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Mutation of the photoreceptor specific homeodomain gene *Pph13* results in defects in phototransduction and rhabdomere morphogenesis

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SUMMARY

The expression and organization of the phototransduction signaling proteins into a specialized light-sensing organelle, the rhabdomere, is required for photoreceptor cells to detect light. We report the characterization of the mutant *Pph13^{hazy}*. Pph13 is a homeodomain transcription factor expressed only in photoreceptor cells. Pph13 expression correlates with the differentiation and not specification of photoreceptor cells. In agreement with its expression profile, we find *Pph13* is required for both rhabdomere morphogenesis and for the proper detection of light. In addition, we demonstrate that Pph13 exerts its effect by the regulation of photoreceptor specific gene expression.

Key words: Homeodomain, Photoreceptor, Rhabdomere, Phototransduction, Terminal differentiation, Hazy, *Drosophila*

INTRODUCTION

Drosophila photoreceptor cell specification and differentiation occurs over a 120 hour period. We have considerable insight into the regulatory mechanisms of photoreceptor cell specification and the mechanisms of phototransduction. However, the events that occur between the specification of a photoreceptor neuron and the final organization and function of the phototransduction signaling cascade are poorly defined. To identify the key components controlling the differentiation of photoreceptor cells, we have screened for mutants that affect the final morphogenetic and functional state of photoreceptor neurons.

The *Drosophila* compound eye consists of an ordered array of 800 individual units known as ommatidium. Each ommatidium contains 20 cells, which include the eight photoreceptor neurons, six outer (R1-R6) and two inner (R7, R8) cells. The adult *Drosophila* eye develops from an epithelial monolayer, the eye imaginal disc. Specification of the photoreceptor neurons begins during the larval third instar and proceeds in a posterior to anterior wave across the eye imaginal disc to create each individual ommatidium (Ready et al., 1976; Wolff and Ready, 1993). The early specification of photoreceptor cells has been well studied (Heberlein and Treisman, 2000) and despite the obvious morphological differences between invertebrate and vertebrate eyes, many of the necessary transcription factors required for eye specification are conserved (Ashery-Padan and Gruss, 2001; Kumar and Moses, 2001). The best example of functional conservation is the role of *Pax6* in eye development. Mutations of *PAX6* in humans (*Aniridia*), mice (*Small eye*) and *Drosophila* (*eyeless* and *twin of eyeless*) all lead to severe defects in eye development (Czerny et al., 1999; Hill et al., 1991; Kronhamn et al., 2002; Quiring et al., 1994; Ton et al., 1991).

Upon specification, photoreceptor neurons immediately send axonal projections into the optic lobe of the *Drosophila* brain. The outer photoreceptor cells project into the lamina whereas the inner photoreceptor cells (R7, R8) send axonal projections deeper into the optic lobe and terminate in the medulla (Meinertzhagen and Hanson, 1993; Wolff et al., 1997). Furthermore, the differentiation of the photoreceptor neurons is not complete until 4 days later, at the end of metamorphosis. One unique feature of vertebrate and *Drosophila* photoreceptor neurons is the creation of a specialized light-sensing organelle on the apical cell surface. In *Drosophila*, the rhabdomere is the photoreceptor light-sensing organelle and is the functional equivalent of the outer segment of vertebrate rod and cone cells. Each rhabdomere consists of 60,000 tightly-packed microvilli, each 50 nm in diameter and 1-2 μm in length (Kumar and Ready, 1995; Leonard et al., 1992). This results in a tremendous increase in surface area to house the tens of millions of rhodopsin molecules and associated signaling molecules that are responsible for the detection of light.

As the rhabdomere develops, the signaling molecules required for the detection and translation of light into a receptor potential are expressed and localized to the rhabdomere. In *Drosophila*, the activation of rhodopsin leads to the activation of Phospholipase C (PLC) via a coupled heterotrimeric G protein. PLC catalyzes the breakdown of phosphatidyl 4,5-bisphosphate [PtdIns(4,5)P_2] into the two intracellular messengers inositol triphosphate [Ins(1,4,5)P_3] and diacylglycerol (DAG). This reaction leads to the opening of light sensitive cation-selective channels (TRP, TRPL and

TRPγ) and the generation of a depolarizing receptor potential (Hardie and Raghu, 2001; Zuker, 1996).

We have isolated a mutation, *hazy*, that represents a null allele of the photoreceptor specific homeodomain gene *Pph13*. *Pph13*^{hazy} mutants are adult viable and each ommatidium contains the appropriate set of photoreceptor and accessory cells. However, *Pph13*^{hazy} mutants have a severe decrease in light sensitivity. We demonstrate *Pph13* is necessary for the terminal differentiation of photoreceptor cells, in particular the morphogenesis of the photoreceptor rhabdomere as well as for the expression of phototransduction signaling proteins.

MATERIALS AND METHODS

ERG recordings

Responses to light were done as previously described (Pak, 1975). Briefly, anesthetized flies were immobilized with myristic acid. Glass electrodes containing 0.7% NaCl were placed into the thorax and onto the surface of the eye. Light generated by a Xenon arc lamp (450 W, Osram) was passed through various intensity and color filters. The data were acquired with Clampex (PCLAMP 6) and the tracings were plotted with BatchPlot.

EM analysis

Electron microscopy of *Drosophila* eyes were carried out as previously described (Baker et al., 1994).

Immunofluorescence stainings

Developing whole retinas were dissected at the appropriate time and processed as described previously (Fan and Ready, 1997; Zelhof et al., 2001). Creation and processing of frozen thin sections were performed as described by Tsunoda et al. (Tsunoda et al., 2001). The primary antibodies used were rabbit anti-TRP, Rh1, TRPL, INAD, Amphiphysin (Zelhof et al., 2001), mouse anti-eye Gβ (Yarfitz et al., 1991), Chaoptin (Van Vactor et al., 1988), 21A6 (Zipursky et al., 1984), sheep anti-Bifocal (Bahri et al., 1997), rabbit anti Rac1 was a gift from Dr L. Luo. If not noted, the primary antibody was created in Dr Charles Zuker's laboratory. Rhodamine conjugated Phalloidin (Molecular Probes) was used for the detection of Actin. FITC and Red-X-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories.

Antibody production

The rabbit polyclonal antibody 677 was created by injecting rabbits with a GST-fusion protein representing amino acids 219-357 of Pph13 and sera was used at a concentration of 1:500.

