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Pph13 and Orthodenticle define a dual regulatory pathway for photoreceptor cell morphogenesis and function

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SUMMARY

The function and integrity of photoreceptor cells are dependent upon the creation and maintenance of specialized apical structures: membrane discs/outer segments in vertebrates and rhabdomeres in insects. We performed a molecular and morphological comparison of *Drosophila Pph13* and *orthodenticle* (*otd*) mutants to investigate the transcriptional network controlling the late stages of rhabdomeric photoreceptor cell development and function. Although Otd and Pph13 have been implicated in rhabdomere morphogenesis, we demonstrate that it is necessary to remove both factors to completely eliminate rhabdomere formation. Rhabdomere absence is not the result of degeneration or a failure of initiation, but rather the inability of the apical membrane to transform and elaborate into a rhabdomere. Transcriptional profiling revealed that *Pph13* plays an integral role in promoting rhabdomeric photoreceptor cell function. Pph13 regulates *Rh2* and *Rh6*, and other phototransduction genes, demonstrating that Pph13 and Otd control a distinct subset of Rhodopsin-encoding genes in adult visual systems. Bioinformatic, DNA binding and transcriptional reporter assays showed that Pph13 can bind and activate transcription via a perfect Pax6 homeodomain palindromic binding site and the Rhodopsin core sequence I (RCSI) found upstream of *Drosophila* Rhodopsin genes. In vivo studies indicate that Pph13 is necessary and sufficient to mediate the expression of a multimerized RCSI reporter, a marker of photoreceptor cell specificity previously suggested to be regulated by Pax6. Our studies define a key transcriptional regulatory pathway that is necessary for late *Drosophila* photoreceptor development and will serve as a basis for better understanding rhabdomeric photoreceptor cell development and function.

KEY WORDS: Pph13, Orthodenticle (Ocelliless), Crx, Pax6, Photoreceptor, Rhabdomere, Rhodopsin, *Drosophila*

INTRODUCTION

Photoreceptor cells have evolved mechanisms to expand the apical membrane that houses the phototransduction machinery required for efficient light capture. Two strategies have emerged, exemplified by the cilia-based outer segment of the vertebrate photoreceptor cell and the microvilli-based rhabdomere of the invertebrate photoreceptor cell (Arendt, 2003; Gehring, 2004; Lamb et al., 2007). Comparative molecular cell biology results (Arendt and Wittbrodt, 2001) and the discovery of both rhabdomeric and ciliary photoreceptor cells in the marine ragworm *Platyneris* (Arendt et al., 2004) suggest that the common ancestor of invertebrates and vertebrates had both cell types present. As a result, the complex visual systems we see today could have evolved from the integration of these two separate populations of photoreceptor cells or the ancestral structure already had a visual system composed of these two fundamental types of photoreceptor cells and associated circuitry (Erclik et al., 2009). In either case, as put forth by Nilsson and Ardent (Nilsson and Arendt, 2008), a critical question in eye evolution is how and when these cell types were incorporated into light-sensing organs. One avenue to address this fundamental question has been to define the transcriptional network responsible for specification of these two cell types. A second and complementary approach would be to understand the transcriptional mechanisms directing the function of photoreceptor cells (i.e. the expression of Rhodopsin) and downstream effectors responsible for the morphological expansion of rhabdomeric and ciliary membranes.

