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REGULAR ARTICLE

Function of *Drosophila mob2* in photoreceptor morphogenesis

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Abstract The *Drosophila* photoreceptor is a highly polarized cell; a mature photoreceptor cell in *Drosophila* contains a photosensitive structure (the rhabdomere) and a supporting membrane (stalk) at its apical membrane. In a screen to isolate genes involved in determining stalk and rhabdomere formation, this study has identified the *Drosophila mob2* (*Dmob2*) gene. Dmob2 belongs to a Mob1/phocein domain protein family whose functions are involved in polarized cell growth and asymmetric cell fate determination in yeast. To study the role of Dmob2 in photoreceptor development, we have raised an antibody against the Dmob2 protein. An immunocytochemical study has shown that Dmob2 is mainly localized in the apical membrane of photoreceptor cells during early development. As development proceeds, Dmob2 is gradually confined to the rhabdomere base of the photoreceptor cells. RNA interference (RNAi) for knockdown Dmob2 expression during eye development impairs rhabdomere formation. Our study further shows that the subcellular localization of phosphorylated Moesin and Crumbs in the developing photoreceptor cell is disrupted in Dmob2 RNAi flies. This work thus reports a novel function of Dmob2 in photoreceptor cell development.

Keywords *Dmob2* · Photoreceptor · Rhabdomere · Morphogenesis · *Drosophila melanogaster* (Insecta)

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Introduction

The *Drosophila* compound eye consists of approximately 750-unit eyes or ommatidia each containing photoreceptor cone and pigment cells (Wolff and Ready 1993). Compound eye development starts in early third instar larvae by sweeping the morphogenetic furrow from the posterior to anterior portion of the eye discs (Ready 1989). Thereafter, cone and pigment cells are differentiated, and eye pattern formation is completed by around 35% of pupal development (% pd; Cagan and Ready 1989; Wolff and Ready 1991). At around 35% pd, the differentiated photoreceptor cells undergo a dramatic shape change by enlarging and extending their apical surface downward to the retinal floor to make contact with the cone cell plate (Longley and Ready 1995). At around 55% pd, two distinct apical domains, an early rhabdomere with short microvilli facing the future interrhabdomere space (IRS) and a smooth stalk membrane extending to the adherens junctions, appear in a subset of photoreceptor cells (Longley and Ready 1995). As development proceeds to ~75% pd, the rhabdomere domain fills with elongated microvilli and becomes more confined at the apical surface. As the IRS opens, the stalk domain juxtaposed apically to the rhabdomere and basally to the adherens junction becomes more prominent (Kumar and Ready 1995).

The rhabdomere and stalk membrane occupy a characteristic compartment in the photoreceptor cell; this raises an interesting question regarding cellular subspecification. Studies have shown that the cell polarity gene, *crumbs* (*crb*), plays a critical role in photoreceptor morphogenesis. Mutation of the *crb* gene results in a wider rhabdomere domain and a shorter stalk domain compared with the wild type (Izaddoost et al. 2002; Pellikka et al. 2002). *Drosophila crb* encodes a transmembrane protein contain-

ing a large extracellular domain and a short cytoplasmic domain (Tepass et al. 1990). The short cytoplasmic domain contains a FERM-binding domain, critical for recruiting Moesin (Moe) and β-spectrin to the plasma membrane (Klebes and Knust 2000; Medina et al. 2002). Another gene affecting rhabdomere morphogenesis is *moesin* (*moe*). Moe belongs to the Ezrin-Radixin-Moesin (ERM) protein family and crosslinks actin filaments with the plasma membrane to determine cell shape and to regulate membrane recycling (Bretscher et al. 2002). *Drosophila* phosphorylated-Moe is known to localize at the rhabdomere base in photoreceptor cells. Depletion of Moe results in severe rhabdomere defects, whereas over-expression of constitutely active Moe disturbs the confinement of Crumbs (Crb) to the stalk membrane (Karagiosis and Ready 2004). Together, these studies suggest that Crb is critical in determining stalk length, whereas Moe is important for rhabdomere formation.

Drosophila mps1-one-binder 2 (Dmob2) protein belongs to a Mob1/phocein domain protein family conserved in yeast, *Trypanosoma brucei*, and humans (Luca and Winey 1998; Salimova et al. 2000; Colman-Lerner et al. 2001; Devroe et al. 2004; Bichsel et al. 2004; Hammarton et al. 2005; He et al. 2005a; Lai et al. 2005). Mob2 is found to bind Cbk1, a conserved serine-threonine kinase, to promote polarized cell growth and to induce asymmetric cell fate in yeast (Racki et al. 2000; Colman-Lerner et al. 2001; Hou et al. 2003; Weiss et al. 2002). In *Drosophila*, the Mob2 protein is shown to co-immunoprecipate with Tricornered (Trc), which encodes *Drosophila* nuclear DBF2-related kinase and is known to regulate actin cytoskeleton arrangement during epidermal cell differentiation (Geng et al. 2000; He et al. 2005b). Other than in biochemical studies, the function of Dmob2 in *Drosophila* remains unknown. This study demonstrates that Dmob2 is predominantly localized at the rhabdomere base of the developing photoreceptor cells. Knockdown of Dmob2 expression by using RNA interference (RNAi) impairs the subcellular localization of Crb and phosphorylated Moe (p-Moe) resulting in a severe rhabdomere phenotype, thereby suggesting a crucial function for Dmob2 in photoreceptor development.

Materials and methods

Drosophila stocks and transgenic constructions

Drosophila melanogaster, w^{1118} was used as the wild type. The *UAS-crb*^{intramyc} fly (Wodarz et al. 1995) was from E. Knust (Institut fur Genetik, Heinrich-Heine-Universität Düsseldorf, Germany). EP lines were from the Szeged *Drosophila* Stock Center and C.T. Chien (Institute of Molecular Biology, Academia Sinica, Taiwan). The *GMR-Gal4* fly was obtained from the Bloomington Stock Center.

To make Dmob2 RNAi flies, we selected three independent sequences from the Dmob2-coding sequence. The desired DNA fragments were amplified by *PfuTurbo* DNA polymerase (Stratagene, La Jolla, Calif.) from the expressed sequence tagged (EST) clone, RE65017 (GeneBank accession number BI 482337, obtained from DGRC, Indiana University, USA). The primers used to amplify the *Dmob2-IR1* (corresponding to positions 1–320 of the *Dmob2*-coding sequences) were 5′-CTAGTCTAGAGAATTCATGAAAGAAACT CTAAGCTCAAAGG-3′ and 5′-CTACTAGCTAGCCT CGAGGCTACGCATAGAAAACTATCAACTG-3′; *Dmob2-IR2* (corresponding to positions 476–921 of the *Dmob2*-coding sequences) were 5′-CTAGTCTAG AGAATTCCGTCACACACCCTCGCTC-3′ and 5′-CTA CTAGCTA GCCTCGAGCTCAAGGTCGCGCAGCAC-3′; *Dmob2-IR3* (corresponding to positions 932–1400 of *Dmob2*-coding sequences) were 5′-CTAGTCTAGAGAA TTCGCCTCACCGACGATACCG-3′ and 5′-CTACTAGC TAGCCTCGAGAAATGTAGTGCGCCAGCG-3′. The polymerase chain reaction (PCR) products were subcloned, in opposite orientation, into the *Eco*RI/*Xho*I and *Nhe*I/*Xba*I sites of a *pWIZ* vector (Lee and Carthew 2003) to generate *pWIZ-Dmob2-IR1*, *pWIZ-Dmob2-IR2*, and *pWIZ-Dmob2-IR3*. To make transgenic flies expressing full-length *Dmob2*, we amplified the *Dmob2* cDNA with *PfuTurbo* DNA polymerase by using two primers (5′-GAAGATCTCA T G A A A G A A A C T C T A A G C T C A A A G G - 3 ′, 5′-CTAGTCTA GACTATGCCGTGGTGGTCGAG-3′) from the EST clone, RE65017. The PCR fragment was subcloned into the *pUAST-Flag* expression vector (kindly provided by Henry Sun at the Institute of Molecular Biology, Academia Sinica, Taiwan) to make *pUAST-Flag-Dmob2*. All constructions were verified by DNA sequencing before further processing for germ-line transformation. After *P-element*-mediated germ-line transformation (Spradling and Rubin 1982), more than five independent lines were obtained for each construct.

