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Short Communication

Rab11 is required during *Drosophila* eye development

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ABSTRACT In an effort to identify the role of Rab11, a small GTP binding protein, during *Drosophila* differentiation, phenotypic manifestations associated with different alleles of *Rab11* were studied. The phenotypes ranged from eye-defects, bristle abnormalities and sterility to lethality during various developmental stages. In this paper, our focus is targeted on eye defects caused by *Rab11* mutations. A novel P-element insertion in the Rab11 locus, *Rab11^{mo}*, displayed characteristic retinal anomalies, which could be reverted by P-element excision and expression of *Rab11^{+}* transgenes. During larval development, Rab11 is widely synthesized in photoreceptor cells and localizes to the rhabdomeres and lamina neuropil in adult eyes. Photoreceptors and associated bristles failed to be formed in homozygous clones generated in *Rab11$^{EP(3)3017}$* eyes. Decreased levels of Rab11 protein and increased cell death in *Rab11^{mo}* third-instar larval eye-antennal discs suggest that the retinal defects originate during larval development. Our data indicate a requirement for Rab11 in ommatidial differentiation during *Drosophila* eye development.

KEY WORDS: *Drosophila, Rab11, photoreceptor cell, rhabdomere*

The regulation of intercellular communication is essential for proper development and survival of all multicellular organisms. This communication is brought about by well coordinated protein transport mechanisms which determine the route by which proteins and peptides are released into its surrounding micro-environment. Eukaryotic cells contain a highly dynamic set of membrane compartments that are responsible for packaging, sorting and recycling of bio-molecules whose transport is mediated by vesicles. Vesicle trafficking is regulated by specific players among which the Rab/Ypt family of proteins plays a major role. These proteins constitute the largest group within the Ras GTPase superfamily and are master regulators of vesicular transport in eukaryotic cells (Urbe *et al*., 1993; Zerial and McBride, 2001). Mammalian genomes contain more than 60 known *Rab* genes (Bock *et al*., 2001), while 29 *Rab* genes have been so far identified in *Drosophila* (Pereira-Leal and Seabra, 2001). Each Rab protein is characterised by a distinctive localisation in the cell and works at a specific stage of vesicular transport pathway.

The vertebrate members of the *Rab11* subfamily - *Rab11a*, *Rab11b* and *Rab25* - have been reported as regulators of endocytic membrane recycling in both polarized and non polarized cells (Casanova *et al*., 1999; Cox *et al*., 2000; Wang *et al*., 2000) and are also involved in exocytosis (Chen *et al*., 1998; Urbe *et al*., 1993; Goldenring *et al*., 1994; Goldenring *et al*., 1996; Calhoun and Goldenring, 1997; Calhoun *et al*., 1998). These studies that were performed on unicellular organisms or isolated cultured cells provided insights into the molecular properties and subcellular localisation of Rab11 as well as on the underlying mechanism of vesicular transport. However, it is only recently that the role of Rab11 during development and differentiation has begun to be appreciated. *Drosophila* has a single *Rab11* gene and studies have shown its requirement during oocyte polarization (Dollar *et al*., 2002; Jankovics *et al*., 2001), cytoskeletal remodeling (Riggs *et al*., 2003) and membrane morphogenesis (Pelissier *et al*., 2003; Zerial and McBride, 2001).

Due to precisely organized architecture, the *Drosophila* eye is an effective model for addressing questions related to processes including cell signaling, neuronal connectivity, control of cell proliferation and vesicular transport. Vesicular transport is undoubtedly critical during eye development. Over the last few years, studies of *Drosophila* Rab1 and Rab6 demonstrated the role of these proteins as important players in processing and/or transport of rhodopsins (Satoh *et al*., 1997, 2005; Shetty *et al*., 1998). In this communication, we show that Rab11 is one of the key players during *Drosophila* eye development.

Results and Discussion

Punctate Rab11 staining in eye imaginal discs

As the C-terminal domains of Rab proteins contain specific motifs directing vesicle targeting (Chavrier *et al*., 1991), we generated Rab11 polyclonal antibodies against a C-terminal 27-