Western analysis

Tissue was placed in extraction/binding buffer (EB; 100 mM KCl, 20 mM HEPES, 5% glycerol, 10 mM EDTA, 0.1% Triton X) with the proteinase inhibitor cocktail mix Complete (Roche Diagnostics), homogenized and sonicated. The mixture was spun and the supernatant was collected and an equal volume of 2× sample/loading buffer was added. All extracts were resolved by SDS-PAGE and then transferred to Immobilon-P (Millipore). Protein detection was done as previously described (Baker et al., 1994). Primary antibodies used in this study were rabbit anti-Rh1, Rh4, TRP, TRPL, TRPγ (Xu et al., 2000), NinaC (Porter et al., 1992), INAD, PLC, Gα, Gγ, RdgC, NinaA, GCα1, Arr2 and mouse anti- eye-Gβ (Yarfitz et al., 1991). If not noted, the primary antibody was created in Dr Charles Zuker's laboratory.

DNA constructs

The expressed sequence tag (EST) GH01528 representing the cDNA for *Pph13* was obtained from Berkeley *Drosophila* Genome Project (via Research Genetics). UAS and heat shock constructs: a *Bgl*II/*Spe*I fragment from GH01528 was cloned into the *Bgl*II/*Xba*I sites of pUAST (Brand and Perrimon, 1993) and the *Bgl*II/*Xba*I sites of pCaSpeR-hs (Thummel et al., 1988) and both were transformed into flies. pcDNA3 construct: a *Bgl*II/*Xho*I fragment from the GH01528 EST was cloned into the *Bam*HI/*Xho*I sites pcDNA3 vector (Invitrogen). The p36 plasmid (Holloway et al., 1995) was obtained from Dr G. Rosenfeld and contains the rat prolactin promoter inserted into pGL2 (Promega). One copy of wild-type and mutated *eye Gβ* upstream regions were cloned into the *Nhe*I site. The mutated *eye Gβ* enhancer used in the transfection assays contained the following sequence 5′-ggcTAATccaATCCgctAGGTgcATTAccg-3′ (the uppercase letters represent the positions of the palindromic Pph13 binding half sites). *eye Gβ-GFP* was constructed by placing 424 nucleotides that are prior to the first ATG of *eye Gβ* into the Green Pelican vector (Barolo et al., 2000). GST fusion protein consisted of amino acids 1-70, representing the homeodomain, cloned into pGEX-4T1 (Pharmacia).

EMSA assays

Electrophoretic mobility shift assays were performed as described previously (Zelhof et al., 1995). In vitro transcribed and translated protein was generated by the TNT Coupled Reticulocyte Lysate System (Promega) according to manufacturer's instructions. Upon annealing of the complimentary oligonucleotides, the oligonucleotides were radiolabeled using a Klenow fill-in reaction. For competition experiments both a 10- and 50-fold excess of cold competitor were used.

Reverse transcriptase and PCR reactions

The following primer sets were used for first-strand synthesis and PCR amplification: Arr1, 5- GAATAAATGGTAGCTCAGCGC-3, 5-CTACATGAACAGGCGTGATTT-3/5-TGTGTCTTTGCGCTTGAT-ATC-3; InaD, 5-TAGAATCATGGTCACTACGCC-3, 5-CAGGC-CAAGAACAAGTTCAAC-3/5-TGTTACATCCTGATTAACGGC-3; NinaE, 5-GGTATTCAGTGGTGTAAGGCC-3, 5-TGGCGTGGTG-ATCTACATATT-3/5-GACATTCATCTTCTTGGCCTG-3; Trpl, 5-AGGGAGCGCATTATATTATCA-3, 5-GAACTACGATCCGCAGA-TGTC-3/5-CATTTCTCGCGTGGTATGTAA-3; Eye Gβ, 5-AAGTG-ATGCGGTTCTCGT, 5-TGTGCCCAAGATTCGATT-3/5-GGTTC-ATACTGGGCGATT-3. Total RNA was isolated from *cn bw* and *Pph13*^{hazy} homozygous heads using Rneasy Mini Kit (Qiagen) and first strand synthesis was accomplished using the Thermoscript RT-PCR System (Invitrogen) and each mRNA target had 35 rounds of PCR amplification.

Transfection assays

Twenty-four hours before transfection NIH 3T3 cells were seeded in 96-well plates. Cells were grown in DMEM supplemented with calf serum. Cells were transfected by using polyfect (Qiagen) according to manufacturer's instructions. Transfection assay mixtures contained 20 ng of reporter with or without 40 ng of pcDNA-*Pph13* per well and assayed for luciferase activity approximately 44 hours post transfection using the Promega Luciferase Assay Kit and a TopCount Scintillation Counter for light detection. Sixteen to 48 wells were transfected for each DNA combination tested per experiment. Three independent transfection experiments were performed per condition assayed.

RESULTS

Pph13^{hazy} mutants have a severe reduction in their response to light and malformed rhabdomeres

To identify genes required for photoreceptor cell differentiation, we screened for the presence or absence of the deep pseudopupil (DPP) in *Drosophila* adult eyes

(Franceschini, 1972; Franceschini and Kirschfeld, 1971). The presence of the DPP is an indication of the overall integrity of the photoreceptor cells and their associated rhabdomeres. Such screens have been effective in isolating mutations that affect eye structure and development (Baker et al., 1992; Banerjee et al., 1987; Pichaud and Desplan, 2001). To limit our search for those mutants that affect aspects of differentiation and not specification, we excluded any mutants that had incorrect external morphology, particularly rough or irregular shaped eyes. Consequently, we screened 6,000 viable second chromosome EMS mutated lines, generated from 38,000 F3 lines (E.K. and C. S. Zuker, unpublished) for the absence of a DPP and isolated 33 mutant stocks which represent 18 complementation groups (data not shown).

A key component of eye function is the organization of the phototransduction machinery into the rhabdomere. As import of the signaling components occurs late in photoreceptor differentiation, we reasoned that flies that lacked a DPP and could not correctly respond to light would be the best candidates for mutants defective in photoreceptor terminal differentiation. Using electroretinogram assays (ERGs) that measure the capacity of photoreceptor cells to convert light into a receptor potential, we tested our collection of 33 mutants for those that had defects in light perception. Our results indicated that among the group that had irregular ERGs, one had a severe deficiency in the detection of light. This mutant responds reproducibly only to long durations of high intensity light. The characteristic on/off transients of wild-type responses are undetectable (Fig. 1C) and by 10 days post eclosion, the mutants appear to lose all responses to light.

While weak or no response to light can be the result of numerous factors (e.g. missing phototransduction molecules or subsequent retinal degeneration), the absence of a DPP in newly eclosed flies suggests that the rhabdomeres did not form. To examine whether the rhabdomeres are present, we performed an EM-ultrastructural analysis on the adult mutant photoreceptor cells. Our examination revealed that all photoreceptor cells and associated rhabdomeres are present. However, the rhabdomeres are severely malformed. They are significantly smaller in size and often the microvilli within each rhabdomere are misaligned (Fig. 1B). In addition, the rhabdomeres do not consistently extend the entire thickness of the retina (data not shown). Given the malformed rhabdomeres and the inability of this mutant to detect light, we tentatively named this mutant *hazy*.

