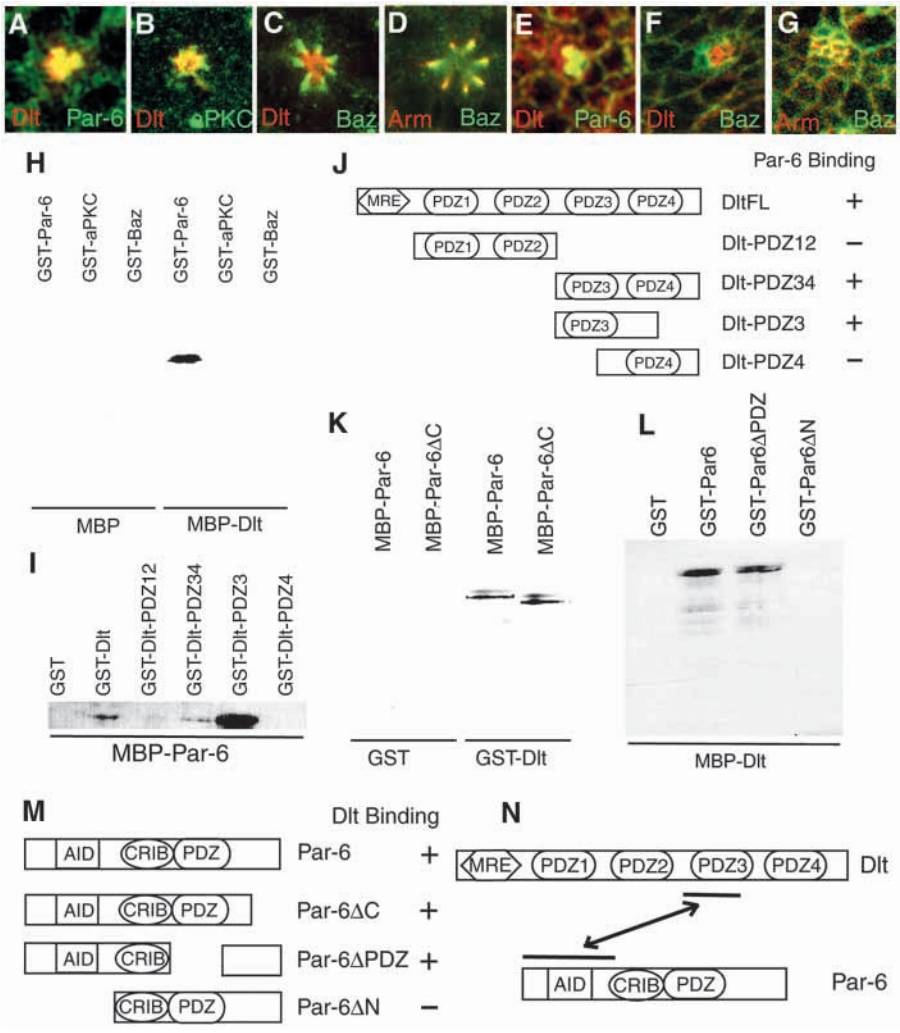


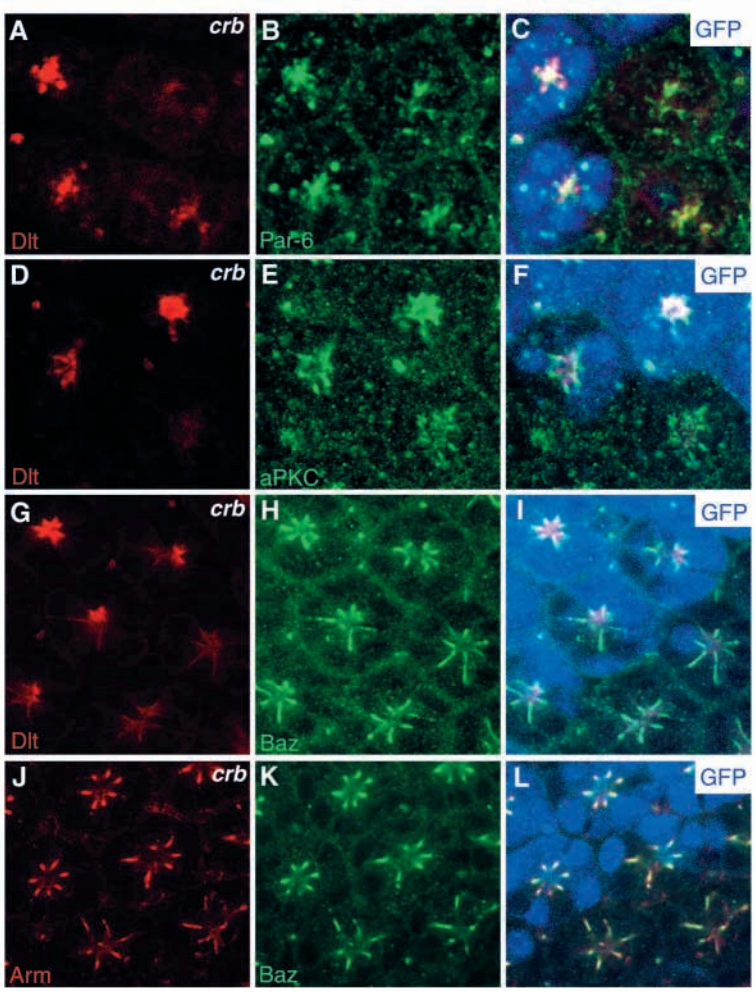

**Fig. 4.** Colocalization of Par-6 complex with Crb complex and direct binding of Dlt to Par-6. (A-D) Both Par-6 (A, green) and aPKC (B, green) colocalize with Dlt (red, A-C) at the apical domain of photoreceptor cluster. aPKC not only colocalizes with Dlt in the apical membrane but also overlaps with AJs (B). Baz does not co-localize with Dlt (C). Baz colocalizes with Arm (D) at the AJ of photoreceptors. (E-G) In eye discs of the third instar larvae, Par-6 colocalizes with Dlt (E) and Baz colocalizes with Arm (G), but not with Dlt (F). (H) Direct binding of Dlt to Par-6, but not to aPKC or Baz. MBP-Dlt fusion protein was attached to amylose bead and used for the binding to GST-Par-6, GST-aPKC or GST-Baz. The blot was probed with anti-GST antibody. (I,J) Direct binding of Dlt by its PDZ3 region with Par-6. Each of GST constructs fused with Dlt deletion mutant proteins was attached to glutathione-agarose bead and used for the binding test with MBP-Par-6. The blots were probed with anti-MBP antibody. (K,L) Deletion of C-terminal PBM motif of Par-6 did not affect the binding of Par-6 to Dlt (K). GST or GST-Dlt was attached to beads and used for binding of MBP-Par-6 or MBP-Par-6ΔC. The blot was probed with anti-MBP antibody (K). The N-terminal region of Par-6 is required for the binding to Dlt, but the PDZ domain is not (L). Each deletion mutant protein of Par-6 was attached to beads and used for binding with MBP-Dlt. The blot was probed with anti-MBP (L). (M,N) Schematic of molecular interaction between Dlt and Par-6. Binding is indicated by two-headed arrow. AID and CRIB are atypical PKC interaction domain and Cdc42-Rac1 interaction domain, respectively.