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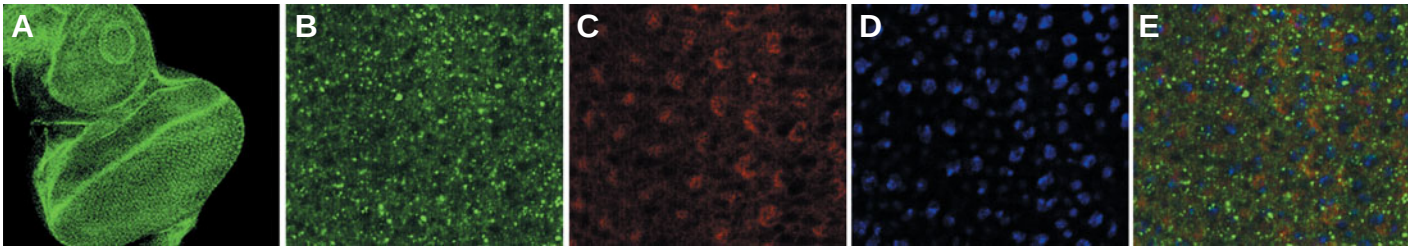
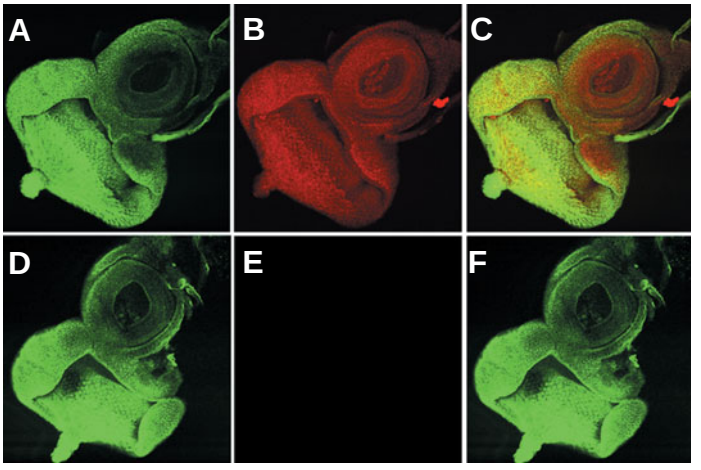


Fig. 1. Distribution of Rab11 on third-instar eye-antennal discs. (A) *The Rab11 protein is widely distributed in all ommatidial and antennal cells.* **(B)** *Higher magnification of the ommatidial cells reveals a puntate staining.* **(C, D)** *Counterstaining of the immunostained eye disc with Rhodamine-Phalloidin and DAPI, which detect F-actin and DNA, respectively.* **(E)** *Merged view of (B, C and D) showing the cytoplasmic localisation of Rab11 and a partial overlap with F-actin.*

mer synthetic peptide. These antibodies were affinity purified and used for localising Rab11 in tissues. Western blot of proteins extracted from various developmental stages showed a single polypeptide of 23 kDa corresponding to the expected molecular mass of Rab11. Immunostaining on eye-antennal discs from third-instar larvae revealed that Rab11 is distributed in the ommatidial precursor cells and in the antennal cells (Fig. 1A). Higher magnification (Fig. 1B) reveals a punctate staining pattern characteristic of a protein involved in vesicular transport. Counterstaining was done with Phalloidin, which specifically stains polymerised actin or F-actin (Fig. 1C) and DAPI for DNA (Fig. 1D). As shown in Fig. 1E, Rab11 predominantly localises in the cytoplasm and occasionally overlaps with F-actin. The punctate staining indicates association with membrane organelles. Specificity of the antibodies was confirmed by competing antibody binding with the 27-mer synthetic peptide. When the eye-antennal discs were immunostained with an overnight pre-incubated mixture of anti-tubulin and anti-Rab11 antibodies, the expected patterns of staining were observed (Fig. 2 A,B). However, when the discs were immunostained with a pre-incubated mixture of anti-tubulin and anti-Rab11 antibodies in the presence of the 27-mer synthetic peptide, no Rab11 staining could be detected (Fig. 2E); although the discs showed expected pattern of staining for tubulin (Fig. 2D). Immunostaining on adult head sections revealed a strong distribution of Rab11 in the rhabdomeres, lamina neuropil and photoreceptor terminals (Fig. 3A) whereas no Rab11 could be detected in the medulla and lobula (Fig. 3B). All together, the localisation of Rab11 in the developing eyes indicates that this protein may play a critical role during the development of this organ in larval or pupal stages. On this basis, we investigated the requirement of Rab11 during eye development in *Drosophila*.

Rab11 is essential for viability of photoreceptors and bristles

Since the mutant animals for the two available alleles of Rab11, namely, $Rab11^{l(3)j2D1}$ and $Rab11^{EP(3)3017}$ - die as embryos and early larvae, we generated $Rab11^{l(3)j2D1}$ somatic clones using *EGUF/hid* method (Stowers and Schwarz, 1999) and analysed their phenotype in adult eyes. We found that all the *FRT-*$Rab11^{l(3)j2D1}$ clones displayed normal ommatidia (Fig. 4B). Scanning electron microscopic analysis of a representative homozygous clone revealed wild type eye architecture (Fig. 4E, H). Since $Rab11^{l(3)j2D1}$ results from a P-element insertion into the second intron of *Rab11*, it is plausible that the insert may specifically affect early development and viability but may not be essential for the eye development. To circumvent the absence of an eye phenotype we used the $Rab11^{EP(3)3017}$ to generate retinal clones. In homozygous *FRT-*$Rab11^{EP(3)3017}$ clones we found that the photoreceptor cells and associated bristles were absent indicating that the mutation induced cell lethality (Fig. 4C). Scanning electron microscopic studies of a representative homozygous clone revealed the absence of photoreceptor cells and bristles (Fig. 4F, I). We concluded that normal function of Rab11 is essential for cell