The transcriptional control of retinal specification has been extensively studied and is known to be conserved between vertebrates and invertebrates (reviewed by Kumar, 2001; Kumar and Moses, 2001). However, this conserved transcriptional network for specification does not help to explain the structural and morphological differences that we see between rhabdomeric and ciliary photoreceptor cells. In addition, vertebrate studies suggest that the transcriptional choice to create a photoreceptor cell with a cilium is separable from the transcriptional network necessary to elaborate the membrane folds/discs of the light-gathering outer segment. For example, the cone-rod homeodomain protein Crx (Chen et al., 1997; Freund et al., 1997; Furukawa et al., 1997) is dedicated to the differentiation/morphogenesis of mammalian photoreceptor cells (reviewed by Hennig et al., 2008; Morrow et al., 1998). Mice lacking Crx specify photoreceptor cells but neither rod nor cone photoreceptor cells form their outer segments or are capable of phototransduction (Furukawa et al., 1999). More importantly, transmission electron microscopy (TEM) studies of *Crx*-null photoreceptor cells reveal that the photoreceptors polarize correctly and produce a connecting cilium but fail to elaborate the characteristic folds/discs that normally populate the outer segment; in other words, outer segment development stalls at the point of elongation and elaboration (Morrow et al., 2005). Subsequent studies have confirmed that Crx directs the expression of numerous

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photoreceptor-specific genes necessary for proper form and function, including those involved in phototransduction (e.g. rod transducin alpha, PDE gamma, arrestin) and factors necessary for the structural integrity of the outer segment (e.g. rhodopsin and peripherin) (Blackshaw et al., 2001; Livesey et al., 2000; Peng and Chen, 2005).

Crx is a member of a subfamily of homeodomain transcription factors known as the Otx family, which in vertebrates consists of Otx1, Otx2 and Crx. Otx family members are defined by a leucine at position 50 (K50) within the homeodomain. Interestingly, *Drosophila* encodes a single Otx family member, Orthodenticle (Otd; Ocelliless – FlyBase), which, like Crx, is crucial for controlling the expansion of the light-gathering apical surface of fly photoreceptors. The role of Otd in rhabdomeric photoreceptor morphogenesis was originally uncovered in a UV phototactic screen for R7 photoreceptor cell development (Vandendries et al., 1996). The viable, eye-specific allele of *otd*, otd^{uvi}, was recovered for its insensitivity to UV light, but further examination of otd^{uvi} photoreceptor cells revealed that *otd* was required for rhabdomere biogenesis. Subsequent studies demonstrated that Otd also regulates numerous photoreceptor-specific genes, including activation of the rhodopsins *Rh3* and *Rh5* as well as *Arrestin 2* (*Arr2*) (Tahayato et al., 2003; Renade et al., 2008). Thus, like Crx, Otd is necessary for the morphogenesis of photoreceptors and regulates similar gene products to Crx, suggesting that rhabdomeric and ciliary photoreceptors share similar genetic pathways for their terminal differentiation. Surprisingly, however, unlike *Crx*, a null mutant of *otd* does not completely eliminate rhabdomere formation or phototransduction, strongly suggesting that additional transcriptional pathways participate in rhabdomere formation and function in *Drosophila*.

In a screen for loci necessary for rhabdomere development, we uncovered a second homeodomain transcription factor necessary for both rhabdomere biogenesis and phototransduction, *Pph13* (Goriely et al., 1999; Zelhof et al., 2003). Pph13 contains a Q50 paired-class homeodomain, and initial characterization revealed a cell biological phenotype similar to that of *otd*: mutant photoreceptor cells are specified normally, but the control of rhabdomere formation and photoreceptor cell function are severely disrupted. Unlike *otd* mutants, adult *Pph13* mutant photoreceptors fail to respond to light, indicating that these factors might regulate overlapping pathways necessary during photoreceptor differentiation and/or maintenance.

Here, to further investigate the transcriptional network(s) controlling rhabdomere biogenesis and photoreceptor cell function, we performed a molecular and morphological comparison of *Pph13* and *otd* mutants. First, our results demonstrate that the absence of both *Pph13* and *otd* in flies mimics the elimination of Crx in vertebrates, resulting in correctly specified photoreceptor cells that are unable to form the light-sensing organelles. Second, transcriptional profiling of *Pph13* mutants revealed that this factor plays an integral role in promoting rhabdomeric photoreceptor cell function, in part through regulation of two Rhodopsin-encoding genes, *Rh2* and *Rh6*, and other phototransduction genes. Finally, we show that Pph13 binds to a conserved element found in all Rhodopsin promoters, the Rhodopsin core sequence I (RCSI), and is essential and sufficient to direct the expression of a reporter controlled by multimerized RCSI sites of photoreceptor cell specificity. Overall, our study has significantly modified the picture of the transcriptional network required for the later stages of photoreceptor cell development in terms of the expansion of the rhabdomere membrane and expression of key factors required for photoreceptor cell function.