Par-6 complex colocalize with Crb complex proteins in photoreceptor cells during pupal stages. As shown in Fig. 4A and B, both Par-6 and aPKC colocalized with Dlt at the rhabdomere stalks, suggesting that the Par-6 complex may play important roles in photoreceptor morphogenesis similar to Crb. By contrast, Baz colocalized with Arm at the AJ of photoreceptors basal to Par-6 and aPKC at the rhabdomere stalk (Fig. 4C,D). We examined third instar eye discs to see whether Baz and Par-6 might colocalize to the apical membrane in earlier stages of eye development. However, even in the third instar eye disc Par-6 and Baz localized separately to the apical Dlt domain and the AJ of photoreceptors, respectively, indicating that Baz is targeted to AJs from the early stage of photoreceptor differentiation (Fig. 4E-G). Interestingly, aPKC not only colocalizes with Dlt in the apical membrane but also overlaps with Arm (Fig. 4B; data not shown). Therefore, in contrast to Crb complex proteins that specifically localized to the apical membrane, Par-6 complex proteins appear to be sorted differently for localizations to the apical Crb domain and/or the AJ. Based on the separate localization of Baz from Par-6 and aPKC, 'Par-6 complex' in photoreceptor membranes will be referred hereafter as a complex of Par-6 and aPKC.

### Direct binding of Dlt with Par-6

Based on genetic interaction between *sdt* and *baz* as well as colocalization of Crb/Sdt/Dlt with Par-6, we speculated that the Crb and Par-6 complex might interact by direct association. Among the protein components in the Crb complex, Dlt may play a role as an adaptor molecule because it has four PDZ domains and one MRE domain to recruit other interacting proteins to the Crb complex. Protein interaction assays using MBP-Dlt fusion protein and purified GST-tagged Par-6, aPKC, or Baz proteins indicated specific binding of Dlt to Par-6 but not to aPKC and Baz (Fig. 4H).

To identify the region of Dlt that binds Par-6, we tested mutant forms of Dlt proteins deleted in different PDZ domains for binding to Par-6. This assay showed that the region of PDZ domain 3 (PDZ3) directly interact with Par-6 (Fig. 4I-J). As Par-6 contains a conserved C-terminal PDZ-domain binding motif (–VLHL) of the class II (Hung and Sheng, 2002), we tested whether Dlt binds to Par-6 via the PBM. Interestingly, a mutated Par-6 protein lacking the C-terminal PBM residues was able to bind Dlt similar to full-length of Par-6 (Fig. 4K). As PDZ domains can interact with other PDZ domains (Hung and Sheng, 2002), we tested whether the Dlt interacts with the PDZ domain of Par-6. Par-6ΔPDZ mutant protein deleted in the PDZ domain still showed binding to Dlt. However, Par-6 protein deleted in the N-terminal domain (Par-6ΔN) failed to bind Dlt (Fig. 4L). This result suggests that the N-terminal region of Par-6 is necessary for binding to the PDZ3 region of Dlt (Fig. 4N).




### Crb, Sdt and Dlt are required for localization of Par-6 complex

We have shown that Par-6 and Crb complexes not only colocalize but also directly interact. To evaluate the physiological significance of this interaction, we examined the effects of *crb* mutation on the localization of Par-6/aPKC. In *crb*$^{-}$ photoreceptors, both Par-6 and aPKC (Fig. 5A-F) were displaced or partially lost from the apical region of the rhabdomere stalks. Therefore, Crb is important for proper localization and maintenance of normal level of Par-6 and aPKC although it may not be required for the formation of Par-6 complex. It is important to note that the pattern of mislocalized Par-6 in *crb*$^{-}$ clones was identical to that of aPKC and Dlt, that is, either mislocalized or lost altogether (Fig. 5A-F). This suggests that Dlt may associate with Par-6/aPKC complex in the absence of Crb.