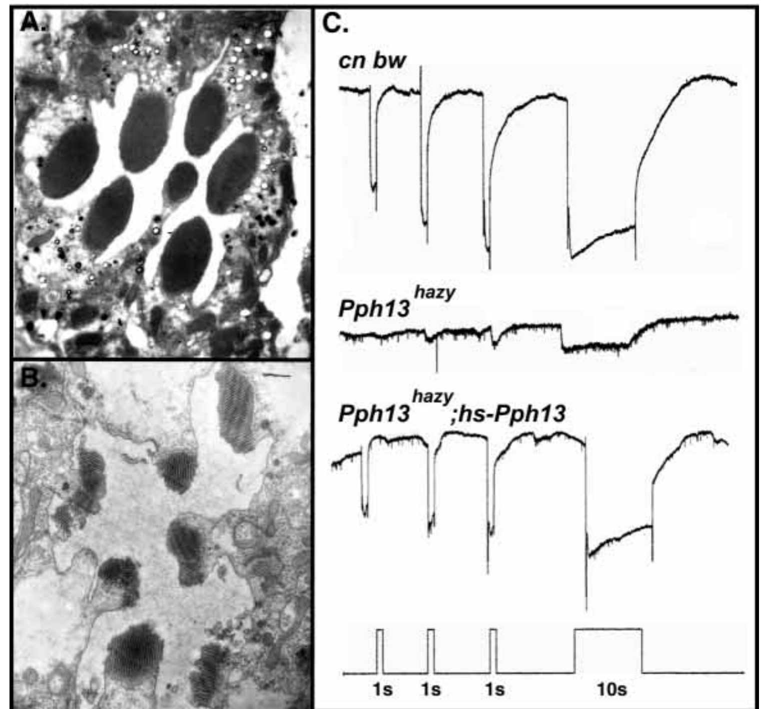

Fig. 1. *Pph13^{hazy}* mutants have malformed rhabdomeres and a dramatic loss in light sensitivity. (A,B) Transmission electron microscopy analysis of *cn bw* (A) and *Pph13^{hazy}* (B) ommatidium. Mutant rhabdomeres are small, malformed and individual microvilli are misaligned. (C) Electroretinograms of *cn bw*, *Pph13^{hazy}* and *Pph13^{hazy}*; *hs-Pph13* (a *Pph13* cDNA under the control of a heat shock promoter). Each consecutive 520 nm light pulse represents a 10-fold increase in light intensity relative to the previous one. The last light pulse is a 10 second pulse of 570 nm light at equal intensity to the last 1 second pulse. The reintroduction of the *Pph13* cDNA rescues all *Pph13^{hazy}* mutant phenotypes.

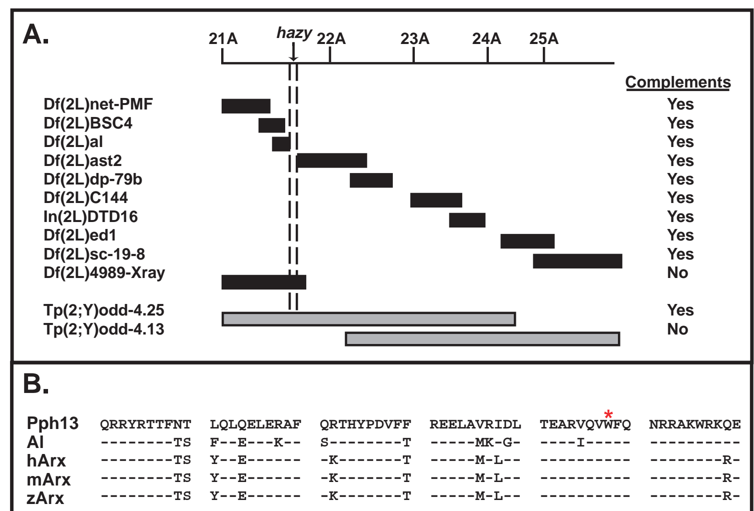

Fig. 2. *Pph13^{hazy}* genetic interval and homeodomain protein comparisons. (A) Recombination and deficiency mapping placed *Pph13^{hazy}* in a small genetic interval 21C7-21D1 on the left arm of the second chromosome. All deficiency and duplication stocks were obtained from the Bloomington Stock Center, except Df(2L)4989, which represents an X-ray induced allele of *Pph13^{hazy}* (A.C.Z., unpublished). (B) Comparison of the Pph13 homeodomain to Drosophila Aristaless (Al) and Aristaless-related homeobox protein (Arx). The red asterisk represents the amino acid change in *Pph13* (W to Stop).

Pph13^{hazy} encodes a paired-class homeobox gene

As a first step in understanding the molecular mechanisms leading to the severe decrease in light sensitivity and rhabdomeric defects in *hazy* mutants, we isolated the responsible gene. Recombination and deficiency mapping placed *hazy* in a small genomic interval of 70 kB of DNA at 21C7-21D1 on the left arm

of the second chromosome (Fig. 2). Sequencing of candidate genes from this interval in both the mutant and isogenic wild-type flies revealed a single base change in the open reading frame of the previously identified homeodomain gene *Pph13* (Dessain and McGinnis, 1993). The nucleotide change results in the introduction of a premature termination codon at amino acid position 58 (W to Stop). The premature stop is within the homeodomain and therefore would also effectively eliminate any DNA binding capacity. More importantly, reintroduction of a wild-type full-length Pph13 cDNA, under the control of a heat shock promoter, by P-element mediated germ line transformation fully rescues all *Pph13^{hazy}* phenotypes (Fig. 1C and data not shown). Rescue is obtained without heat shock, suggesting only a basal level of expression is required for proper photoreceptor cell morphogenesis.

Pph13 is expressed in photoreceptor cells

Using an antibody raised against the C-terminal region of Pph13, we examined the spatial and temporal expression of Pph13 throughout *Drosophila* development. In agreement with *Pph13* mRNA localization (Goriely et al., 1999), Pph13 expression during embryogenesis is limited to the cells of Bolwig's organ, the larval photoreceptor center (Fig. 3A). The adult photoreceptor cells are specified during the third larval instar. However, we do not detect any Pph13 staining in the eye imaginal disc or any other imaginal tissue. Pph13 expression is first detected in at 36 hours APF prior to the detection of the membrane folds that will give rise to the rhabdomeres (Fig. 3B). Expression is limited to only photoreceptor cells and expression is maintained during adult life. As expected, Pph13 localization is nuclear as compared to a nuclear *lacZ* marker (Fig. 3E-G) and immunoreactivity is not detected in *Pph13^{hazy}* mutant embryos or photoreceptor cells (data not shown).