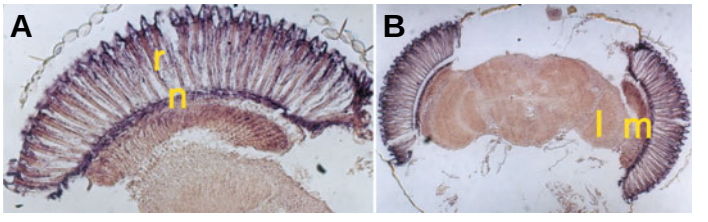


Fig. 2 (Left). Specificity of the Rab11 antibody. (A-C) *Eye-antennal disc stained with anti-tubulin and anti-Rab11 antibodies (control) showing normal pattern of distribution of tubulin* **(A)** *and Rab11* **(B)** *proteins.* **(D-F)** *Eye-antennal disc stained with a pre-incubated mixture of anti-tubulin, anti-Rab11 antibodies. and the 27-mer synthetic peptide used for generating the rabbit anti-Rab11 antibodies, showing normal staining for tubulin* **(D)** *and absence of staining for Rab11* **(E)**. *(C) and (F) are merged pictures of (A,B) and (D,E) respectively.*

Fig. 3 (Right). Rab11 distribution in adult head and eye sections. (A) *Rab11 is detected in the photoreceptors, primarily in the rhabdomeres (r) and the lamina neuropil (n).* **(B)** *No significant Rab11 staining could be detected in medulla (m) and lobula (l). (A) is an enlargement of eye shown to the right in (B).*

viability in eyes. In order to complement the observations made with *Rab11*$^{EP(3)3017}$, it was desirable to have an adult viable allele of Rab11, which could be directly used for such phenotypic studies. One such insertion was recovered during a P-element mutagenesis screen in our laboratory and was utilized for this study.

Ommatidia defects linked to P-insertion in the Rab11 *gene*

By mobilizing a P-element in a nearby locus, we generated an insertion in Rab11 gene. *In-situ* localization of this P-element on the polytene chromosomes mapped the insertion to 93B region. Sequence analysis of a 1.9kb (1.1 kb and 0.8 kb) plasmid rescued fragments revealed a P-element insertion in the 5' regulatory region of the *Rab11* gene (Fig. 5). This mutation produced viable adults. The mutant animals displayed low viability and some homozygotes survived only till larval, pupal or pharate adult stages. The pharate adults as well as adults showed dark patches in the ommatidia, which upon close examination proved to be malformed ommatidia, thus this allele was named as *Rab11*^{mo}. *Rab11*$^{l(3)j2D1}$, an insertion in second intron and *Rab11*$^{EP(3)3017}$, an insertion in second exon of *Rab11* gene die as embryos and during first instar stage of development, respectively (Fig. 5). *Rab11*^{mo} homozygotes were also sterile and showed antennal bristle abnormalities, phenotypes that have been noted in other Rab11 alleles (Abdelilah-Seyfried *et al*., 2000; Jankovics *et al*., 2001; Dollar *et al*., 2002).

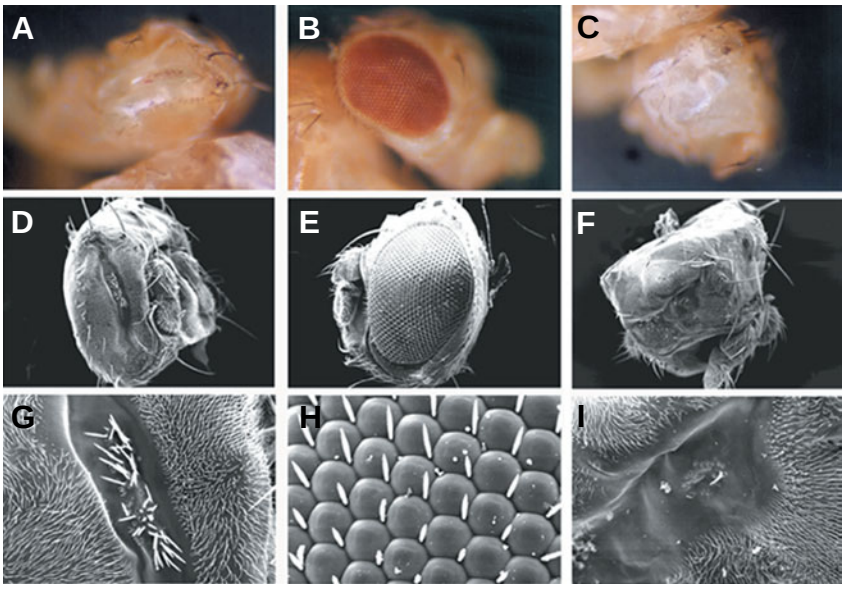


Fig. 4. Light and scanning electron microscopic analysis of *Rab11* mutant somatic clones. (A,D,G) *In yw; EGUF/+; FRT82B GMR-hid3R CL/FRT82B GMR-hid3R CL eyes photoreceptor cells were destroyed due to the induction of cell death by GMR-hid.* **(B,E,H)** *In yw; EGUF/+; FRT82BGMR-hid3R CL3R/FRT82B Rab11*$^{l(3)j2D1}$ *the eye organization is similar to that of wild type showing a regular pattern in the ommatidial distribution.* **(C,F,I)** *In yw; EGUF/+; FRT82B GMR-hid3R CL3R/FRT82BRab11*$^{EP(3)3017}$ *eyes all photoreceptor cells are missing indicating that Rab11 is required for photoreceptor cell differentiation and viability.*