AJs are mislocalized in the absence of Crb. As Baz protein localizes to AJs, it is possible that Baz and AJ are mislocalized together in *crb*$^{-}$ clones. As expected, Baz and the AJ marker Arm showed an identical pattern

**Fig. 5.** Mislocalization of Par-6, aPKC, and Baz in *crb*$^{-}$ photoreceptors. Localization of Par-6 complex proteins was examined in *crb*$^{-}$ mutant clones (No GFP colored in blue). (A-C) Dlt (A, red) is greatly reduced or mislocalized in *crb*$^{-}$ mutants. Par-6 (B, green) is co-mislocalized with Dlt (C). (D-F) aPKC (E, green) is also reduced and co-mislocalized with Dlt (D, red). (G-I) Baz (H, green) is expanded basolaterally and remains basal to Dlt (G, red). (J-L) Baz (K, green) is co-mislocalized with Arm in the basolateral membrane (J, red). Defects in *crb*$^{-}$ cells are cell-autonomous based on the phenotypes specifically associated with *crb*$^{-}$ cells in mosaic photoreceptor clusters.

of mislocalization in the photoreceptors, further supporting that Baz is an AJ component (Fig. 5G-L). As seen with apical markers such as Dlt and Sdt, mislocalization of Baz was also age dependent, showing more severe defects in older pupal eyes (data not shown).

As shown earlier, the localization of Crb and Sdt is interdependent. Therefore, we also examined whether the localizations of Par-6-aPKC-Baz are similarly altered in *sdt*$^{-}$ mutant cells (Fig. 6). Dlt was almost entirely lost in the absence of Sdt, but Par-6 and aPKC were mislocalized and significantly reduced (Fig. 6A-F). This phenotype appeared to be very similar to the mislocalization of Par-6 and aPKC in *crb*$^{-}$ clones. The pattern of abnormal Baz localization in *sdt*$^{-}$ and *crb*$^{-}$ clones was also comparable (Fig. 5G-L and 6G-I). Therefore, loss of either component of the Crb complex results in similar effects on the localization of Par-6 complex proteins. As no *dlt* null mutation is available, we used *dlt*$^{MY10}$, a ~7.5kb deletion that uncovers *dlt* and other flanking genes, α-*spectrin*, *cdc37* and *JTBR* (*Jumping Translocation Breakpoint*). *dlt*$^{MY10}$ clones showed nearly complete loss of Sdt from the apical membrane (Fig. 6J-L). Moreover, *sdt* (Fig. 3D-F; Fig. 6A-I) and *dlt*$^{MY10}$ (data not shown) clones showed essentially indistinguishable pattern of mislocalization of Crb and Par-6 proteins, consistent with the interdependent requirement of Dlt and Sdt for apical organization of photoreceptors.

To determine whether the Crb complex is not only required but also sufficient for localization of Par-6 complex, we misexpressed Crb$^{PBM}$ [Crb$^{intra}$ domain with mutated JM but normal PBM (Izaddoost et al., 2002; Klebes and Knust, 2000)] using *GMR-GAL4* to induce mislocalization of Sdt and Dlt to the basolateral membrane. Under this condition, Par-6 and aPKC were ectopically localized together with Sdt and Dlt all around the cell membranes (Fig. 7A,B), but Baz was not (Fig. 7D). Misexpression of Crb$^{JM}$ (Crb$^{intra}$ with normal JM but PBM deletion) using *GMR-Gal4* can ectopically recruit AJs in basolateral membranes (Izaddoost et al., 2002; Klebes and Knust, 2000). In the same situation, Baz was also ectopically recruited together with Arm (Fig. 7E), but Dlt and Par-6 were not (Fig. 7C,F). Therefore, the Crb complex is necessary and sufficient for targeting of Par-6 complex.

## Localization of Crb complex and AJ depends on Par-6 complex

Direct interaction between Crb and Par-6 complexes suggests that Crb complex is not only required for Par-6 localization but also dependent on the Par-6 complex for its localization to the rhabdomere stalk. To test the function of Par-6 complex, we examined the localization of Crb complex proteins in clones of *par-6*$^{\Delta 226}$ (Petronczki and Knoblich, 2001), *aPKC*$^{K06304}$ (Wodarz et al., 2000) and *baz*$^{Xi106}$ (Wodarz et al., 1999) null mutants. Most clones of *par-6*$^{-}$ and *aPKC*$^{-}$ mutations were very small consisting of a few cells, suggesting that Par-6 and aPKC are important for proliferation and/or survival of retinal cells.