Antibody production and Western blotting

To generate antibody against the Dmob2 protein, we used PCR to amplify 284 amino acids of the Dmob2 C-terminus by using two primers: 5′-ATCTAGGGATCCCAATT TCATGTGATAGCGCATCTG-3′ and 5′-ATCT AGAAGCTTCTATGCCGTGGTGGT CGAGG-3′. The PCR product was cloned to pQE-30 vector (Qiagen, Valencia, Calif.). After iso-propyl β-D-thioglactopyranoside induction, we isolated the 35-kDa Dmob2 recombinant protein from *Escherichia coli* and used it as an antigen. To generate antibody, 500 μg bacterial expressed Dmob2 protein was mixed with a complete adjuvant and injected into a rabbit.

After several boosts, the serum was collected and tested for immunoreactivity. For Western blotting, adult fly heads were collected and homogenized with a homogenization buffer (50 mM HEPES, 50 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, 10% glycerol) with protease inhibitors. The cell lysates were centrifuged at 25,000*g* for 10 min at 4°C to remove insoluble material. Following SDS-polyacrylamide gel electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane. To process for immunoblotting, the membrane was blocked with 5% non-fat milk in TRIS-buffered saline with Tween (TBST). The membrane was then incubated with anti-Dmob2 antiserum (1:8000) at 4°C overnight. During the following day, the membrane was washed three times with TBST and then incubated with peroxidase-conjugated goat anti-rabbit IgG (1:10,000). After the secondary antibody incubation, the membrane was washed and processed for chemiluminescent reaction (Pierce, Rockford, Ill.). Signals were detected with a cooled charge-coupled device camera (Fuji film, Japan).

Immunocytochemistry and histology

For immunocytochemistry, dissected fly eyes were fixed in 4% paraformaldehyde for 20 min. After three washes and a blocking step, the eye discs were incubated with primary antibodies. The primary antibodies used in this study included rabbit anti-Dmob2 (1:1000), rat anti-Crb (1:500; Pellikka et al. 2002), and rabbit anti-phosphorylated Moe (1:100; Karagiosis and Ready 2004). Rhodamine- or fluorescein-isothiocyanate-conjugated phalloidin (Sigma-Aldrich, St. Louis, Mo.), which stained the actin in the cell cortex, was used to label the cell boundary. Stained eyes were washed three times with phosphate-buffered saline with Tween (PBST) and incubated with secondary antibodies. The secondary antibodies used in this study were conjugated with Alexa 488 (Invitrogen Molecular Probes, Carlsbad, Calif.), Texas red or Cy5 (Jackson ImmunoResearch Lab, West Grove, Pa.). After three washes, eyes were mounted in mounting medium (0.25% n-propyl gallate, 50% glycerol in PBS, pH 8.6) and examined on a Zeiss LSM 510 confocal microscope. Images were processed with Adobe Photoshop 6.0 software.

For scanning electron microscopy, adult eyes were dehydrated through serial ethanol and acetone before being processed for critical point drying. After being coated with gold, the eyes were examined by a scanning electron microscope (Hitachi S2300). Images were acquired with a Digital Image Acquisition System (GW Electronics, Norcross, Ga.) and were processed with Adobe Photoshop 6.0. For transmission electron microscopy, the eyes were dissected, fixed in 2% paraformaldehyde and 1.75% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4) for 1 h, incubated in 1% tannic acid in fixative for another hour, and postfixed with 2% osmium tetroxide (Electron Microscopy Sciences, Fort Washington, Pa.) in 0.1 M sodium cacodylate. Following several washes with 0.1 M sodium cacodylate buffer, the eyes were incubated overnight with 2% uranyl acetate, serially dehydrated in ethanol, and embedded in Epox 812 (Electron Microscopy Sciences, Fort Washington, Pa.). Tissues were sectioned on a Reichert ultramicrotome and observed by using a Hitachi H-600 transmission electron microscope.

Results

Subcellular localization of Crb and Moe dynamically changes during photoreceptor development

Studies have shown that p-Moe and Crb act as key regulators for organizing the stalk and rhabdomere during photoreceptor morphogenesis (Izaddoost et al. 2002; Pellikka et al. 2002; Karagiosis and Ready 2004). To investigate further the role of p-Moe and Crb in photoreceptor morphogenesis, we systematically examined the subcellular localization of p-Moe and Crb in developing photoreceptor cells. At 35% pd of the wild type, when photoreceptor differentiation was completed, p-Moe and Crb were mainly concentrated at the center of the ommatidia, a region that develops into the future rhabdomere (Fig. 1a′-a″). At 45% pd of the wild type, the majority of p-Moe and Crb staining remained at the ommatida center (Fig. 1b′-b″). In higher magnification images, we found that p-Moe and Crb staining overlapped in most photoreceptor cells, except at R4 (Fig. 1b‴, arrowhead). At 50% pd of the wild type, most p-Moe and Crb staining remained at the ommatidia center (Fig. 1c-c″). In higher magnification images, we found that p-Moe and Crb staining began to separate into the stalk and rhabdomere domain in a majority of cells, but some remained overlapping at the apical surface of R2, R3, and R5 (Fig. 1c‴, arrowheads). At 75% pd of the wild type, p-Moe and Crb appeared as non-overlapping and occupied specifically the stalk and rhabdomere domains, respectively (Fig. 1d-d‴). This specific pattern could be seen more clearly in higher magnification images (Fig. 1d‴).

Identification of Dmob2 gene as a *GMR>Crb^{intramyc}* suppressor

The above observations prompted us to study whether genes that regulated the subcellular localization of Crb and Moe during photoreceptor development were present. To search for such genes, we conducted an EP-modifier screen to seek genes that could modify the *GMR-Gal4/UAS-crb^{intramyc}* (*GMR>crb^{intramyc}*) eye phenotype.