MATERIALS AND METHODS

Microarray analysis

The platform for all the microarray experiments was the DGRC-2 oligonucleotide spotted arrays. The DGRC-2 arrays contain DNA fragments corresponding to ~93% of the genes in the *D. melanogaster* genome annotation version 4.3. The microarray experimental procedures followed the standard protocols available at the Drosophila Genomics Resource Center (DGRC) website (https://dgrc.cgb.indiana.edu/microarrays/support/protocols.html) that are optimized for handling *Drosophila* RNAs and the DGRC-2 arrays. Total head RNA for each developmental time point and genotype (*cn bw*, $Pph13^{hazy}$ *cn bw*, otd^{uvi} and otd^{uvi}; $Pph13^{hazy}$ *cn bw*) was isolated using Trizol and then reverse transcribed (Superscript III, Invitrogen) to cDNA. The cDNA was labeled using the Array 50 Dendrimer Kit (Genisphere) with Cy3 and Cy5 dyes. Following hybridization, the slides were scanned using an Axon GenePix Scanner 4200A. Image processing and generation of the GenePix results (GPR) file were performed using GenePixPro 6.0 (Axon). GPR files containing raw intensities were loaded into Bioconductor (release 2.5.1; http://www.bioconductor.org) for further analysis. Each slide was normalized individually using optimized local intensity-dependent normalization (OLIN) to correct for dye bias and to remove any artifacts. For each developmental time point, four slides were analyzed with two dye swaps. The normalized intensity values from all slides were then used to identify differentially expressed genes using linear models for microarray data (LIMMA). The resulting set of *P*-values was used as a measure of confidence for the observed intensity change between the two channels. Those genes that showed a greater than 2-fold change and had a *P*-value of less than 1% were considered as potential targets. Downregulated and upregulated genes are listed for each microarray experiment in Tables S1-S8 in the supplementary material. The accession number for the microarray data is GSE22613.

RT-PCR validation

Total head RNA from the appropriate genotypes and developmental time points was isolated using Trizol and first-strand synthesis was accomplished using Superscript III reverse transcriptase (Invitrogen) with both oligo(dT) and random hexamers as primers. PCR amplification was performed and the number of cycles was optimized for each set of primers. The list of primers used can be found in Table S9 in the supplementary material.

Pph13 binding site identification

The twelve genes downregulated in a $Pph13^{hazy}$ mutant at 72 hours after puparium formation (APF) were considered for identification of a Pph13 binding site. A 500 bp region upstream of each of the twelve genes was retrieved from FlyBase (version 4.3) and scanned for a conserved motif using MEME (Bailey and Elkan, 1994) using the following parameters: mod anr; minw 6; maxw 15; nmotifs 3; revcomp. The most significant motif matched a previously characterized Pph13-dependent regulatory region in the *G*β promoter (Zelhof et al., 2003) and thus was characterized further as a potential consensus Pph13 binding motif.

Electrophoretic mobility shift assay (EMSA)

EMSAs were performed as described (Zelhof et al., 2003; Zelhof et al., 1995). To generate a smaller version of Pph13, termed Pph13-S, the cDNA was digested with *Stu*I and religated. The removal of the *Stu*I fragment results in a protein of 208 amino acids (versus 358 amino acids) and contains the entire homeodomain. The sequences of the DNA oligonucleotides are included in Fig. 6. For Otd, we used a version of the protein that contains amino acids 1-393. The results obtained were identical as for the full-length protein, but the smaller version was more robustly produced in reticulocyte lysates.