In *par-6*$^{-}$ photoreceptors, Dlt and Arm were strongly reduced and/or mislocalized (Fig. 8A-B). Dlt and Arm were also mislocalized in *aPKC*$^{-}$ mutant photoreceptors (Fig. 8C-D). Interestingly, Dlt failed to localize to the membrane, resulting in a diffused distribution from the apical membrane (Fig. 8C). In *baz*$^{-}$ mutants, Arm was mislocalized (Fig. 8F) consistent with the localization of Baz to the AJ. The mislocalized Arm was detectable in proximal region, but completely lost in distal region (data not shown). Surprisingly, the *baz*$^{-}$ mutation caused almost complete loss of Dlt which is localized apical to the AJ in normal photoreceptors (Fig. 8E).

These results demonstrate that Par-6 complex proteins are essential for proper localization of Crb complex and AJ components. Notably, Crb complex was lost or unable to localize to the membrane in the absence of Par-6 complex. By contrast, Par-6 complex remained in the membrane although mislocalized, in the absence of Crb complex. This phenotypic difference suggests that Par-6 complex may play primary role for membrane targeting of the Crb complex, and Crb complex proteins might be essential for maintaining the Par-6 complex.

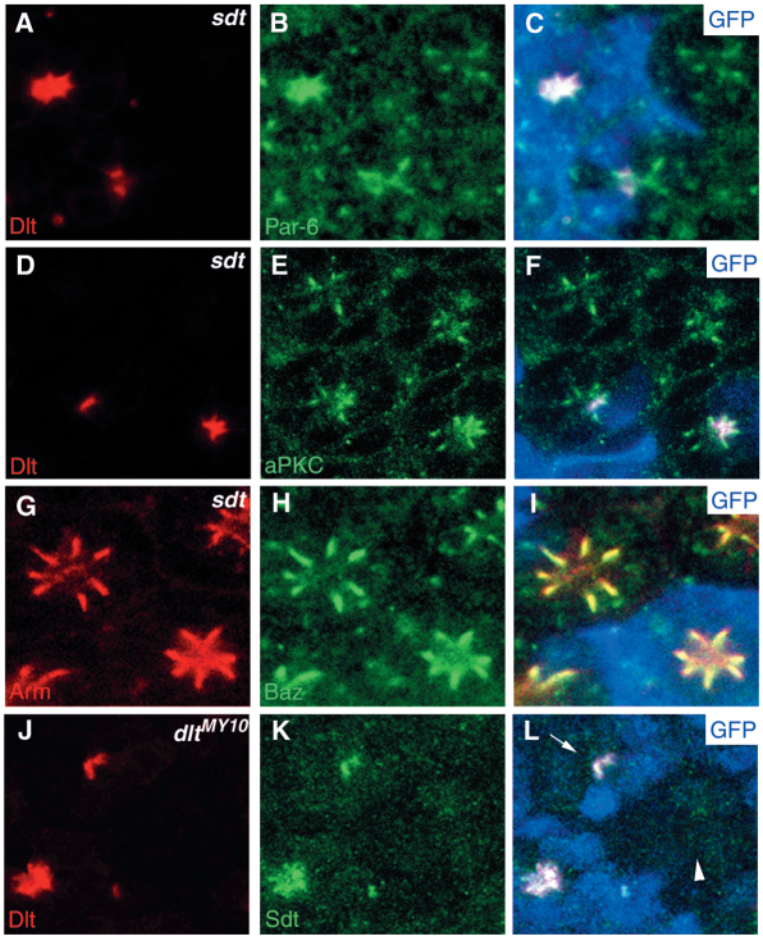



**Fig. 6.** Mislocalization of Par-6, aPKC and Baz in *sdt*$^{-}$ and loss of Sdt in *dlt*$^{MY10}$. The localization of Par-6, aPKC, and Baz was examined in *sdt*$^{-}$ mutants (no GFP in blue). (A-C) Par-6 (B, green) is mislocalized whereas Dlt (A, red) is undetectable in *sdt*$^{-}$ mutants. (D-F) aPKC (E, green) is mislocalized in *sdt*$^{-}$ mutants. (G-I) Baz (H, green) is basolaterally expanded and colocalized with