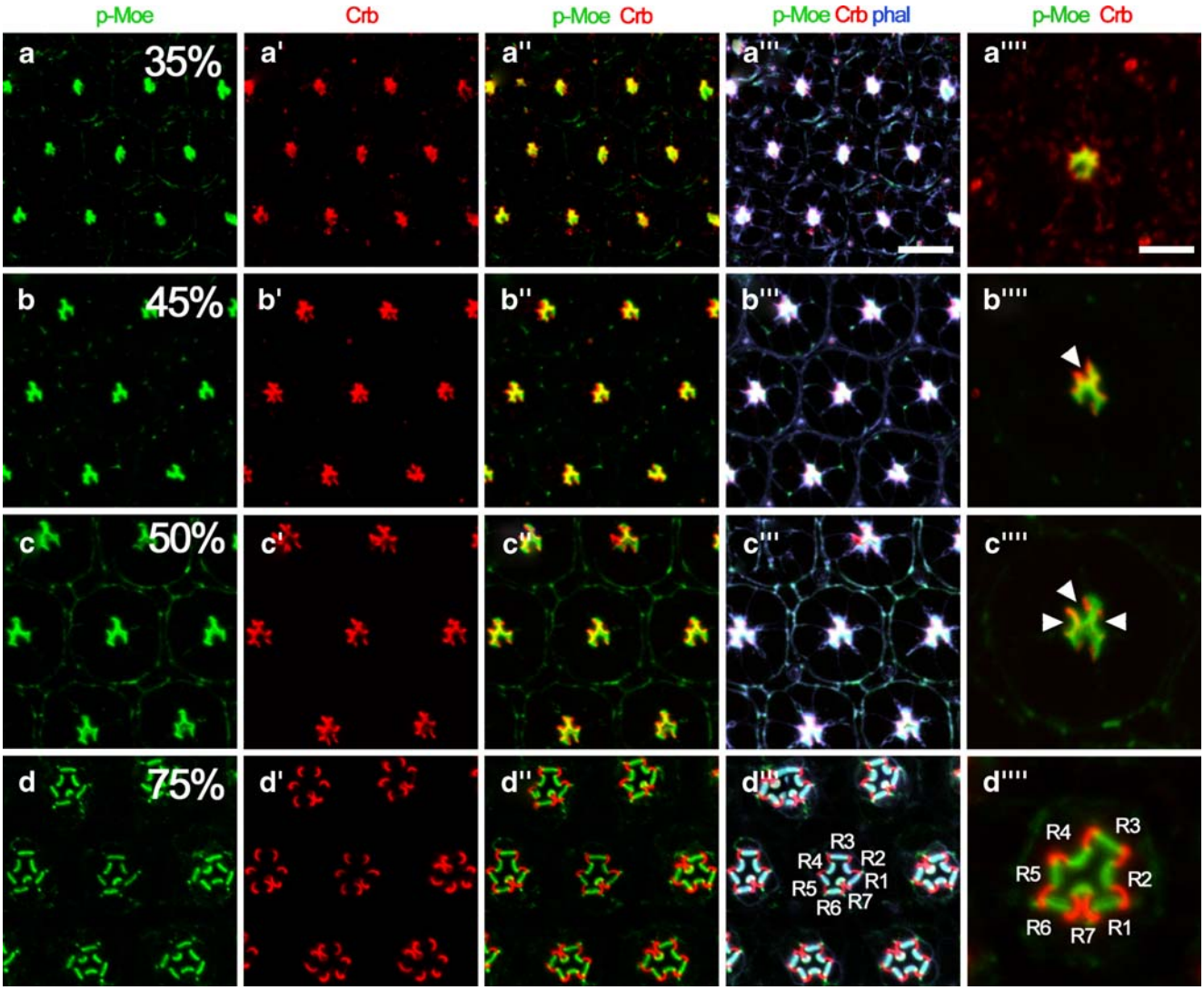

Fig. 1 Subcellular localization of p-Moe and Crb during photoreceptor development; confocal images revealing the subcellular localization of p-Moe (*green*) and Crb (*red*) at selected stages of eye development. **a-a′′′′** By 35% pd of wild type eyes, p-Moe and Crb are mainly localized at the apical membrane of photoreceptor cells. **a′′′** is a merged image showing that the eye stains with anti-p-Moe, anti-Crb, and rhodamine-phalloidin. **a′′′′** Higher magnification image showing colocalization of p-Moe and Crb. **b-b′′′′** At 45% pd of wild type eyes, p-Moe and Crb staining are localized at the apical surface of photoreceptor cells. **b′′′′** Higher magnification image showing colocalization of p-Moe and Crb in most photoreceptors except R4 (*arrowhead*). **c-c′′′′** Confocal images reveal the subcellular localization of p-Moe and Crb at 50% pd of wild type eyes. At this stage, the p-Moe remains at the apical membrane, but Crb begins to be confined at the stalk domain, except in R2, R3, and R5 (**c′′′′**, *arrowheads*). **d-d′′′′** Confocal images reveal the subcellular localization of p-Moe and Crb by 75% pd of wild type eyes. **d′′′′** At this stage, p-Moe and Crb are separated and localize at the distinct rhabdomere and stalk domain, respectively (*R1-R7* photoreceptors 1–7). *Bar* 10 μm (**a′′′**), 5 μm (**a′′′′**)

The *UAS-crb*^{intramyc} fly expresses a membrane-bound cytoplasmic domain of Crb. When activated, this works as a dominant mutation that disrupts epithelial polarity during embryogenesis and eye development (Wodarz et al. 1995; Fan et al. 2003; Grzeschik and Knust 2005). The rationale of this type of screening is to express an additional gene in the *crb*^{intramyc} background in which the expressed genes can rescue the *GMR>crb*^{intramyc} eye phenotype.

Ectopic expression of the Crb cytoplasmic domain by using the *GMR-Gal4* activator caused severe external (Fig. 2a) and internal (Fig. 2c) eye phenotypes. In this study, we screened roughly 1000 EP lines and identified four EP lines (0.4%) that could mitigate the *GMR>crb*^{intramyc} eye phenotype. All four EP lines activated different transcripts. Here, we report that one of the EP lines (EP3497), which activates the expression of the CG11711 (*Dmob2*) gene, could mitigate both external (Fig. 2b) and internal (Fig. 2d) eye phenotypes of the *GMR>crb*^{intramyc} fly. Thus, we were now interested in studying the way that this gene participated in eye development. The *CG11711* gene encodes a Mob1/

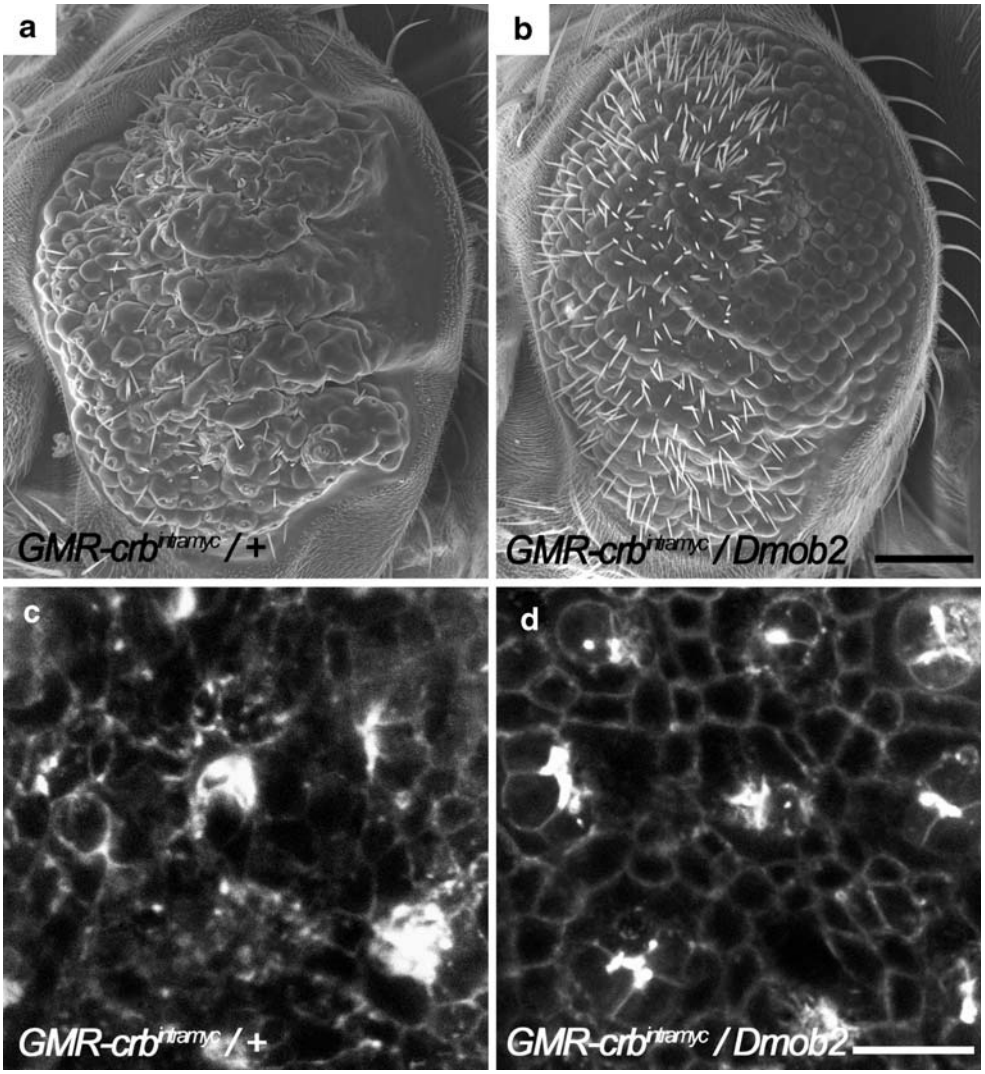

Fig. 2 Overexpression of Dmob2 mitigates the severe eye phenotype in the *GMR>crb*^{intramyc} fly. Scanning electron micrographs of adult eyes in (**a**) *GMR>crb*^{intramyc} and (**b**) *GMR>crb*^{intramyc}*/UAS-Dmob2*. Confocal images showing 75% pd of (**c**) *GMR>crb*^{intramyc} eyes and (**d**) *GMR>crb*^{intramyc}*/ UAS-Dmob2* eyes stained with rhodamine-phalloidin to reveal the internal eye structure. *Bar* 100 μm (**a**, **b**), 10 μm (**c**, **d**)

phocein domain protein, previously named *Dmob2* by He et al. (2005b). We followed their nomenclature and used the *Dmob2* designation in the following study.