To determine whether the P-insert in *Rab11* is responsible for these defects we carried out excision of the P-element in *Rab11*^{mo} and generated revertants. Out of 53 revertants, one pure excision line exhibiting a wild type phenotype was obtained. This indicates that the phenotype/s were only due to the Rab11 mutation and no second site mutation was responsible. To further test this conclusion, we constructed *pCasper-Rab11* transgene and following transformation we obtained two independent Rab11 transgenic lines *(pCasper-Rab11a* & *pCasper-Rab11b)*. Enhanced level of Rab11 protein of the expected size was obtained on Western blots of protein extracts from *Rab11* transgenic larvae in comparison to wild type, hence indicating them to be functional transgenes. The *pCasper-Rab11a; Rab11*^{mo}*/Rab11*^{mo} flies showed nearly wild type eye morphology indicating that the mutant phenotype resulted from a mutation in *Rab11*. Apart from the eye defects, the transgene also abolished all other mutant phenotypes including antennal bristle abnormalities and sterility. We even found that a single copy of the *Rab11* transgene could restore viability of *Rab11*$^{EP(3)3017}$ and *Rab11*$^{l(3)j2D1}$ homozygous animals.

In this communication, we particularly focused our attention on the eye defects produced by *Rab11*^{mo}. Numerous dark patches, which were variable in size, could be detected in *Rab11*^{mo} eyes (Fig. 6B) and showed no site-specificity. Some eyes had only 2-3 small isolated dark patches while others contained hardly any normal ommatidia. A representative wild type eye is compared with the mutant eye in Fig. 6 A,C, respectively. Adult escapers of *Rab11*^{mo}*/Rab11*$^{EP(3)3017}$ transheterozygotes, which were primarily larval lethal, also showed eye defects while the eyes of *Rab11*^{mo}*/Rab11*$^{l(3)j2D1}$ individuals were normal with no obvious defects (Fig. 6D).

Scanning electron microscopic (SEM) studies revealed that the dark patches were made of deformed ommatidial cells. In comparison to the wild type regular ommatidial arrangement (Fig. 6 E,I), fused ommatidia and missing bristles were detected in *Rab11*^{mo}*/Rab11*^{mo} and *Rab11*^{mo}*/Rab11*$^{EP(3)3017}$ (Fig. 6 F,G,J.K). No alteration could be detected in *Rab11*^{mo}*/Rab11*$^{l(3)j2D1}$ (Fig. 6 H,L). We wondered if the dark patches, fused ommatidia and missing bristles correspond to something deep within the ommatidia.

Histology contributed to our understanding of the nature of

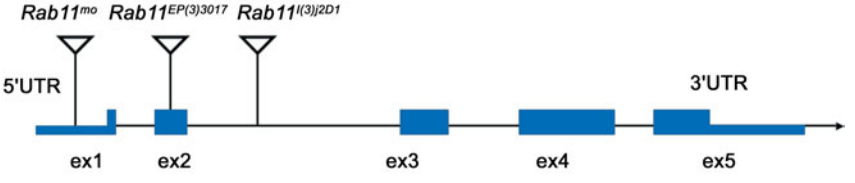


Fig. 5. Schematic representation depicting the position of P-element insertions in Rab11 mutants. *The exons have been represented as 'ex' and the introns are shown as thin lines. The 5'UTR and 3'UTR are marked. Rab11*^{mo} *is an insertion in the 5' regulatory region, Rab11*$^{l(3)j2D1}$ *is an insertion in the second intron and Rab11*$^{EP(3)3017}$ *in the second exon of Rab11 gene, respectively.*