Transfection assays

The *Rh5*-Luc and *Rh6*-Luc reporters and the pAc-lacZ construct used for transfection normalization have been described previously (Xie et al., 2007). *Rh1*-Luc was constructed by subcloning a *Bgl*II/*Hin*dIII fragment carrying the –253/+68 *Rh1* promoter fragment into pGL3basic (Promega). *Drosophila* S2 cells (Invitrogen) were maintained in HyQ SFX-Insect

media (Hyclone) at room temperature (RT, 20-22°C). 1×10^6 cells in 1 ml HyQ SFX were plated in 12-well tissue culture dishes (Corning) 24 hours prior to transfection with 1.5 μl Fugene HD (Roche). Cells were transfected with 200 ng each of a pGL3 promoter construct, pAc-lacZ, armadillo-Gal4 (kindly provided by Xinhua Lin, Cincinnati Children's Hospital Medical Center), UAS attB-Pph13, and/or pAc-Otd, and brought to a final concentration of 1 μg with either pAc5.1-HisA (Invitrogen) or pUAST (Brand and Perrimon, 1993) vectors. Luciferase assays were performed 48 hours post-transfection as previously described (Xie et al., 2007).

Transmission electron microscopy

Fly heads were dissected and fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) overnight at 4°C. The heads were then washed with 0.1 M cacodylate buffer three times and postfixed in 2% OsO_4 solution in 0.1 M cacodylate buffer for 2 hours. After fixation, heads were rinsed three times in 0.1 M cacodylate buffer, twice in distilled water, and then passed through a graded series of ethanol washes before incubation in 1:1 propylene oxide:Epon overnight at RT. The next day, tissues were incubated in pure Epon-812 resin for 6 hours, embedded and hardened at 60°C overnight. Ultrathin sections were placed on uncoated 300 mesh copper grids and stained with Reynold's lead citrate for 20 minutes and with 2% aqueous uranyl acetate for 15 minutes. Samples were observed under TEM operated at 60 KV and digital images were captured and imported into Adobe Photoshop.

Immunofluorescent stainings

Cryosections (12 μm) from the appropriate genotypes were sectioned, fixed and processed as previously described (Zelhof et al., 2003). The following stocks were used: *cn bw*, *Pph13^{hazy} cn bw*, *otd^{uvi}* and *otd^{uvi}; Pph13^{hazy} cn bw*. For 3XP3 detection, the following stocks were obtained from the Bloomington Stock Center: 3XP3-RFP (M{3×P3-RFP.attP}ZH-86Fb) and 3XP3-GFP (y^1 M{vas-int.Dm}ZH-2A w) and crossed into a *Pph13^{hazy}* mutant background or pCaSpeR-*hs-Pph13*. GFP and RFP were visualized directly, without fixation. All flies were kept at 22°C and heat shocks were performed at 37°C. The following primary antibodies were used: rabbit anti-Rh1 (1:500), anti-Rh4 (1:200), rabbit anti-InaD (1:400), mouse anti-Rh2 (1:100) (all from Dr C. Zuker, UCSD), and rabbit anti-Rh6 (1:2500; from Dr C. Desplan, NYU). Rhodamine-conjugated phalloidin (Molecular Probes) was used to detect F-actin. FITC-conjugated secondary antibodies were obtained from Jackson ImmunoResearch. Digital images were captured on a Leica SP5 scanning confocal microscope at the IU-Bloomington LMIC facility and imported into Adobe Photoshop.