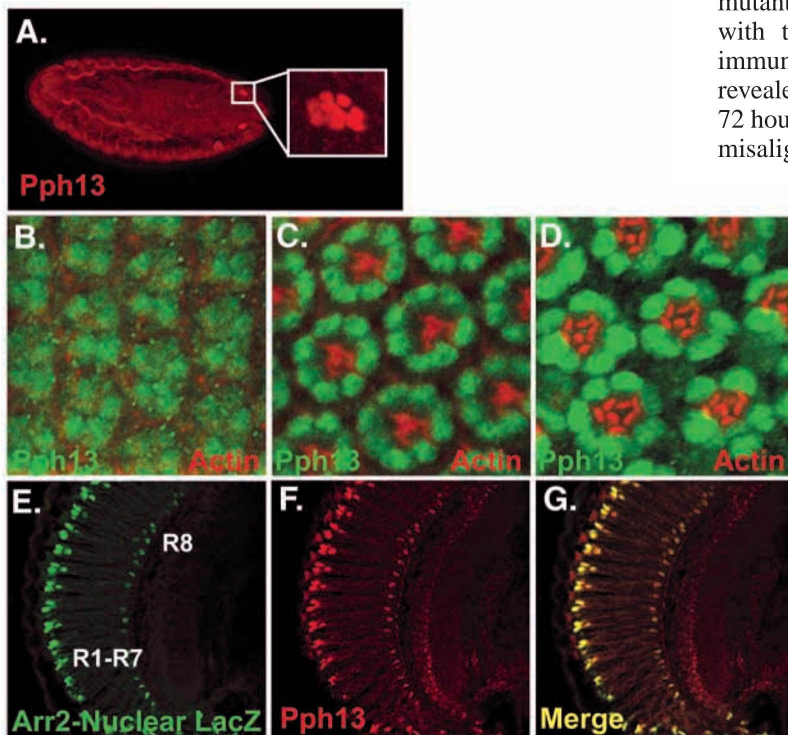

Fig. 3. Pph13 is expressed in *Drosophila* photoreceptor cells. Spatial and temporal expression profile of Pph13. (A) Stage 16 embryo. Pph13 is only detected in the bilateral cluster of cells that constitute Bolwig's organ. The box represents a higher magnification view. The staining of the embryonic trachea is non-specific. (B-D) Pph13 (green) and F-actin (red) staining of developing photoreceptor cells: (B) 36 hours APF; (C) 48 hours APF; (D) 72 hours APF. Pph13 expression is detected in all eight photoreceptor cells. At 72 hours APF, not all of the photoreceptor nuclei are shown in this optical section. (E-G) Pph13 (red) and Arr2-nuclear *lacZ* (green) expression in a cryostat-section of a 3-day-old adult head. Pph13 localization is maintained in adult *Drosophila* photoreceptor cells.

Rhabdomere development stalls at 72 hours APF in *Pph13^{hazy}* mutants

How does Pph13 control the morphogenesis of rhabdomeres? The small malformed rhabdomeres observed in *Pph13^{hazy}* mutants may either be the result of retinal degeneration or a flaw in their biogenesis. Our EM ultrastructural analysis of mutant photoreceptor cells did not reveal any clear signs of degeneration. The rhabdomere terminal web, which is responsible for maintaining the separation and support of the rhabdomere from the cell cytoplasm, forms in *Pph13^{hazy}* flies (Fig. 1B). We do not observe a large concentration of vesicles or multivesicular bodies (MVBs) in the cell cytoplasm associated with degenerating rhabdomeres or the characteristic involuting of the rhabdomere membrane, as seen in *ninaE* mutants (Kumar and Ready, 1995).

If the malformed rhabdomeres were the result of improper morphogenesis, an examination of the spatial and temporal appearance of rhabdomeric proteins during the steps of microvilli formation would not only define the developmental time of the defect but also identify possible Pph13 transcriptional targets. The exact mechanism of microvilli initiation and elongation are not known but there are a few protein markers that can be used to gauge the progression and structure of the developing rhabdomere. At 48 hrs after pupariation (APF), when the initial microvilli folds are forming, we find the accumulation of rhabdomeric proteins, Chaoptin (Van Vactor et al., 1988), Bifocal (Bahri et al., 1997), Amphiphysin (Zelhof et al., 2001), 21A6 (Zipursky et al., 1984), in mutants is indistinguishable from wild type (data not shown). At 72 hrs APF, the progression of rhabdomere development and localization of rhabdomeric proteins appears to be normal in *Pph13^{hazy}* mutants (Fig. 4). However, at 96 hours APF, the staining of F-actin clearly reveals a severe lack of growth and elongation of the rhabdomere microvilli in mutants (Fig. 4H). Staining for F-actin demonstrates that the mutant rhabdomeres are not full and round when compared with their wild-type counterparts. In agreement with our immunofluorescent studies, EM ultrastructure analysis revealed rhabdomere biogenesis is not proceeding correctly by 72 hours APF. The microvilli of mutant photoreceptor cells are misaligned and loosely packed, as seen in adult mutant rhabdomeres (Fig. 5C,D). Whereas at 60 hours APF no discernible ultrastructure defects are observed between wild-type and mutant photoreceptor cells (Fig. 5A,B), suggesting Pph13 function is crucial

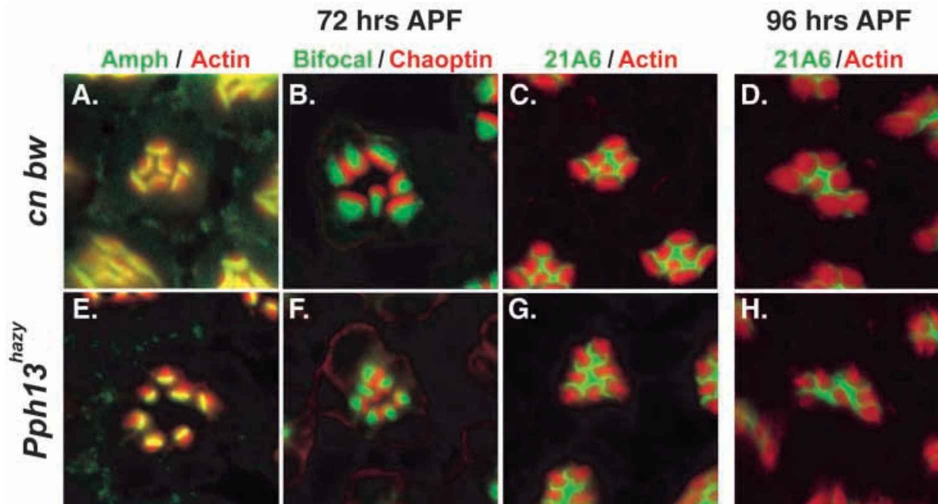

Fig. 4. *Pph13*^{hazy} mutants are stalled in development at 72 hours APF. (A-C) Immunofluorescence of rhabdomeric proteins in *cn bw* ommatidia at 72 hours APF. (D) Actin and 21A6 staining in *cn bw* ommatidium at 96 hours APF. (E-G) Immunofluorescence of rhabdomeric proteins in *Pph13*^{hazy} ommatidia at 72 hours APF. (H) Actin and 21A6 staining in a *Pph13*^{hazy} ommatidium at 96 hours APF.

between 60 and 72 hours APF for proper rhabdomere formation.