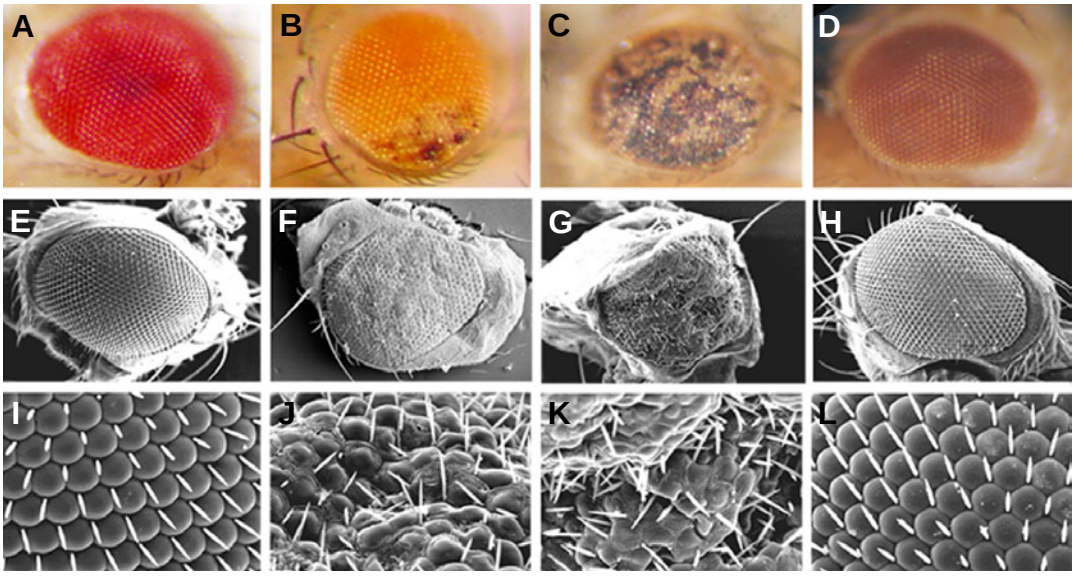


Fig. 6. Eye defects associated with *Rab11* alleles. (A,E,I) *Eyes from wild type flies showing an organised ommatidial architecture.* **(B,F,J)** *and* **(C,G,K)** *Eyes of Rab11^{mo}/Rab11^{mo} showing abnormalities from minor* **(B)** *to greater severity* **(C)**. **(F,G,J,K)** *SEM pictures show fusion of ommatidia and missing bristles in mutant individuals. Rab11^{mo}/Rab11$^{EP(3)3017}$ adult esacpers also displayed retinal defects.* **(D,H,L)** *Eyes of Rab11^{mo}/Rab11$^{l(3)j2D1}$ flies showed no obvious defect and were similar to wild type.*

retinal anomalies. Toluidine blue stained sections of adult eyes from flies of different genotypes were examined. In comparison to the wild type regular ommatidial arrangement (Fig. 7A), photoreceptor cells could be hardly seen in the severely affected *Rab11^{mo}/Rab11^{mo}* mutant eyes (Fig. 7B). In these individuals, only residual cell fragments appeared to be present in the retina. The drastic loss of retinal structures could be related to the frequent collapse of the eyes noticed during critical point drying for SEM studies. Eye sections of the *Rab11* revertant and of transgenic *pCasper-Rab11a; Rab11^{mo}/Rab11^{mo}* flies displayed a regular ommatidial arrangement and preservation of the photoreceptor rhabdomere structure (Fig. 7 C,D).

Since a single *Rab11* transgene could abolish the defects associated with the *Rab11^{mo}* mutations, it is possible to envisage that a decrease in the level of Rab11 protein in *Rab11^{mo}* could lead to retinal defects. A comparison of the amount of Rab11 proteins between wild type and *Rab11^{mo}* flies revealed only a decrease of 10-50% in mutant individuals (Fig. 8A). Similarly, the body extracts showed 20% decrease in Rab11 level in the mutants. In contrast, when fly heads were separated from the rest of the body, we noticed an eight-fold decrease in Rab11 protein in mutant heads compared to wild type heads (Fig. 8A). This suggests that *Rab11^{mo}* mutation is eye specific but the interpretation is confounded by the loss of ommatidia in *Rab11^{mo}* mutants. It raises the concern whether the Rab11 decrease in adult eyes would result from the loss of ommatidia or correspond to a genuine decrease which had occurred during earlier developmental stages. It is plausible that a decrease in Rab11 during larval and pupal stages could be the cause of the deformed ommatidia seen in *Rab11^{mo}* flies. To confront this we performed immunostaining on eye-antennal discs from third-instar *Rab11^{mo}* larvae and found a noticeable reduction in Rab11 protein level in the *Rab11^{mo}* eye-antennal discs (Fig. 8C) in comparison to that in wild type discs (Fig. 8B) even though the number of ommatidial cells were similar to wild type individuals (as seen by Elav antibody staining in the later section), indicating that a decrease in Rab11 protein during third-instar larval development leads to retinal defects in adults.