Expression of Dmob2 in developing photoreceptor cells

To study the possible function of Dmob2 in photoreceptor development, we raised a specific antibody to Dmob2 and examined its subcellular localization in developing photoreceptor cells. After injection of a bacterial expressed Dmob2 recombinant protein into rabbits, we were able to isolate a polyclonal antibody that recognized a 57-kDa Dmob2 protein and another 72-kDa protein, possibly the long form of the Dmob2 protein (Fig. 3a). When we incubated the antibody with Dmob2 recombinant protein, both bands disappeared, suggesting that this antibody is specific to the Dmob2 protein (Fig. 3a). Using this antiserum, we studied the subcellular localization of Dmob2 at the selected stages of developing eyes. By 35% pd of wild type eyes, Dmob2 was concentrated at the center of the ommatidia, a region further differentiating into rhabdomeres (Fig. 3c). At 50% pd of wild type eye, Dmob2 appeared throughout the cytoplasm and the plasma membrane, with an accumulation of apical staining at photoreceptors R1, R3, and R5 (Fig. 3d, d″, arrows). At 75% pd of the wild type eye, Dmob2 expression appeared predominant in the rhabdomere base of all photoreceptor cells, although some punctuate immunostaining was seen in the cytoplasm of photoreceptor cells (Fig. 3e). In a control experiment, we used antibody, which had been preincubated with Dmob2 recombinant protein, to stain 35% pd of wild type eyes. We found no specific pattern in the photoreceptor cells, suggesting the antibody generated in this study was specific to Dmob2 protein (Fig. 3b). The specific pattern of Dmob2 in developing photoreceptor cells suggested that this protein played an important role in photoreceptor development.

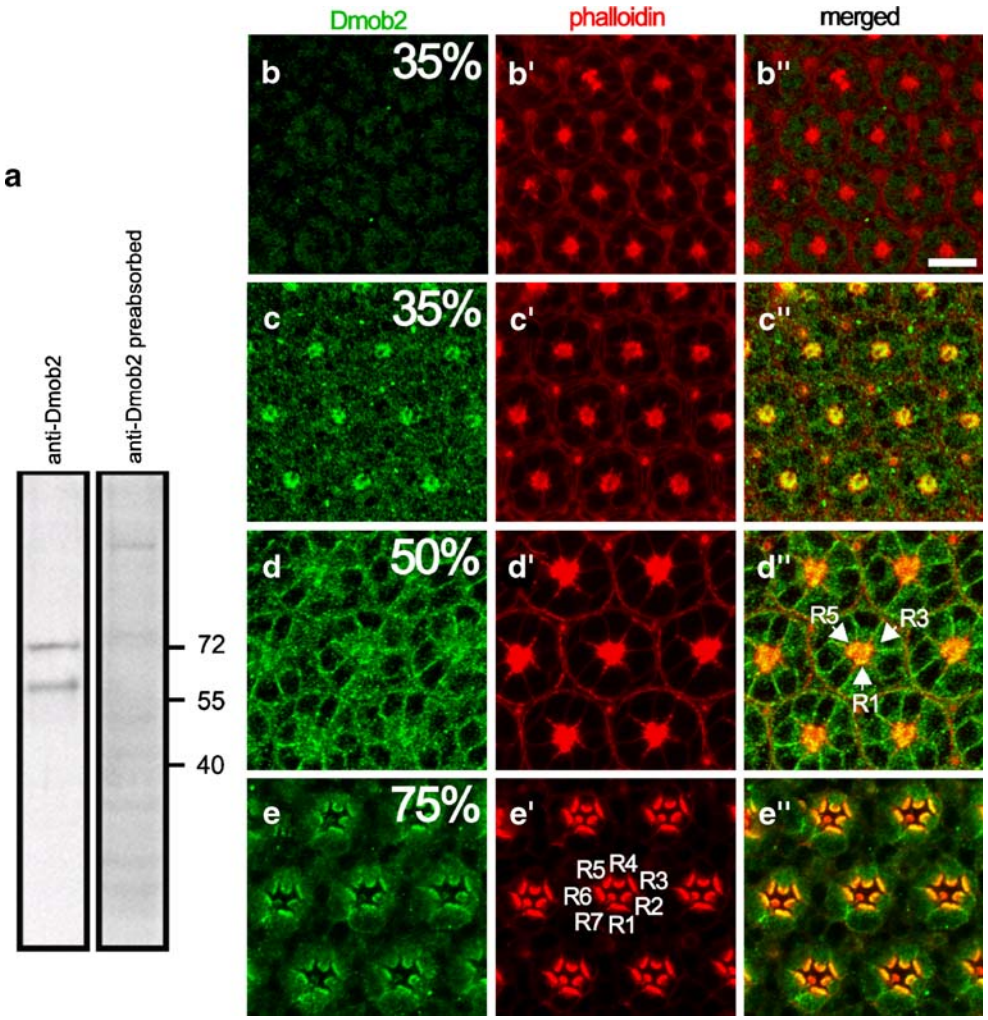

Fig. 3 Subcellular localization of the Dmob2 in developing photoreceptor cells. **a** Western blots revealing that anti-Dmob2 antibody recognizes a 57-kDa Dmob2 protein and another long form of Dmob2 protein at 72 kDa. When the antibody is incubated with Dmob2 recombinant protein before and during Western analysis, the two bands are diminished, suggesting that Dmob2 antibody specifically recognizes Dmob2 protein. **b–e** Confocal micrographs showing wild type eyes stain for Dmob2 (*green*) and phalloidin (*red*). **c** By 35% pd of wild type fly, Dmob2 staining is concentrated at the center of the ommatidium, a region that is the precursor of future rhabdomeres. **d** At 50% pd of wild type eye, Dmob2 appears throughout the cytoplasma and plasma membrane but with an accumulation of apical staining at photoreceptors R1, R3, and R5 (**d**′′, *arrows*). **e** At 75% pd of wild type, Dmob2 expression is mainly in the subrhabdomere domain (*R1-R7* photoreceptors 1–7). **b** When the antibody is incubated with Dmob2 recombinant protein before sections are processed for immunocytochemistry, only background signals are detected. *Bar* 10 μm

Dmob2 is required for eye development

To analyze further the function of Dmob2 in photoreceptor morphogenesis, we tried to isolate the *Dmob2* null alleles but failed in our attempt. Thus, we used an alternative strategy by generating transgenic flies to express *Dmob2* double-stranded RNA (dsRNA). After downregulating Dmob2 expression, we investigated its affect on eye development. A total of three distinct transgenic flies that targeted different *Dmob2* RNA fragments (*pWIZ-Dmob2-IR1*, *pWIZ-Dmob2-IR2*, *pWIZ-Dmob2-IR3*) were generated (Fig. 4a). To assay the ability of RNAi flies to knockdown Dmob2 expression, we crossed each RNAi transgenic fly to flies with *GMR-Gal4*, an eye-specific activator, to drive the transcription of *Dmob2* dsRNA. We then isolated pupal eyes and assayed for their Dmob2 expression by using Western blot. The results showed that Dmob2 expression in *GMR>Dmob2-IRs* was reduced by roughly 90% as compared with that in the wild type (Fig. 4b). The immunocytochemical evidence was consistent with the immunoblot result showing that *GMR>Dmob2-IRs* flies downregulated Dmob2 expression in photoreceptor cells (Fig. 4c–j).

Using these transgenic flies, we investigated whether the down-regulation and over-expression of *Dmob2* expression affected eye development by using scanning electron microscopy and transmission electron microscopy. Scan-