RESULTS

Pph13 and Otd are essential for, and cooperate in, rhabdomere elaboration

The removal of either Pph13 or Otd does not affect specification of photoreceptor cells within an ommatidium, but all photoreceptor cells show an acute defect in rhabdomere morphogenesis (Vandendries et al., 1996; Zelhof et al., 2003). Despite the general similarities in rhabdomeric phenotypes between these mutants, the genes that are affected in *Pph13^{hazy}* versus *otd^{uvi}* mutants are distinct (Ranade et al., 2008; Zelhof et al., 2003). The mutants also have different physiological characteristics: *otd^{uvi}* flies are UV insensitive, but otherwise maintain an intact electroretinogram (ERG), whereas *Pph13^{hazy}* mutants do not respond to any light. Thus, these data suggest that Otd and Pph13 might regulate separate pathways during photoreceptor differentiation.

To better define the genetic relationship between these two factors during rhabdomere biogenesis, we performed a detailed TEM analysis of rhabdomere formation in various genetic combinations of *Pph13* and *otd*. Consistent with these factors functioning in separate pathways, we did not detect an enhancement in rhabdomere phenotype with the removal of one copy of either transcription factor in the homozygous background of the other (*otd^{uvi}/y; Pph13^{hazy}/+* or *otd^{uvi}/+; Pph13^{hazy}/Pph13^{hazy}*), and did not observe rhabdomere defects in the transheterozygote (*otd/+; Pph13^{hazy}/+*) (data not shown). However, newly eclosed flies from *otd^{uvi}; Pph13^{hazy}* double mutants showed a complete loss of rhabdomere formation: in the majority of ommatidia, there were no detectable rhabdomeres, and in some ommatidia only rudimentary structures containing some juxtaposed membrane were seen distally, but clearly nothing resembling a mature rhabdomere was observed (Fig. 1).

Although the rhabdomeric phenotypes for both *Pph13* and *otd* mutants have been shown to be a result of a developmental defect in morphogenesis, and not degeneration, Pph13 and Otd do regulate the transcription of factors that when absent can lead to retinal degeneration (Kumar and Ready, 1995) (see below). Furthermore, there is no doubt that the photoreceptor cells of the double mutant are not healthy and undergo degeneration. As such, we needed to address whether the absence of rhabdomeres observed in the double mutant is the result of degeneration or truly represents a failure of the rhabdomeres to initiate or elaborate into the correct structure. To test this, we compared the morphogenesis of rhabdomeres in *Pph13*, *otd*, and the double mutant to *cn bw* at three developmental time points: 60, 72 and 96 hours APF. At 60 hours APF, ~12 hours after the first detection of changes in the apical membrane of wild-type photoreceptor cells, we assayed whether or not the process of rhabdomere morphogenesis had initiated. We observed, in all genetic combinations (Fig. 2A-D), that the photoreceptor apical membranes had separated, primordial microvilli-like projections were present and the inter-rhabdomeral space was forming (Fig. 2D). This was further confirmed by the finding that Spacemaker (Eys), a marker of rhabdomere initiation (Husain et al., 2006; Zelhof et al., 2006), is also secreted in these mutants (data not shown). However, it was evident that the organization of the apical membranes was not identical between

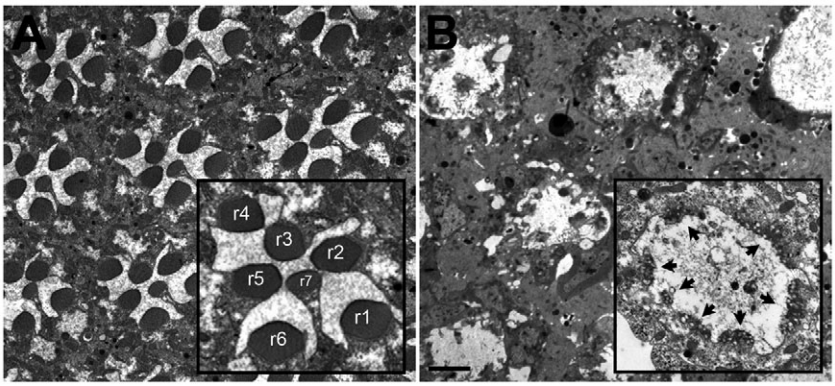

Fig. 1. Rhabdomeres are absent in the *otd; Pph13* double mutant. (**A**) Transmission electron microscopy (TEM) of wild-type *cn bw* ommatidia. A rhabdomere (r) is present for each photoreceptor cell in this section: six outer and one inner rhabdomere. (**B**) TEM of *otd; Pph13* double-mutant ommatidia. There is a complete absence of rhabdomeres in the mutant photoreceptor cells (arrows) and all photoreceptor cells are present. Samples are from newly emerged adult *Drosophila*. Scale bar: 5 μm.