Retinal deformities originate during late larval development

In *Drosophila* eye, cell differentiation begins with the progression of the morphogenetic furrow across the field of progenitor cells, such that cells at the furrow are just beginning to differentiate, whereas those situated more posteriorly are progressively developing (Ready *et al.*, 1976). Immuno-fluorescence staining of the Elav protein in third-instar larval eye discs revealed an abnormal ommatidial arrangement in *Rab11^{mo}/Rab11^{mo}* (Fig. 9A, B). Similarly, immunostaining with the monoclonal anti-Mab22C10 antibody showed disorganised bundles of axons between the brain and the eye discs in *Rab11^{mo}* (Fig. 9C, D). Finally acridine orange staining revealed an increased number of dying cells below the morphogenetic furrow in *Rab11^{mo}* by comparison to wild-type (Fig. 9E, F). Taken together, these data suggest that the retinal defects could be traced to ommatidial differentiation which

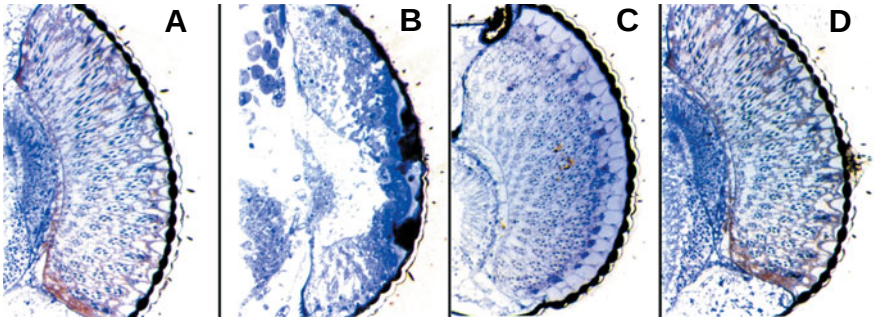


Fig. 7. Toluidine blue stained retinal sections (A) *Horizontal section of a wild type fly head showing the regular arrangement of ommatidial cells.* **(B)** *Horizontal section of a Rab11^{mo}/Rab11^{mo} eye showing a disorganised ommatidial cell arrangement. Note the severe loss of retinal structures.* **(C)** *Horizontal section of a Rab11 revertant eye showing the regular wild type ommatidial and* **(D)** *horizontal sections of Pcasper-Rab11a; Rab11^{mo}/Rab11^{mo} flies showing a normal ommatidial arrangement.*

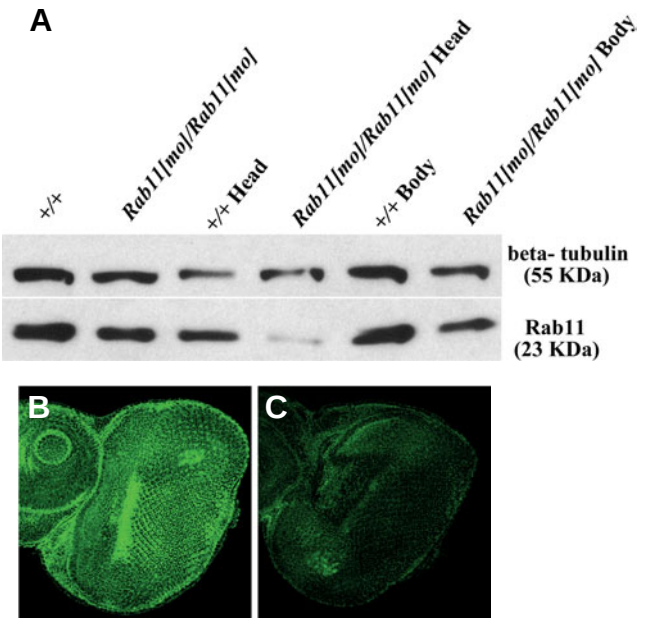


Fig. 8. Levels of Rab11 protein in *Rab11^{mo}/Rab11^{mo}* tissues. (A) *Western analysis of adult extracts showing a decrease in Rab11 protein in Rab11^{mo}/Rab11^{mo} individuals in comparison to wild type. Note the pronounced decrease of Rab11 level in head in comparison to body. Control loading was performed by comparing the amount of beta-tubulin.* **(B)** *Immuno-staining of Rab11 protein in wild type and* **(C)** *Rab11^{mo}/Rab11^{mo} third-instar larval discs. A decrease in the intensity of of Rab11 staining is less intense in (C) compared to (B).*

takes place during late larval development and to the death of numerous neuronal cells during late third instar and early pupal stages. As ommatidial morphogenesis is a complicated process, inadequate membrane growth and recruitment, as well as anomaly in rhodopsin transport due to altered Rab11 protein may lead to eye degeneration in mutant eye discs. In support of our conclusion, a very recent article also describes the requirement of Rab11 for the transport of rhodopsin in *Drosophila* photoreceptor cells (Satoh *et al.*, 2005)

In summary, our results demonstrated the role of *Drosophila* Rab11 during eye morphogenesis. We showed that the *Rab11^{mo}* P-element insertion induce retinal deformities which could be abolished with a *Rab11* transgene. Precise excision of the P-element from *Rab11^{mo}* also leads to reversion of the mutant phenotype. We found that a reduced level of Rab11 protein is associated with cell death in *Rab11^{mo}* eye-antennal discs and show that these retinal defects originate during larval development. Our findings reveal that Rab11 is a key player during ommatidial differentiation in *Drosophila*.