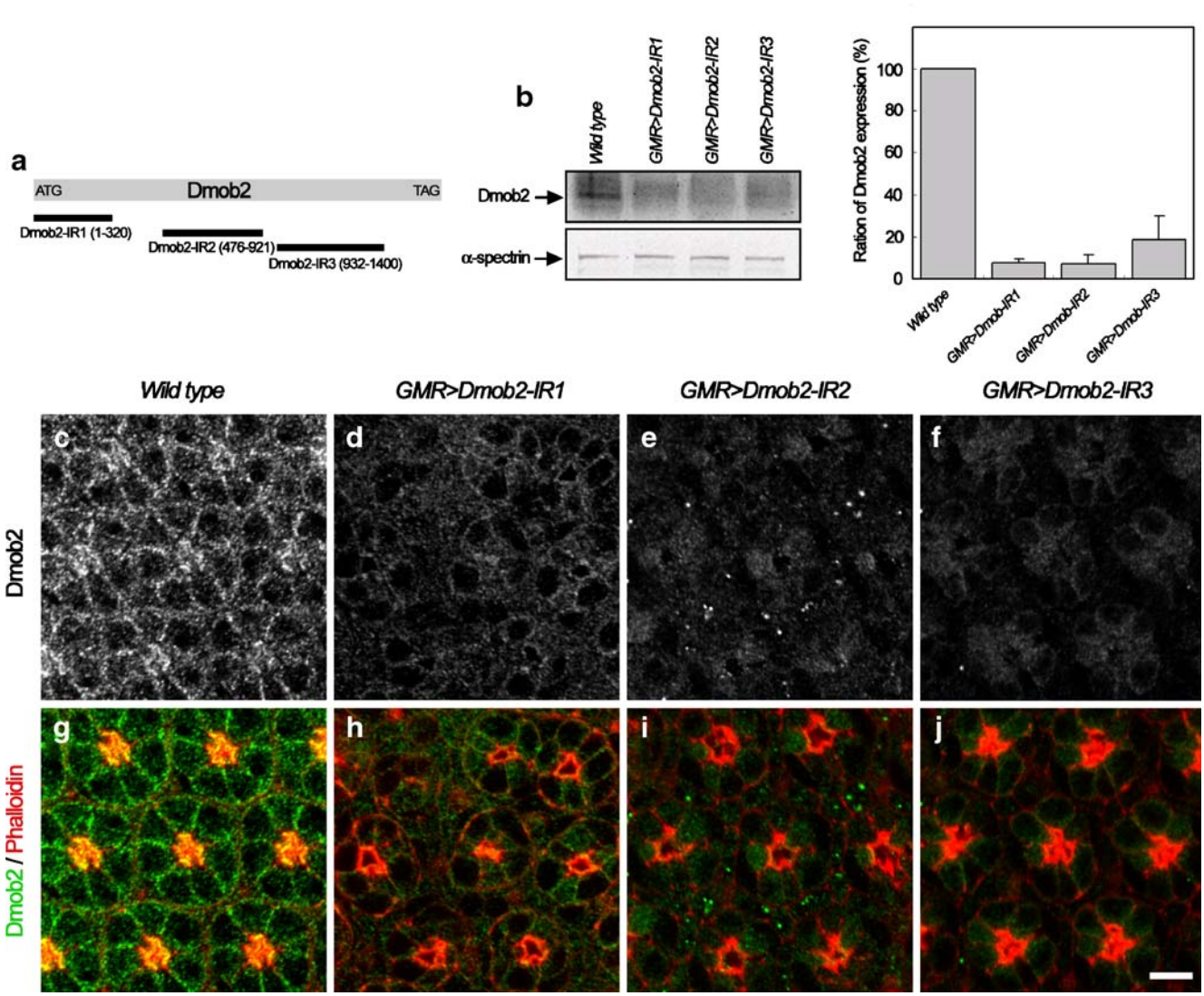

Fig. 4 Down-regulation of Dmob2 expression in *GMR>Dmob2-IR* flies. **a** Representation of three double-stranded RNAs used to disrupt Dmob2 expression. **b** Western blot analysis showing the expression of Dmob2 in wild type and *GMR>Dmob2-IRs* flies. Quantitative analysis demonstrates that Dmob2 expression decreases roughly 90% compared with that in wild type. **c–j** Confocal micrographs showing the expression of Dmob2 by 50% pd of wild type and *GMR>Dmob2-IRs* photoreceptor cells. Immunocytochemical evidence is consistent with the Western blot indicating that *GMR>Dmob2-IRs* flies down-regulate Dmob2 expression in photoreceptor cells. *Bar* 10 μm

ning electron microscopy revealed that the eye of control adults (*GMR-Gal4/+*) consisted of a hexagonal array of approximately 750 dome-shaped ommatidia; small mechanosensory bristles projected from alternate vertices over most of the eye (Fig. 5a). When we crossed *GMR-Gal4* to *pWIZ-Dmob2-IRs* (*GMR>Dmob2-IRs)* flies, the regular array of ommatidia disappeared; a cavernous structure often appeared in the center of the ommatidia, and the bristles projected randomly on the eyes. Since all *Dmob2-IRs* showed similar eye phenotypes, we only present the *GMR>Dmob2-IR1* phenotype here (Fig. 5b) and for further analysis. In addition, we also examined the eye phenotype produced by over-expression of Dmob2 in developing eyes by using the *GMR-Gal4* activator. In *GMR>Dmob2* flies, the change in external eye phenotype was subtle, showing only a slightly abnormal array of ommatidia (Fig. 5c). Transmission electron microscopy demonstrated that photoreceptor cells in the control eyes (*GMR-Gal4/+*) were arrayed as a typical trapezoid (Fig. 5d). The rhabdomeres appeared as oval and faced toward the IRS (Fig. 5d, star). Our findings also showed that rhabdomeres were confined to the center of the apical surface of photoreceptor cells. In *GMR>Dmob2-IR1* adults, the array of photoreceptor cells was not trapezoid (Fig. 5e). The shape and size of rhabdomeres became irregular. Some rhabdomeres were missing (Fig. 5e, arrowheads), and some were widely expanded (Fig. 5e, arrows). In *GMR>Dmob2* adults, the array of photoreceptor cells was irregular (Fig. 5f). Some

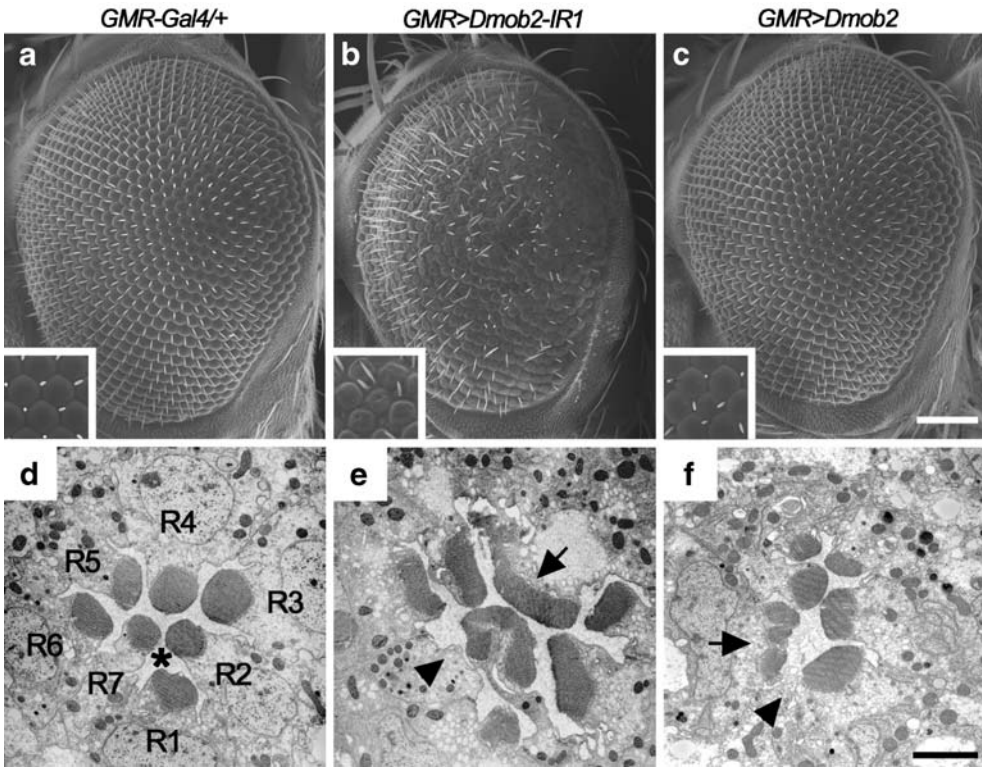

Fig. 5 Dmob2 is required for normal eye development. **a–c** Scanning electron micrographs showing control (*GMR-Gal4/+*), *GMR>Dmob2-IR1*, and *GMR>Dmob2* adult eyes. **a** In the *GMR-Gal4/+* fly, regular dome-shaped ommatidia make up the entire eye field. The mechanosensory bristles project from alternate facet vertices over most of the eye. *Inset*: Higher magnification image of compound eye. **b** In the *GMR>Dmob2-IR1* fly, the regular array of ommatidia is absent, with a cavernous structure often occurring in the center of ommatidia. *Inset*: Mechanosensory bristles project randomly on the eyes. **c** In the *GMR>Dmob2* fly, the external eye resembles that of controls. **d–f** Transmission electron micrographs showing internal eye structure of *GMR-Gal4/+*, *GMR>Dmob2-IR1*, and *GMR>Dmob2* adults. **d** In the *GMR-Gal4/+* fly, the rhabdomere appears oval and is composed of a stack of microvilli. The rhabdomere localizes specifically in the center of apical membrane, which is marked by the two adherens junctions. All rhabdomeres face each other toward the IRS (*star*). **e** In the *GMR>Dmob2-IR1* fly, the shape of the rhabdomere is irregular; some are widely expanded (*arrow*), and some are missing (*arrowhead*). **f** In the *GMR>Dmob2* fly, the shape of the rhabdomere becomes irregular, some appearing with multiple domains (*arrow*) and others being greatly reduced (*arrowhead*). *Bars* 100 μm (**a**, **b**), 20 μm (**c**, **d**)

photoreceptors had multiple rhabdomere domains (Fig. 5f, arrow) or lacked a rhabdomere at their apical surface (Fig. 5f, arrowhead).