Arm (G, red) in *sdt*$^{-}$ mutants. (J-L) Sdt (K, green) is lost in *dlt*$^{MY10}$ mutant photoreceptor cluster (arrowhead) indicated by the absence of Dlt (J, red). The arrow indicates cell-autonomous loss of Sdt in *dlt*$^{MY10}$ cells in a mosaic cluster.

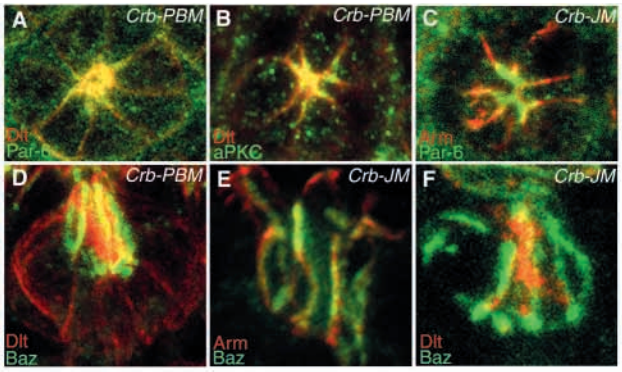



**Fig. 7.** $Crb^{intra}$ is sufficient to recruit Par-6 complex and AJs. (A,B) Overexpression of $Crb^{PBM}$ causes ectopic co-localization of Dlt (red), Par-6 (A; green) and aPKC (B; green). (C) Arm (red) is displaced by the overexpression of $Crb^{JM}$, but Par-6 (green) is not. (D-F) The localization of Baz was examined using $Crb^{PBM}$ (D) or $Crb^{JM}$ (E,F) overexpression. Dlt (red) was ectopically mislocalized by the $Crb^{PBM}$ expression (D), but Baz (green) was not. Baz (E,F) was ectopically displaced by the $Crb^{JM}$ overexpression. In this situation, Arm (E; red) was mispositioned with Baz, but Dlt (F; red) was not. A-C are tangential sections; D-F are longitudinal sections.

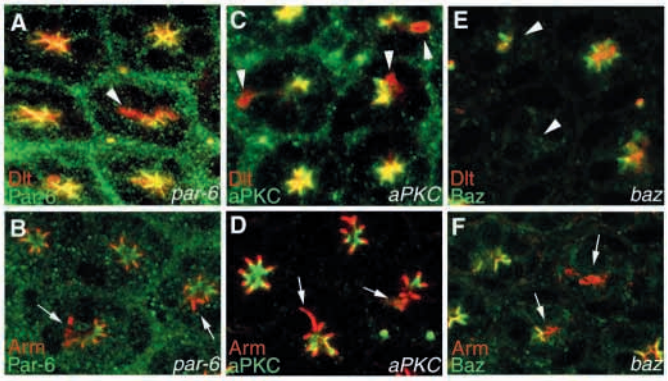



**Fig. 8.** Par-6 complex is required for localization of Crb complex and AJ. (A,B) Dlt (arrowhead; A, red) and Arm (arrows; B, red) are mislocalized in *par-6*$^{-}$ mutant cells marked by loss of Par-6 (green) staining. (C,D) Dlt (arrowheads; C, red) is diffused away from apical membrane, and Arm (arrows; D, red) is displaced in *aPKC*$^{-}$ mutants marked by the absence of aPKC (green) staining. (E,F) In *baz*$^{-}$ clones indicated by loss of Baz (green) staining, Dlt (arrowheads; E, red) is lost and Arm (arrows; F, red) is mislocalized at proximal section. But Arm is not detected at distal section (data not shown).