Little is known about the molecular events that occur during this 12 hr period of development. Nevertheless, at this time Rac1 is expressed. Rac1 has been implicated in the formation of the rhabdomere terminal web (Chang and Ready, 2000) and Rac, as seen in lamellipodia formation, has the ability to reorganize the actin cytoskeleton to create and support membrane protrusions (Etienne-Manneville and Hall, 2002; Hall, 1998). Since the terminal web forms in *Pph13*^{hazy} mutants, we expected to see normal Rac1 expression and localization in our mutants. First, our data indicate that the temporal expression of Rac1 is normal in but surprisingly the accumulation of Rac1 at the interface between the rhabdomere and the cell cytoplasm is very irregular in mutants (Fig. 6). First, as early as 72 hours APF, we see ectopic spots of Rac1 in the photoreceptor cell body and by 96 hours APF, there is a grossly abnormal amount of Rac1 accumulating at the rhabdomere cell cytoplasm interface and within *Pph13*^{hazy} photoreceptor cells. Interestingly, this misregulation of Rac1 activity or accumulation may be contributing to the rhabdomere defects observed in *Pph13*^{hazy} photoreceptor cells.

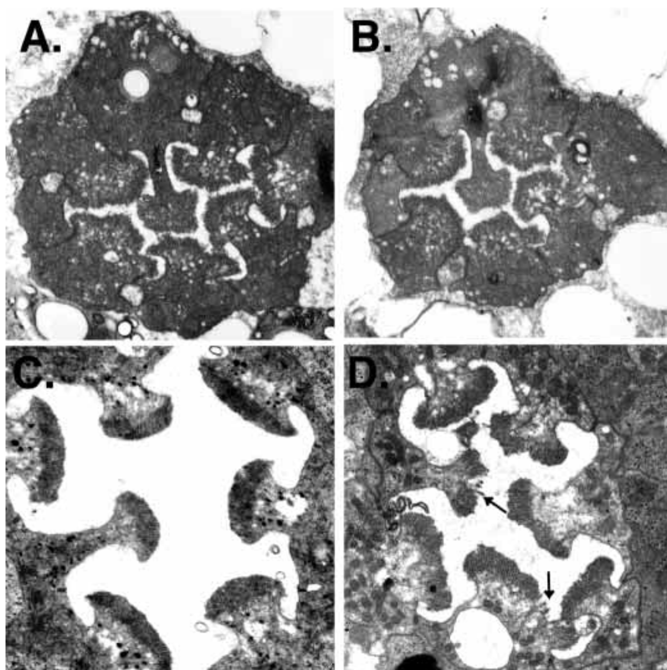

Fig. 5. Transmission electron microscopy of a *cn bw* (A,C) and *Pph13*^{hazy} (B,D) ommatidium at 60 hours APF (A,B) and at 72 hours APF (C,D). Arrows denote irregular shape and size of the mutant rhabdomeres.

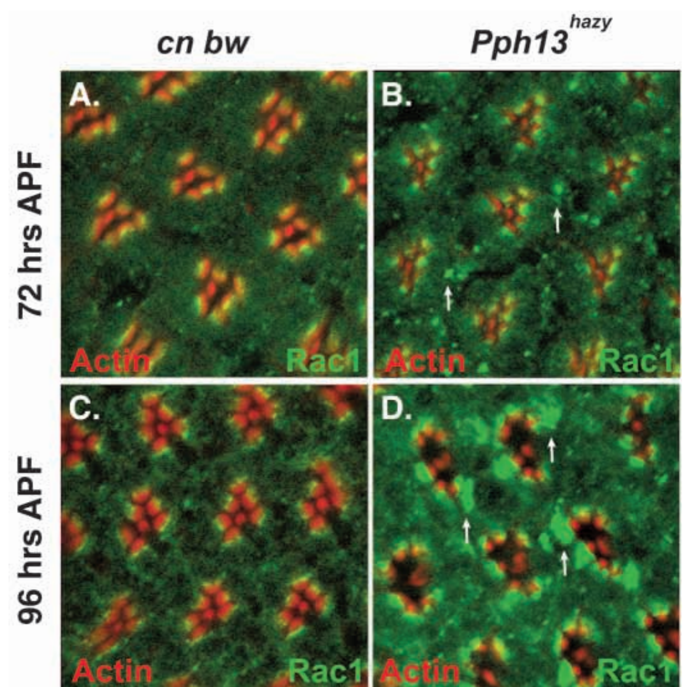

Fig. 6. Rac1 expression and localization in wild-type and Pph13^{hazy} mutant photoreceptor cells. (A-D) Wild-type (A,C) and mutant (B,D) photoreceptor cells expressing Rac1 (green) and F-actin (red) at 72 hours APF (A,B) and at 96 hours APF (C,D). The preparations were scanned at the same intensity of light. Arrows indicate the ectopic and grossly abnormal accumulation sites of Rac1 localization in mutant photoreceptor cells.

Pph13 is not required for cell identity

Does *Pph13* have a role in photoreceptor cell fate specification? To address this possibility, we examined several features associated with changes in photoreceptor cell fate. First, we checked the expression and distribution of photoreceptor cell specific rhodopsins. We do not detect the misexpression or absence of the unique rhodopsins of the outer and inner photoreceptor cells (Fig. 7D and data not shown). In addition, there are no changes in the stereotypical position of the rhabdomeres as present in the late specification mutant *spalt* (Mollereau et al., 2001). Nor do we observe any pathfinding or target choice mistakes of the photoreceptor axons (data not shown). Last, unlike transcription factors involved in the early specification of photoreceptor cells [e.g. *eyeless* (Halder et al., 1995), *eyes absent* and *sine oculis* (Pignoni et al., 1997)], misexpression of *Pph13* in other tissues does not lead to the appearance of ectopic eyes.