The adult phenotype seen in *GMR>Dmob2-IR1* and *GMR>Dmob2* flies encouraged us to study the time at which the phenotype appeared during eye development. To achieve this goal, we stained the developing eye with rhodamine-phalloidin and used a confocal microscope to examine the compound eye structures during development. In the third instar eye disc, photoreceptor differentiation was marked by the morphogenetic furrow (Fig. 6a, arrow). Anterior to the furrow, the cells remained undifferentiation. Posterior to the furrow, the cells began to differentiate and to form arrays as regular photoreceptor clusters. When comparing the eye morphology of control (*GMR>Dmob2-IR1*) and *GMR>Dmob2* flies at this stage, we found no obvious difference among them (Fig. 6a–c). By 35% pd of control (*GMR-Gal4/+*) eyes, the regular array of photoreceptor cells was enclosed by a thin layer of pigment cells (Fig. 6d). At 35% pd of *GMR>Dmob2-IR1* eyes, the array of photoreceptor cells became irregular (Fig. 6e). Our finding also revealed that the number of pigment cells increased and the array of pigment cells appeared as multiple layers (Fig. 6e, stars). At 35% pd of *GMR>Dmob2* flies, the array of photoreceptor cells and pigment cells was normal (Fig. 6f). By 50% pd of *GMR-Gal4/+* flies, the photoreceptor cells were arrayed as a trapezoid, enclosed by a thin layer of pigment cells (Fig. 6g). At 50% pd of *GMR>Dmob2-IR1* eyes, the typical array of photoreceptor cells was disturbed (Fig. 6h); the number of pigment cells increased, and the array of pigment cells became multiple layers (Fig. 6h, stars). At 50% pd of *GMR>Dmob2* flies, the array of photoreceptor cells was slightly abnormal (Fig. 6i). Occasionally, the number of pigment cells increased, and the size of pigment cells was variable (Fig. 6i, stars). By 75% pd of *GMR-Gal4/+* flies, the photoreceptor cells were arrayed as a trapezoid. Phalloidin staining revealed the rhabdomeres as oval and faced each

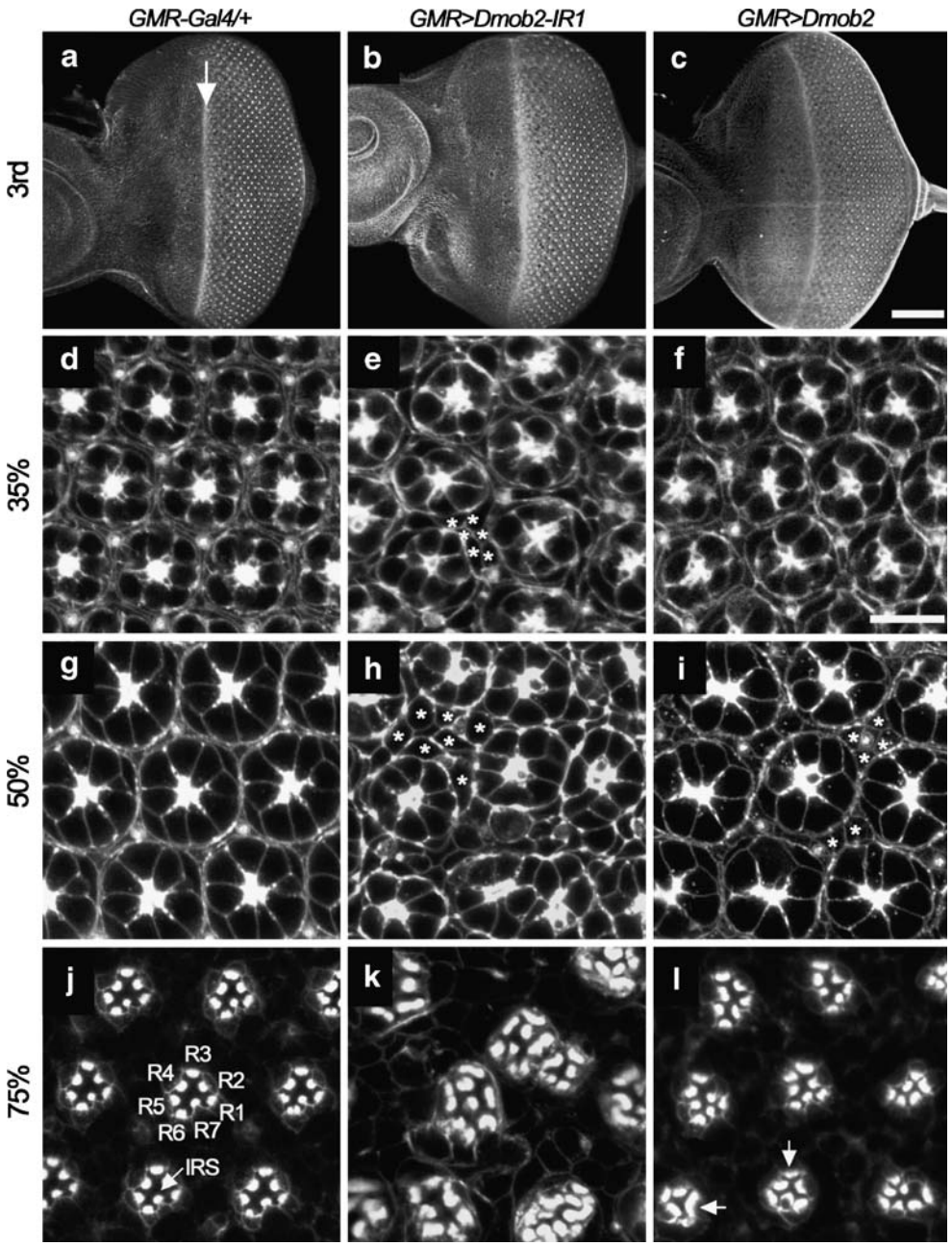

Fig. 6 Staining of selected stages of developing eyes with rhodamine-phalloidin to study whether alterations in Dmob2 expression affect eye development. **a–c** In third instar (*3rd*) eye discs, no obvious eye defects are seen among control (*GMR-Gal4/+*), *GMR>Dmob2-IR1*, and *GMR>Dmob2* flies (*arrow* morphogenetic furrow). **d** At 35% pd of control eyes, the regular array of photoreceptor cells is enclosed by a thin layer of pigment cells. **e** By 35% pd of *GMR>Dmob2-IR1* eyes, the array of photoreceptor cells become slightly irregular. The number of pigment cells increases, and the array of pigment cells forms multiple layers (stars). **f** By 35% pd of *GMR>Dmob2* flies, the array of photoreceptor cells and pigment cells resemble those of the control fly. **g** At 50% pd of control flies, the photoreceptor cells are arrayed as a trapezoid and enclosed by a thin layer of pigment cells. **h** By 50% pd of *GMR>Dmob2-IR1* eyes, the typical array of photoreceptor cells is disturbed. The number of pigment cells increases, and the array of pigment cells become multiple layers (*stars*). **i** At 50% pd of *GMR>Dmob2* flies, the array of photoreceptor cells is slightly irregular. The number of pigment cells increases occasionally (*stars*). **j** At 75% pd of control flies, the photoreceptor cells are arrayed in trapezoid shape (*R1-R7* photoreceptors 1–7). Phalloidin staining reveals rhabdomeres as being oval and facing each other toward the IRS (*arrow*). **k** By 75% pd of *GMR>Dmob2-IR1* flies, the array of photoreceptor cells, and the shape of rhabdomeres becomes irregular. The space between ommatidia is irregular. **l** At 75% pd of *GMR>Dmob2* flies, the array of photoreceptor cells is no longer trapezoid, and the shape of some rhabdomeres is slightly abnormal (*arrows*). *Bar* 10 μm

other toward the IRS (Fig. 6j, arrow). By 75% pd of *GMR>Dmob2-IR1* flies, the array of photoreceptor cells and the shape of rhabdomeres had become irregular (Fig. 6k). The space between the ommatidia was also irregular. At 75% pd of *GMR>Dmob2* flies, the array of photoreceptor cells had lost its trapezoid form and the shape of some of the rhabdomeres had become slightly abnormal (Fig. 6i, arrows). Together, these morphological studies suggested that Dmob2 played an important role in eye development.