## DISCUSSION

Crb and Par-6 form two distinct protein complexes involved in the establishment of embryonic epithelial cell polarity. In this study, we examined whether these complexes are required for the organization of rhabdomeres and AJs of photoreceptors, and whether this process is controlled by interaction of the two complexes.

### Interdependency of Crb-Sdt-Dlt for their apical membrane localization

In the absence of Crb, Sdt is mislocalized together with Dlt from the rhabdomere stalk. By contrast, both Crb and Dlt are almost absent in *sdt* mutants. Our results provide in vivo evidence for the inter-dependent function of Crb complex proteins in the developing retina. The strong dependence of Crb localization on Sdt and Dlt suggests that Crb may be destabilized or may not be targeted to the membrane in the absence of Sdt or Dlt. It is intriguing that Sdt and Dlt are lost only partially in the absence of Crb. Our findings of a direct interaction between Dlt and Par-6 suggest that Sdt-Dlt can still be targeted to the membrane in the absence of Crb through the binding of Dlt to the Par-6 complex. However, it is important to note that Dlt is essentially lost in *sdt* mutant clones and *vice versa*. This raises an intriguing possibility that Dlt or Sdt are dependent on each other in vivo to be targeted to the apical membrane via binding to either Crb or Par-6. This mutual dependency between Dlt and Sdt may explain why Dlt and Sdt are lost in the absence of the other, rather than being associated with the Par-6 complex.

### Direct interaction of Crb and Par-6 complexes

The interaction between the Crb and Par-6 complexes is mediated by the PDZ3 region of Dlt and the N-terminal domain of Par-6 (Fig. 4N). The N-terminal domain of Par-6 (Fig. 4L) is also used for binding aPKC (Joberty et al., 2000; Lin et al., 2000; Qiu et al., 2000). Therefore, a potential function of Dlt is to bind Par-6 in competition with aPKC or to facilitate the interaction of Par-6 with aPKC or other Par-6 binding proteins. Our mutant analysis indicates that loss of Dlt and Sdt in *sdt*$^{-}$ clones causes mislocalization of both Crb and Par-6 complex proteins (Fig. 3D-F; Fig. 6A-I). This suggests that Sdt-Dlt interaction provides a scaffold to recruit Crb complex to the Par-6 complex and enhance the stability of these two complexes rather than functioning as a competitor for aPKC.

Proteins in Crb and Par-6 complexes consist of multiple functional domains which may be involved in diverse protein-protein interactions. A recent study has shown that in mammalian cell culture systems the PDZ domain of Par-6 binds not only Par-3 but also the N terminus of Pals1 (Hurd et al., 2003). These results suggest that the crosstalk between the Crb and Par-6 complexes is mediated by multiple domain-specific interactions. Evidence from our genetic analysis using mutants suggests that the crosstalk between the two complexes is mutually required for normal organization of apical membranes and AJs in vivo, and also provides a basis for partial redundancy of these complexes in the organization of photoreceptor cell polarity. Interestingly, when either Crb or Sdt is lost, mislocalization or elimination of other associated components including Par-6 complex proteins becomes more severe in the age-dependent manner. This suggests that the Crb complex may be required for the maintenance rather than the formation of the Par-6 complex. The age-dependent degenerative phenotype may be related to the requirement of extensive apical membrane growth to make rhabdomeres and AJs along the growing axis of photoreceptors during pupal stage. Loss of any one component of the Crb complex is likely to be increasingly more detrimental as the process of membrane reorganization proceeds. In *crb*$^{-}$ or *sdt*$^{-}$ mutants, significant fractions of Par-6 complex proteins remain in the membrane despite the age-dependent and progressive mislocalization of apical markers. By contrast, loss of Par-6 or aPKC resulted in mislocalization of Dlt from the apical membrane (Fig. 8). This suggests that the Par-6 complex plays

essential functions for membrane localization of Crb complex proteins. Furthermore, both Par-6 and aPKC seem to be important for survival and/or proliferation of retinal cells as mutant clones were very small compared with adjacent twin spots and often completely disrupted probably due to cell death. This is consistent with the findings of frequent apoptosis in *aPKC*$^{-}$ or *par-6*$^{-}$ embryos (Petronczki and Knoblich, 2001; Wodarz et al., 2000).