Pph13 is necessary for the expression of photoreceptor specific genes

Given that *Pph13* is a transcription factor, we reasoned that the nature of the signaling defect is due to the loss or misexpression of known phototransduction proteins. To test this hypothesis, we checked for the presence of phototransduction proteins in adult head extracts isolated from wild-type and *Pph13*^{hazy} mutants. Western analysis revealed undetectable levels of four phototransduction proteins – eye Gβ, TRPL, TRPγ and Arr1 in *Pph13*^{hazy} mutants (Fig. 7A). Eye Gβ with Gγ is responsible for the coupling of Gα to rhodopsin. In addition, strong hypomorphic alleles of Gβ greatly reduce the ability of photoreceptor cells to respond to light (Dolph et al., 1994). The activation of Gα ultimately leads to the regulation of light sensitive cation-selective channels. In *Pph13*^{hazy} mutants the cation-selective channels TRPL and TRPγ (Xu et al., 2000) are missing. Arr1, which has a role in the deactivation of rhodopsin and termination of the light response (Dolph et al., 1993) is also undetectable. Based on previous genetic and molecular studies of the missing proteins, none of them alone is sufficient to explain the severe loss of light sensitivity in *Pph13*^{hazy} mutants. Nonetheless, the defect we observe in *Pph13*^{hazy} mutants maybe a cumulative effect of all the missing proteins identified.

The majority of the proteins we examined are present in *Pph13*^{hazy} mutant photoreceptor cells but our Western analysis did not address the question of whether these proteins are localized correctly in photoreceptor cells. To check for proper subcellular localization, we examined the expression of proteins via immunofluorescence techniques on frozen thin sections of adult eyes. Our results confirm the absence of TPRL and eye Gβ in *Pph13*^{hazy} mutants. Our western blot data suggested an absence of Arr1 expression but our immunofluorescent data demonstrates that Arr1 levels are only diminished and not absent in mutant photoreceptors. We can detect Arr1 in mutant R7/R8 photoreceptor cells and in the outer photoreceptor cells (R1-R6) the levels of expression are probably below our level of detection, suggesting *Pph13* is only required for full expression Arr1 in all photoreceptor cells (Fig. 7). Last, molecules that show normal or reduced levels of expression, such as with INAD or TRP, show correct subcellular localization to the malformed rhabdomeres in

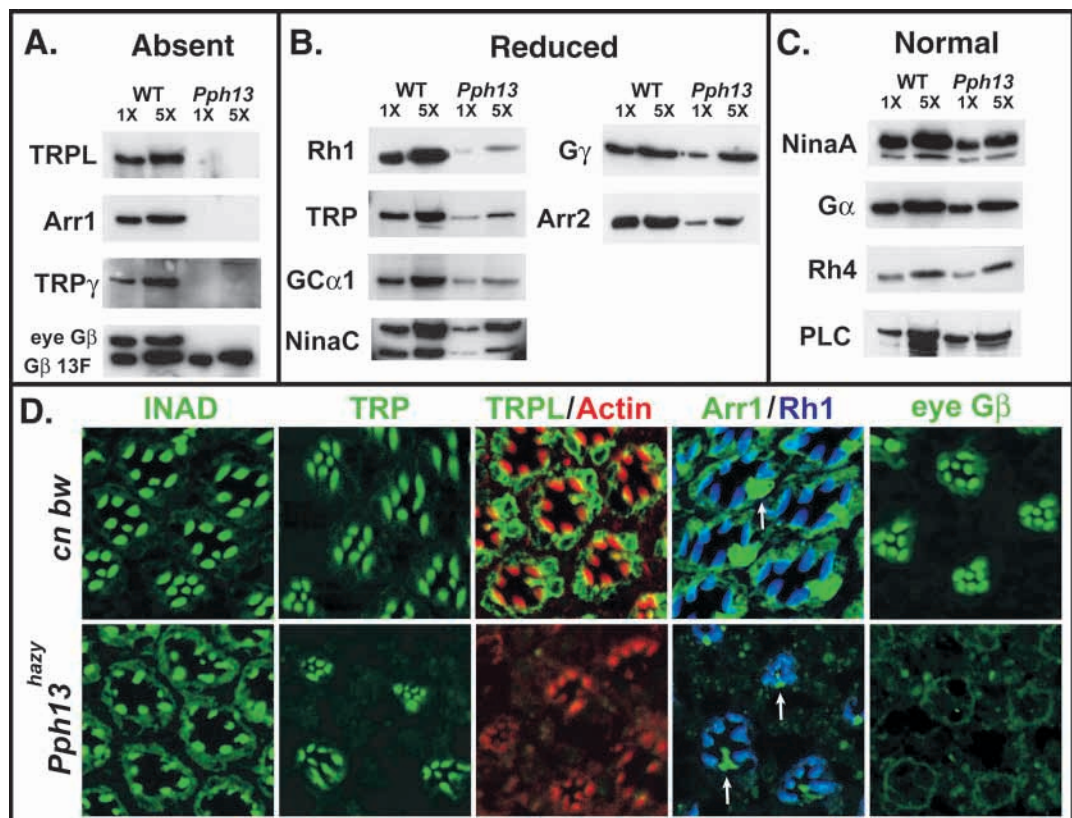

Fig. 7. Pph13 is required for the expression of photoreceptor specific genes. (A-C) Western analysis of photoreceptor specific proteins in cellular extracts isolated from *cn bw* and *Pph13*^{hazy} adult heads. There is no detection of TRPL, Arr1, TRPγ and eye Gβ in *Pph13*^{hazy} mutant extracts. Both NinaC isoforms (p132 and p174) are shown. (D) Immunofluorescence of photoreceptor specific proteins of 1 μm frozen sections of light-exposed *cn bw* and *Pph13*^{hazy} eyes. INAD and TRP localize to the rhabdomeres in both wild-type and mutant cells. TRPL and eye Gβ are not detected in *Pph13*^{hazy} mutants. Arr1 expression is only detected in the inner photoreceptor cells (R7, R8, arrow) and rhodopsin (Rh1) is detected only in the six outer photoreceptor cells (R1-R6).

Pph13^hazy mutants (Fig. 7D). Overall, these results suggest *Pph13* is downstream of the genes required for eye specification and Pph13 transcriptional targets are necessary for the proper detection of light.

Eye Gβ is a transcriptional target of Pph13

Our results suggest that *Pph13* exerts its affect on photoreceptor differentiation by regulating transcription. If this is the case, we predict that the mRNAs for the missing proteins would not be detectable. RT-PCR reactions confirm that the transcripts for *trpl* and *eye Gβ* are absent while the transcripts for *arr1* are present (Fig. 8A). We also predict that if *trpl* or *eye Gβ* represents Pph13 transcriptional targets, potential binding sites for Pph13 should exist in their promoter regions. The consensus DNA binding site for a Paired class homeodomain protein containing a glutamine at amino acid position 50 of the homeodomain is a palindrome of TAAT separated by three nucleotides (Fortini and Rubin, 1990; Wilson et al., 1993; Wilson and Desplan, 1995). Scanning the transcriptional units of *eye Gβ* and *trpl* revealed one element containing strong potential binding sites for Pph13 upstream of the transcriptional start of *eye Gβ*. Within a span of 25 nucleotides, we find two palindromes spaced by three nucleotides and a third overlapping palindrome separated by two nucleotides (see Fig. 8B).