Down-regulation of Dmob2 expression alters subcellular localization of p-Moe and Crb in photoreceptor cell

To investigate further the reason that causes the eye phenotype on the down-regulation of Dmob2, we studied the subcellular distribution of p-Moe and Crb in Dmob2 RNAi flies. At 75% pd of *GMR-Gal4/+* flies, p-Moe was specifically localized at the subrhabdomere base of photoreceptor cells (Fig. 7a′). With down-regulated Dmob2 expression, p-Moe staining became diffuse (Fig. 7b′, arrows) or was even missing in some photoreceptor cells (Fig. 7b′, arrowheads). In *GMR>Dmob2* flies, most p-Moe localization remained at the subrhabdomere domain, as in control flies, but we occasionally found some diffuse p-Moe expression (Fig. 7c′, arrows). By 75% pd of *GMR-Gal4/+* flies, Crb was specifically localized at the stalk domain; little or no Crb staining appeared in the subrhabdomere base (Fig. 8a′). When Dmob2 expression was downregulated, the pattern of Crb expression was perturbed (Fig. 8b′, arrows). In *GMR>Dmob2* flies, most Crb remained at the stalk domain, although exceptions were seen (Fig. 8c′, arrows). These observations showed that the down-regulation of Dmob2 altered the localization of p-Moe and Crb in photoreceptor cells. Whether Dmob2 altered p-Moe and Crb localization directly or through some unknown mechanisms is currently unclear and requires more study.

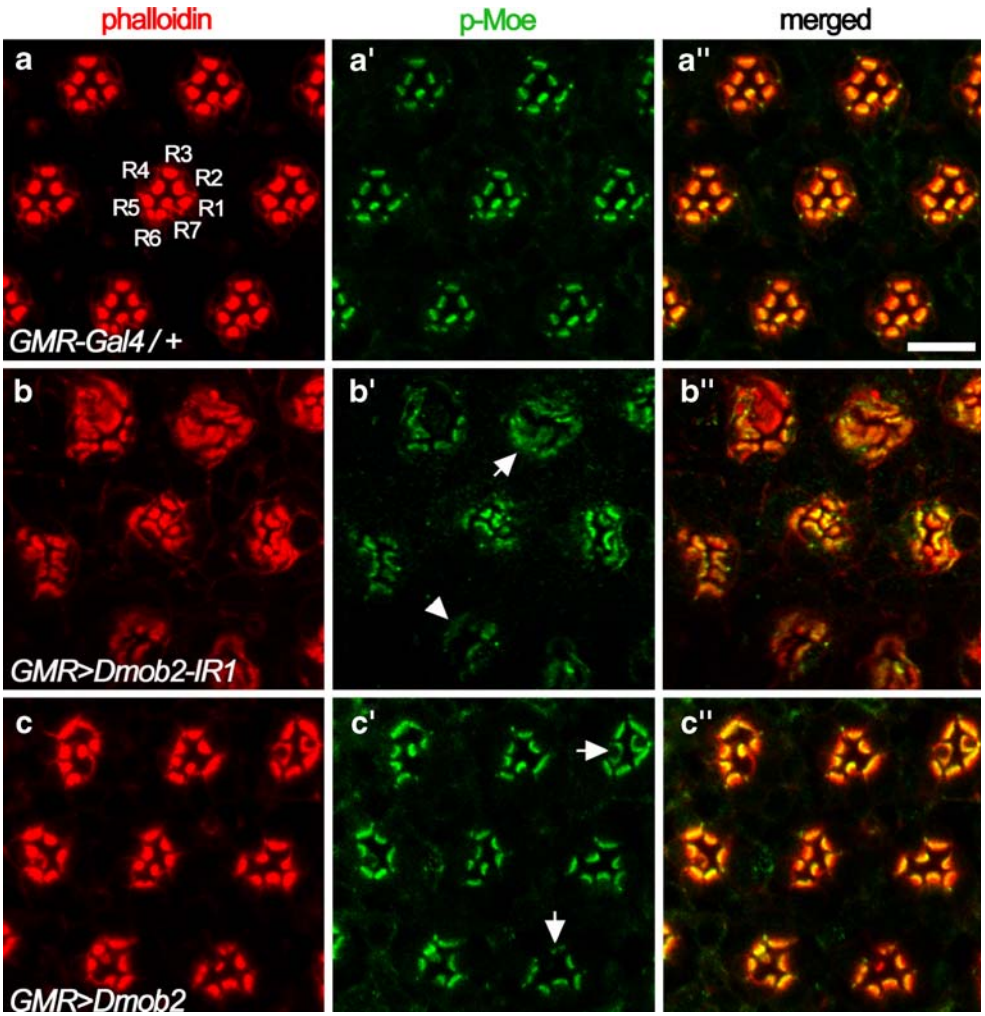

Fig. 7 Dmob2 alters the subcellular localization of p-Moe in developing photoreceptor cells; confocal micrographs showing subcellular localization of p-Moe in photoreceptor cells. **a–a″** In a control (*GMR-Gal4/+*) fly, p-Moe is specifically localized at the subrhabdomere base. **b-b″** In the *GMR>Dmob2-IR1* fly, p-Moe staining fails to appear at the defined domain and instead is diffuse in the cytoplasm (**b′**, *arrow*) or even absent in some photoreceptor cells (**b′**, *arrowhead*). **c–c″** In *GMR>Dmob2* flies, most p-Moe localization remains at the subrhabdomere domain, but some aberrant p-Moe staining patterns are occasionally found (**c′**, arrows). *Scale bar* 10 μm