Materials and Methods

Fly stocks

Rab11$^{EP(3)3017}$/TM6B (Abdelilah-Seyfried *et al.*, 2000) and *Rab11$^{l(3)j2D1}$/TM6B* (Bloomington Stock Centre) are existing P-insertions in the *Rab11* gene. *Rab11^{mo}/TM6B* is a P-element mutation generated in our lab and caries a P-element inserted in the 5' regulatory region of *Rab11* gene. *y w; Δ2-3, Sb/TM6B* (JSK-17, Robertson *et al.*, 1988) and *y w; TM3, Sb/TM6B* (JSK-3, Bier *et al.*, 1989) were the strains used for P-element mobilization. P-element mobilization was done essentially as described by Cooley *et al.* (1988). The *P{ry+ t7.2=neoFRT} 82B* line (Xu and Rubin, 1993) was used for generating a third chromosome *FRT-Rab11.* These *FRT-Rab11* and *yw; EGUF/EGUF; FRT GMR-hid 3R CL/TM2* (Stowers and Schwarz, 1999) were used for producing eye specific somatic clones. All flies were reared on standard yeast supplemented food at 22±1ºC.

Antibodies

A 27 amino acid synthetic peptide (CEGDVIRPSNVEPIDVKP TVTADVRKQ) corresponding to the C-terminal sequence of Rab11 was coupled to maleimide activated Keyhole Limpet Haemocyanin (KLH) and injected into rabbits for raising polyclonal antibodies. The cysteine residue was introduced as the first residue to facilitate tagging of the peptide to KLH. The antibodies were purified using Protein-A agarose column and the absorbance at 280 nm for each fraction was determined. The antibodies were then purified using a sulpholink-27-mer-peptide column. To obtain the desired working titre of anti-Rab11 antibodies, different antibody dilutions were subjected to western blot analysis using total proteins extracted from different stages of fly development. Dilutions of 1:20,000 for western blot analysis and 1:200 for

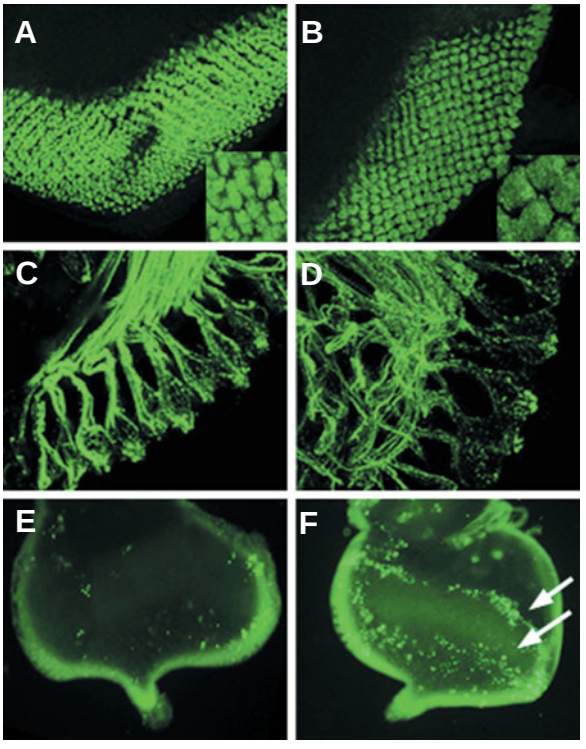


Fig. 9. Organisation and cell death in wild-type and *Rab11^{mo}/Rab11^{mo}* developing eye-discs. (A) *Wild type and* **(B)** *Rab11^{mo}/Rab11^{mo} eye imaginal discs of late third instar larvae stained for the Elav protein, showing the ommatidial organisation. Insets display enlargements of single ommatidium.* **(C)** *Ordered axonal connection between the brain and eye disc in wild type and* **(D)** *disorganized bundles of axons in Rab11^{mo}/Rab11^{mo} larvae as seen by immunostaining with the mouse monoclonal 22C10 antibody. Detection of cell death by Acridine Orange staining in* **(E)** *wild type and* **(F)** *Rab11^{mo}/Rab11^{mo} eye imaginal discs. An increased number of dying cells is detected posterior to the morphogenetic furrow in the mutant disc (arrow).*

immunostaining on intact tissues were routinely used.

Rab11 clonal analysis

Homozygous somatic clones of *Rab11* alleles were specifically induced in the eyes using the *EGUF/hid* technique. The *Rab11* alleles, $Rab11^{EP(3)3017}$ and $Rab11^{l(3)j2D1}$ were recombined to the *P{ry+t7.2=neo FRT}82B* chromosome. The *FRT82B Rab11* recombined arm was selected on the basis of neomycin resistance conferred by the FRT construct and the mutant phenotype due to the presence of P-element marker *w+*. Individual FRT flies were crossed with *y w; EGUF/EUGF; FRT GMR-hid 3R CL/TM2* flies. Eye clones were examined in *y w; EGUF/+; FRT82B Rab11 allele/ FRT GMR-hid 3R CL* males. Adult heads were dissected and subjected for histological and electron microscopic examinations.