To demonstrate binding specificity of Pph13 to this element, we used both a full-length copy of Pph13 and a GST fusion protein containing only the homeodomain of Pph13 (data not shown) in electrophoretic mobility shift assays (EMSA). In our assays, both protein forms specifically bind to this element. Mutation of the palindromic sites results in the elimination of Pph13 binding and, as expected, the mutated element could not compete for Pph13 binding (Fig. 8B). On the other hand, cold competitor of the wild-type element does result in a dose-dependent inhibition of Pph13 binding.

Based on our findings, we would predict that Pph13 acts as an activator and not a repressor of gene transcription. To test whether Pph13 has the ability to activate transcription we created a reporter construct containing the *eye Gβ* enhancer, nucleotides –323 to –105, upstream of the minimal rat *prolactin* promoter controlling luciferase expression (Holloway et al., 1995). In transient transfection assays, upon the co-transfection of Pph13 we see an average of a thousand fold activation of transcription specifically from the reporter containing the *eye Gβ* enhancer as compared to the parental vector. Mutation of the palindromic binding sites within the response element eliminates the transcriptional activation seen with the addition of Pph13 (Fig. 8C), confirming the role of Pph13 as a potential activator of photoreceptor specific gene expression.

To further prove a direct regulation of *eye Gβ* by Pph13, we asked whether a transgenic construct containing the *eye Gβ* enhancer is expressed in photoreceptor cells and requires Pph13 for expression. As such, we placed GFP immediately downstream of first 424 nucleotides that are prior to the first ATG of *eye Gβ*. As the expression of GFP is low in all of our transgenic lines and combined with the auto-fluorescence of pigmented eyes, we could not say with absolute certainty that this genomic region of *eye Gβ* limits GFP expression only to photoreceptor cells. However, using western blot analysis, we detect GFP only in head extracts; more importantly, GFP expression is dependent on the presence of Pph13. When the transgenic construct is recombined into a *Pph13^hazy* mutant background, GFP expression is greatly diminished (Fig. 8D).

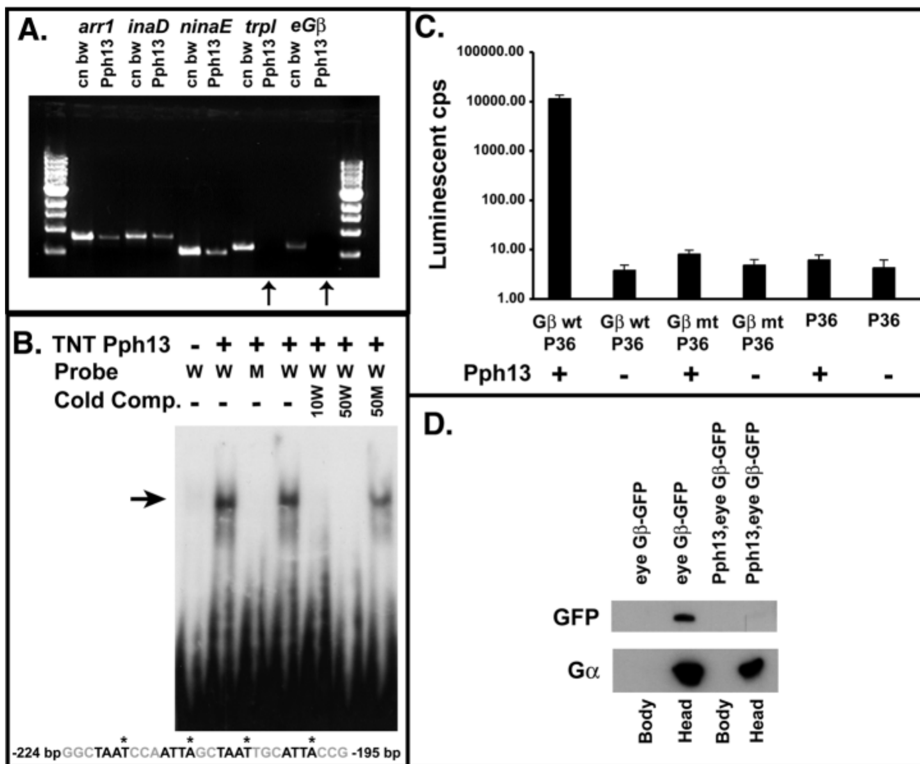

Fig. 8. *eye Gβ* is a transcriptional target of Pph13. (A) Reverse transcriptase reactions coupled to PCR for the detection of photoreceptor specific mRNAs. Transcripts for *trpl* and *eye Gβ* are not detected in *Pph13^hazy* mutants. (B) Potential Pph13 binding sites in the *eye Gβ* enhancer and electrophoretic mobility shift assay. Full-length Pph13 binds specifically to the palindromic sites located in the *eye Gβ* enhancer. Arrow indicates shifted complex and asterisks denote the mutated base pairs. W, wild-type enhancer; M, mutated enhancer. (C) Transient transfection assay. The bar graph shows the data from one experiment that is representative of all the transient transfection experiments performed in this study. Each point represents the average of 16 transfected wells (error bar indicates s.d. of each data set). (D) Western analysis of *eye Gβ-GFP* expression in wild-type and mutant *Pph13^hazy* flies. Protein extracts were isolated from both heads and bodies of wild-type and *Pph13^hazy* mutant flies that contained two copies of the *eye Gβ* enhancer driving GFP expression. Antibodies against Gα were used as a loading control for the head extract lanes.

DISCUSSION

Pph13 is only required for photoreceptor terminal differentiation

To date the molecular mechanisms responsible for photoreceptor cell differentiation remain largely undefined. We have characterized a null mutant for the homeodomain gene *Pph13*. Pph13 is expressed only in photoreceptor cells, the larval cells of Bolwig's organ and the adult R1-R8 photoreceptor cells, and we have not detected any defect in specification of these cells. Pph13 does not have the ability to activate transcription outside the context of a photoreceptor cell (data not shown) nor does ectopic expression of *Pph13* affect the fate of any of the photoreceptor or associated accessory cells (data not shown). The only phenotypes observed are in photoreceptor cell morphogenesis and function, suggesting Pph13 function is restricted to photoreceptor cell terminal differentiation.

*Pph13^hazy^* mutants have two striking defects: the ability of the photoreceptor cell to detect light and the biogenesis of the light sensing organelle, the rhabdomere. Are the two phenotypes connected? We cannot eliminate the possibility that the malformed rhabdomeres are contributing to the inability of these mutants to detect light or vice versa. However, the severity of the rhabdomere defect cannot be solely responsible. For example, from our own screen we have isolated mutants that result in malformed rhabdomeres equal to those seen in *Pph13^hazy^* but have a normal ERG (data not shown). In addition, the loss of Chaoptin and NinaC both result in a considerable loss of rhabdomeric size and rhodopsin levels but they have a better response to light then *Pph13^hazy^* mutants (Matsumoto et al., 1987) (data not shown).