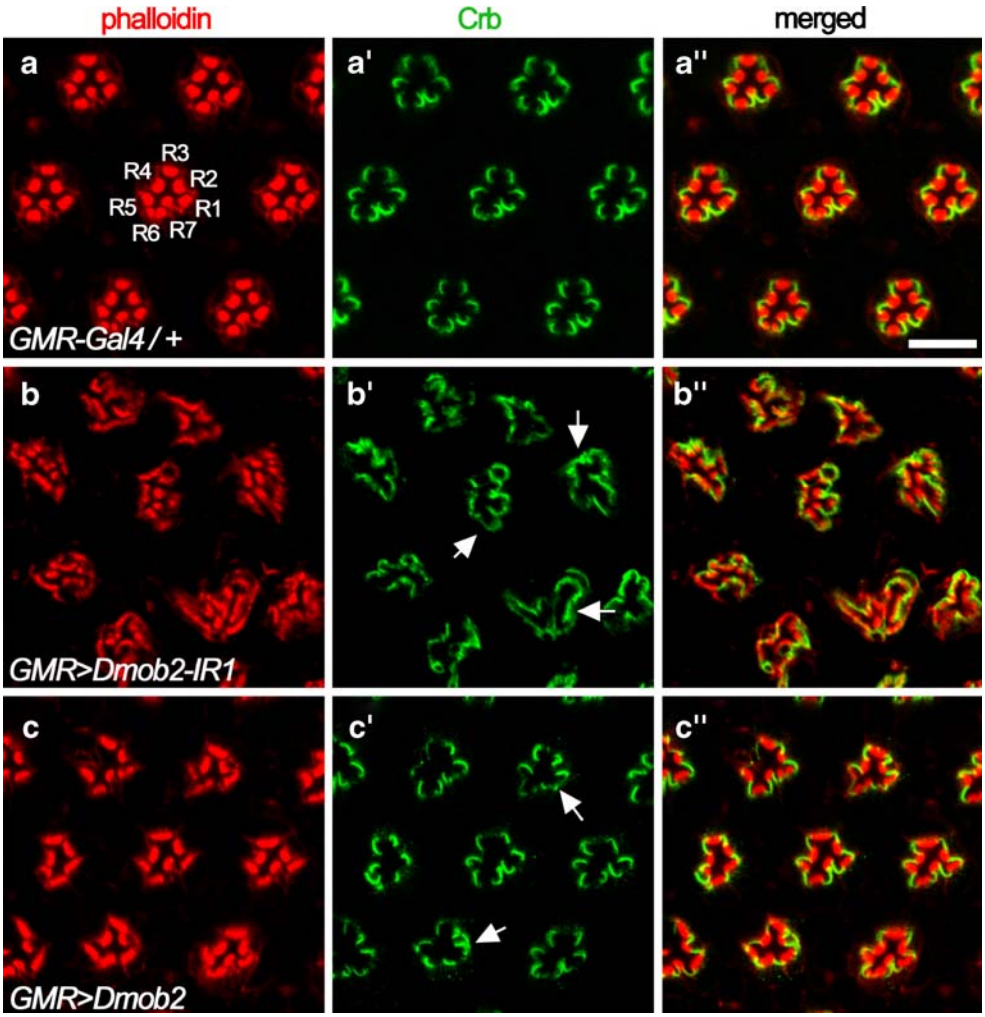

Fig. 8 Dmob2 alters the subcellular localization of Crb in developing photoreceptor cells. **a–a″** In a control (*GMR-Gal4/+*) fly, Crb specifically localizes at the stalk domain. No or little Crb staining lies at the subrhabdomere base. **b–b″** In a *GMR>Dmob2-IR1* fly, Crb fails to localize at a defined domain and appears at the subrhabdomere base (**b′**, *arrows*). **c–c″** In *GMR>Dmob2* flies, most Crb remains at the stalk domain, with some exceptions (**c′**, *arrows*). *Bar* 10 μm

Discussion

The apical surface of the *Drosophila* photoreceptor cell comprises the photosensitive rhabdomere domain and the supporting stalk domain. On a molecular basis, the way that proteins influence cellular subspecification are unknown. Studies have shown that the Crb complex is required for the determination of the stalk domain (Izaddoost et al. 2002; Pellikka et al. 2002; Hong et al. 2003; Nam and Choi 2006), whereas Moe is required for the determination of the rhabdomere domain (Karagiosis and Ready 2004) during photoreceptor morphogenesis. Currently, the way that Crb and Moe coordinate with each other in defining stalk and rhabdomere domains is unclear. This study shows that p-Moe and Crb are colocalized at the apical membrane of photoreceptor cells in the early pupal stage. As development proceeds, p-Moe and Crb gradually separate and become specifically localized at the stalk and the subrhabdomere base. These observations suggest that a dynamic balance between p-Moe and Crb localization in photoreceptor cells is critical for the determination of stalk and rhabdomere formation during photoreceptor development.

Our work demonstrates that the subcellular localization of p-Moe and Crb changes from the apical surface to the specific stalk and subrhabdomere domains during photoreceptor morphogenesis (Fig. 1). The question as to which mechanisms regulate the subcellular relocalization of p-Moe and Crb is worthy of investigation. A previous study has shown that β_H-spectrin, Crb, and Dpatj form a stable complex in the apical membrane of photoreceptor cells (Pellikka et al. 2002). Another study has further shown that *Drosophila* Moe links to Crb and β_H-spectrin in the polarized epithelium (Medina et al. 2002). Based on immunostaining results (Fig. 1), we hypothesize that the Moe, Crb, and β_H-spectrin form a protein complex in the apical membrane of photoreceptor cells in early pupae. As development proceeds, the Moe-Crb-β_H-spectrin complex disintegrates. After disintegration, Moe and Crb might degrade specifically at the stalk or subrhabdomere domains. Thus, the final pattern of p-Moe at the subrhabdomere and Crb at the stalk domain is formed. Alternatively, the

separation of Crb and p-Moe may not go through the protein degradation pathway but might simply translocate each other to the stalk and subrhabdomere domains, respectively.

The observation of Dmob2 staining in this study might provide a clue for the confinement of Crb to the stalk membrane during development. At 50% pd of wild type eyes, we have observed that Dmob2 staining is excluded from the apical surface of photoreceptors R4 and R7 (Fig. 3d). At the same time, Crb staining is confined at the stalk membrane in photoreceptors R4 and R7 (Fig. 1c′). The correlation between the missing Dmob2 staining and the restriction of Crb staining at the stalk membrane at photoreceptors R4 and R7 suggests that Dmob2 participates in excluding Crb from the apical microvilli domain during development. Here, we do not provide strong enough evidence to define the mechanism of Dmob2 in Crb and p-Moe separation. However, these observations are interesting and well-worth pursuing in the future.

In systematic studies of the function of Dmob2 in eye development by means of RNAi, our findings show that specific eye phenotypes only appear in pupal stages, but not in the third instar eye disc, suggesting that Dmob2 functions in pupal eye development (Fig. 6). Among these defects, we have found that the down-regulation of Dmob2 leads to two major defects in rhabdomere formation and pigment cell differentiation. Rhabdomere morphogenesis is a developmental process in which the photosensitive structure is built on the apical surface of photoreceptor cells. Currently, the molecular regulation of rhabdomere biogenesis remains unclear. However, growing evidence indicates that cell polarity determination and actin cytoskeleton organization are two crucial events occurring during rhabdomere formation (Li et al. 2007; Pham et al. 2008). Studies have shown that Mob proteins interact with the nuclear Dbf-2-related (NDR) protein kinase, Cbk1, and participate in regulating cell division and maintaining polarized cell growth (Colman-Lerner et al. 2001; Weiss et al. 2002). In budding yeast, the *Drosophila* Furry-like protein, Pag1 forms a complex with NDR kinase and regulates cell morphogenesis (Du and Novick 2002). Similarly, another *Drosophila* Furry-like protein, Mor2, regulates cell polarity and is required to localize F-actin at the cell ends in fission yeast (Hirata et al. 2002). This suggests that one of the functions of the Mob protein in organizing cell polarity acts through the regulation of the actin cytoskeleton. In *Drosophila*, mutation of *furry* results in the formation of branched bristles, branched arista, and multiple hair cell phenotypes in the wing (Cong et al. 2001). Mutation of *tricornered*, a *Drosophila* homolog of NDR kinase, results in a similar multiple hair phenotype as seen in the *furry* mutant, suggesting that *tricornered* and *furry* are functional in the same pathway in mediating hair development (He et al. 2005a). Co-immunoprecipitation assay has shonw that Tricornered physically interacts with Furry in *Drosophila* (He et al. 2005a). Furthermore, studies have also demonstrated that Tricornered negatively regulates Rac signaling to control dendritic branching suggesting a role for Tricornered in the organization of the actin cytoskeleton (Emoto et al. 2004). Biochemical studies have further demonstrated that *Drosophila* Mob1 (Mats) and Mob2 are co-immunoprecipitated with Tricornered (He et al. 2005b). Based on these studies, Mob protein, NDR kinase, and Furry appear to form a complex to regulate actin assembly during cellular morphogenesis. In this investigation, we show that the down-regulation of Dmob2 often causes malformation of the rhabdomere. These observations suggest that Dmob2 is linked to the Tricornered or Furry pathway in organizing actin filaments during rhabdomere biogenesis. Thus, an examination of whether mutations in *furry* and *tricornered* have similar eye phenotypes as seen in the *Dmob2-RNAi* fly will be of interest in the future.

The reason for the increase in the number of pigment cells in the *Dmob2-RNAi* fly is unclear. Studies have shown that Mob family proteins, Mob1 (Mats), act as tumor suppressors to regulate cell proliferation. When *mats* is mutated, cell proliferation increases and results in tumor formation (Lai et al. 2005). Since Dmob2 and Mats share a similar Mob1/phocine domain, Dmob2 might play multiple roles in organizing the actin cytoskeleton during rhabdomere biogenesis and in controlling cell proliferation during pigment cell development. Taken together, the results in this study suggest the importance of Dmob2 in eye development. Although these data are mainly based on RNAi but not on *Dmob2* null alleles, they still provide significant information for the elucidation of the function of Dmob2 in developing photoreceptor cells and give further insights into the molecular mechanisms of photoreceptor morphogenesis.

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