Generation of* Rab11^{mo} *and revertant

A P-element insertional mutagenesis screen was initiated in the 93B region using a *pLacw+* insertion line in *mvl* gene (Bier *et al.*, 1989). A series of insertions were generated and phenotypically analysed. One of the insertion mutant line showed abnormal ommatidia. A congenic line of the insertion was generated by crossing the flies for several generations to w^{1118} eliminating background mutations. The resulting insertion was adult viable and displayed abnormal ommatidia. *In-situ* localisation mapped the P-element to chromosomal region 93B8-13. In order to identify the gene in which P-element was inserted in this line, the genomic DNA was digested by EcoR1 and standard plasmid rescue protocol (Pirrotta. 1986) was followed. It is expected that the plasmid DNA thus obtained will contain 3' region of P-element having *AmpR* gene, *ori*, polylinker 2 (containing a Pst1 site) and the flanking genomic DNA. With the aim to eliminate the portion of P-element from the rescued genomic DNA, the plasmid DNA was digested with EcoR1 and Pst1 and three fragments of 1.9, 1.1 and 0.8 kb were obtained. Subsequent analysis revealed that 1.1 and 0.8 kb fragments were from genomic DNA almost devoid of P-element and hence they were used for sequencing and cloning experiments. Sequence analysis of the plasmid rescued fragments showed that the P-element was inserted in *Rab11*, hence we named the new mutation under the designation $Rab11^{mo}$ *[mo = malformed ommatidia]*.

Cloning of the* Rab11 *gene and construction of a* Rab11^{+} *transgene

A λFIXII *Drosophila* genomic library (Stratagene, La Jolla, CA) was screened using either a labeled 1.1 kb plasmid rescued genomic DNA fragment or a PCR amplified 661bp from a Rab11 cDNA (EST GH10576) as probes. A 6.3 kb XhoI-KpnI genomic DNA fragment containing the entire *Rab11* gene was cloned into the *pCasper4* vector (Pirrotta, 1988). Transgenic flies were generated by microinjecting *pCasper4-Rab11* DNA into w^{1118}*; Δ2-3, Sb/+* flies according to standard protocol (Rubin and Spradling, 1982; Spradling and Rubin, 1982). Two out of six transgenic lines with an insertion in the second chromosome and producing wild type Rab11 protein were named as *pCasper-Rab11a* and *pCasper-Rab11b*. These two lines were individually crossed with the available Rab11 alleles, $Rab11^{EP(3)3017}$, $Rab11^{l(3)j2D1}$ and $Rab11^{mo}$ which were examined for developmental rescue in mutant homozygous and in trans-heterozygous conditions.

Immunostaining, scanning electron microscopy and toluidine blue staining

Whole organ staining was carried out as described by Patel, 1994 and immunostaining of paraffin embedded adult head sections was done according to Ausubel et. al., 1994. Plastic sections of adult eye were stained in a 1% toluidine-1% borate aqueous solution for 2 min at 60ºC (Cagan and Ready, 1989). The slides were rinsed in water to remove excessive stain and examined under light microscope. For SEM studies, adult flies of desired genotypes were decapitated, the proboscis was removed and both eyes were separated with the help of a sharp razor to facilitate accessibility of internal head structures for proper fixation and mounting in the appropriate orientation. Samples were fixed in 2.5% gluteraldehyde for overnight at 4ºC, washed 3 x 30 min with 0.1M PBS, dehydrated in ascending acetone grades and then critical point dried. They were then mounted on studs in desired orientation under the stereo-binocular microscope and coated with gold (thickness 30 to 35 nm). Scanning was done on SEM mode in a LEO 435VP electron microscope at 15 kV.

Western analysis

Ten adult heads of the desired genotype were dissected in PBS, transferred to a microfuge tube containing 30µl protein sample buffer (100mM Tris, pH6.8, 1M DTT, 10% SDS, 100 mM PMSF, pH 6.8, 1% bromophenol blue and 1% glycerol) and boiled for 10 min followed by quick chilling and centrifugation at 5000 rpm for 10 min at 4ºC. Proteins in the supernatant were separated by SDS-PAGE (12.5%) and blotted on Immobilon-P membrane (Millipore). The blot was first incubated with rabbit anti-Rab11 polyclonal antibodies (dilution 1: 20,000) and then with a mouse anti-β tubulin monoclonal antibody (dilution 1:50). The blots were developed using horseradish peroxidase labeled anti-rabbit and anti-mouse antibodies in conjunction with the ECL detection system (Amersham Pharmacia Biotech, UK) and quantitation was done with NIH Image J software.

Acridine Orange staining

Eye discs from the late third instar larvae were dissected out in Poels' Salt solution (PSS, Lakhotia and Tapadia, 1998), incubated in 1µg/ml acridine orange (Sigma) solution in PSS for 3 min, washed three times, mounted in PSS and immediately observed under a fluorescence microscope (Nikon E800) using a B-2A filter.

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