In addition, our results demonstrate that Pph13 is required for the transcription of phototransduction proteins. Clearly, *trpl*, *trp*γ and $G\beta$ are not expressed in mutant photoreceptor cells, and the absence of Pph13 affects the full expression of several other signaling components. This is clearly observed with Arr1 expression. First, our data demonstrates that in the inner (R7/R8) wild-type photoreceptor cells have a considerable higher expression of Arr1 when compared with the outer photoreceptor cells (R1-R6). Second, the loss of Pph13 does not eliminate expression of Arr1 in photoreceptor cells. Arr1 expression can be seen in mutant R7/R8 photoreceptor cells and the lack of signal in the outer photoreceptors is not due to the absence of Arr1 expression but rather the fact that these cells start out with lower levels of Arr1. Taken together, while all of the detected protein aberrations can explain the severe loss of light sensitivity, our results do not eliminate the possibility of a yet unidentified molecule required for proper phototransduction. Selective rescue and identification of any other missing components will be needed to explain the complete molecular mechanisms responsible for the decrease in light sensitivity.

The molecular mechanisms for rhabdomere biogenesis are for the most part unknown. Nonetheless, our data do provide a few insights into rhabdomere biogenesis.

Our results demonstrate that *Pph13* is required for the generation or execution of a late acting signal necessary for the elaboration and growth of the microvilli into a rhabdomere. Immunofluorescent and EM analyses demonstrate that the defects observed in *Pph13^hazy^* mutants are the result of a developmental flaw and not of retinal degeneration. The disorganized rhabdomeres do not show any of the characteristic signs of degeneration and more significantly we detect a clear halt in rhabdomere development by 72 hours APF. In addition, by all measurements, the early events (36 to 60 hours APF) of rhabdomere biogenesis occur normally.

Our data also indicate the failure of growth is not due to the improper localization or delivery of proteins to the rhabdomere. For example, Chaoptin, which is required for the cross-linking of microvilli still localizes to the developing rhabdomere before and after (data not shown) the rhabdomere has stalled in development. In addition, the proteins composing the phototransduction machinery, especially rhodopsin which has a role in phototransduction and in maintaining the structural integrity of the rhabdomere (Kumar et al., 1997; Kumar and Ready, 1995), are imported and stabilized within the malformed rhabdomeres. We also do not observe the characteristic expansion of the endoplasmic reticulum associated with defects in rhabdomeric protein cell trafficking (Baker et al., 1994; Colley et al., 1995; Sang and Ready, 2002).

What is responsible for the flaw in rhabdomere biogenesis? Most notably for a transcription factor believed to be necessary for the activation and not repression of gene transcription, we see a grossly abnormal accumulation of Rac1 in *Pph13^hazy^* mutant photoreceptor cells. However, the presence of Rac1 is in agreement with the fact that the terminal web does form in *Pph13^hazy^* mutants. Given that the exact function of Rac1 has not been resolved in rhabdomere biogenesis and that small Rho GTPases have been implicated in mediating signals required for actin reorganization (Etienne-Manneville and Hall, 2002; Hall, 1998), future experiments will address the function of Rac1 in photoreceptor terminal differentiation and determine how the misregulation of Rac1 accumulation and activity may be contributing to the *Pph13^hazy^* rhabdomere phenotypes.

Homeodomain proteins and photoreceptor terminal differentiation

Our molecular cloning of *Pph13^hazy^* has identified another homeodomain gene required for photoreceptor morphogenesis. Previous reports have established or implicated *eyeless* (Pax6), *orthodenticle* (*otd*) and *Onecut* homeodomain genes in eye development (Nguyen et al., 2000; Quiring et al., 1994; Vandendries et al., 1996). What is the relationship between these various homeodomain transcription factors and how do they coordinate photoreceptor terminal differentiation? Numerous possibilities exist in which each of these transcription factors could control a unique subset of molecular mechanisms required for a functional photoreceptor cell; alternatively, they could act in concert on the same genes to promote differentiation. To eliminate or confirm any one of these possibilities would be premature and further extensive characterization of each of these genes in photoreceptor development is necessary.

Nevertheless, our preliminary data does allow for some speculation. First, it is clear that *eyeless* is required for photoreceptor cell specification and without it a photoreceptor cell a gene like *Pph13* could not function. Besides its early role in photoreceptor cell specification, *eyeless* is also necessary for rhodopsin expression (Papatsenko et al., 2001; Sheng et al., 1997) and superficially, characterization of the late

transcriptional targets of Eyeless and Pph13 appear to be different. First, Pph13 is absolutely required for *trpl*, *trpγ* and *Gβ* expression but is not necessary for rhodopsin expression. This result would suggest that once a cell has committed to a photoreceptor cell fate, both Eyeless and Pph13 have separate and distinct molecular pathways that contribute to photoreceptor differentiation.

However, comparison of *otd* and *Pph13* mutants suggest a more complex mode of coordination for photoreceptor differentiation. First, the rhabdomere defects observed otd^{uvi} and $Pph13^{hazy}$ mutants are similar (Vandendries et al., 1996). In each case, the defects appear not to be the result of degeneration but a failure in their biogenesis. The rhabdomere terminal web does form in both cases but the overall size and morphology are abnormal. Both Otd and Pph13 are required in the same developmental time window for rhabdomere morphogenesis, but neither is necessary for the expression of the other (data not shown). Whether Otd and Pph13 represent two parallel pathways directing the expression of the same genes or two distinct pathways with different genetic targets to promote rhabdomere biogenesis will require further investigation. In addition, they do not share a defect in phototransduction. otd^{uvi} photoreceptor cells exhibit normally ERGs and we do not detect the loss of phototransduction proteins downstream of rhodopsin as seen in Pph13 mutants (data not shown). Clearly, Pph13 is responsible for two aspects of photoreceptor cell differentiation: phototransduction and rhabdomere morphogenesis.

Given that the molecular mechanisms orchestrating the differentiation of photoreceptor cells remain largely undefined, the goal of our genetic approach was to isolate genes required for photoreceptor terminal differentiation. Our work with $Pph13^{hazy}$ has shed some light on the regulation of this process. However, additional studies that combine the accessibility and genetic amenability of *Drosophila* eye development, with whole genome expression profiling techniques in both wild-type and $Pph13^{hazy}$ mutant photoreceptor cells, should identify additional transcriptional targets necessary for photoreceptor cells to achieve and maintain a functional state.

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