### Localization of Baz at AJs of photoreceptors

An important distinction of Par-6 complex in the photoreceptors from other epithelia is the localization of Baz. Baz localizes with Crb complex in the subapical membrane (Kuchinke et al., 1998; Wodarz et al., 2000) or both the subapical region and AJ (Bilder et al., 2003) in the *Drosophila* embryonic epithelia. Vertebrate Par-3 also localizes to the apical tight junction in vertebrate epithelial cells (Izumi et al., 1998; Suzuki et al., 2001). By contrast, Baz in the photoreceptors are specifically positioned in the AJs basal to the all other proteins in the Crb/Par-6 complexes. As shown in Fig. 7E, Baz and Arm are recruited together to ectopic membrane sites by misexpression of Crb$^{JM}$, suggesting that Baz is an integral component of AJ. However, Baz is not recruited by Crb$^{PBM}$, whereas Par-6 and aPKC can be ectopically recruited by Crb$^{PBM}$ rather than Crb$^{JM}$. Therefore, Baz appears to be recruited to AJ independently of Par-6/aPKC.

Intriguingly, despite its specific localization to AJs, loss of Baz resulted in most severe disruption of AJ as well as the more apical Dlt domain. It has been proposed that the Par-6/aPKC cassette is recruited to the site of cell-cell contact and then moves along the most apical zone of the developing cell-cell contact. In this process, an important step for cell polarity formation is to tether the cytoplasmic Par-6/aPKC complex to the site of cell-cell contact at the membrane which is mediated by the interaction of Par-3 and a membrane protein JAM (Ohno, 2001). Therefore, our results that *baz* mutation causes loss of Dlt and AJs support the crucial role of Baz in the initial step of cell polarization. However, the distinct localization of Baz from Par-6 and aPKC in the photoreceptors suggests that the mode of Baz localization varies in different systems. In photoreceptors, Baz may be targeted to the membrane with Par-6 but be sorted out from Par-6 in subsequent steps of polarization to remain in the AJs, whereas Par-6-aPKC-Baz cassette remains together in the complex in other epithelia. In contrast to Baz, aPKC localizes to both rhabdomere stalk and AJ (Fig. 4B), suggesting that Baz and Par-6 are completely separated during polarization while aPKC is not sorted from both Par-6 and Baz. The critical function of Baz in the localization of Crb complex in the rhabdomere stalk is consistent with the requirement of Baz for Crb localization in embryonic epithelia (Bilder et al., 2003). However, the requirement of Baz in the embryo appears to be dependent on the stage of development as Crb distribution in the absence of Baz becomes normal in late embryos (Tanentzapf and Tepass, 2003). On the contrary, such stage-dependent recovery of Crb complex localization has not been observed in *baz*$^{-}$ photoreceptor cells.

Recent studies have shown that mutations in human *CRB1* cause RP12 and LCA, severe recessive retinal diseases, emphasizing the importance of Crb family proteins in the eyes of mammals including humans. The *Drosophila* Crb and human CRB1 are localized in analogous subcellular membrane domains of photoreceptors, the rhabdomere stalk and the inner segment in *Drosophila* and human photoreceptors, respectively. Besides similar subcellular localization, Crb and human CRB1 are functionally conserved (den Hollander et al., 2001b; Izaddoost et al., 2002; Roh et al., 2002). Age-dependent photoreceptor defects in *crb* mutant also provide analogy to age-dependent retinal degeneration in RP12/LCA patients. Our studies here imply that hCRB1 may function as a protein complex with homologs of Sdt and Dlt and such complex may interact with a homologous Par-6 complex. Whether such homologous human genes are the targets of inherited retinal diseases such as RP remains to be studied.

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