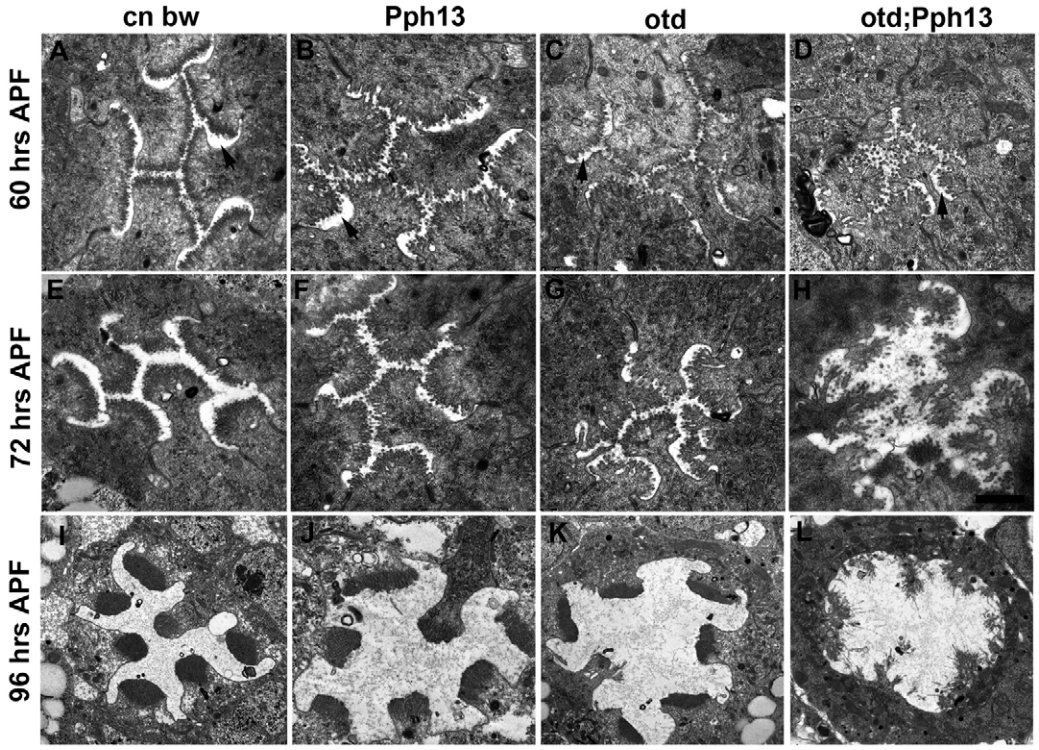

Fig. 2. Developmental profile of rhabdomere morphogenesis. (**A-L**) TEM of rhabdomere formation in *cn bw* (A,E,I), *Pph13* (B,F,J), *otd* (C,G,K) and *otd; Pph13* (D,H,L) genetic backgrounds at 60, 72 and 96 hours APF. (A-D) In all genetic backgrounds, including the double mutant, the process of rhabdomere initiation has begun. The apical membranes have separated, microvilli-like projections are present and an extracellular space is observed (arrows). (E-H) However, by 72 hours APF, rhabdomeric defects are observed in all mutant phenotypes, with the *otd; Pph13* double mutant being most severe. (I-L) By 96 hours, there are discernible rhabdomere structures in each of the single mutants and a clear absence and loss of rhabdomeric structures in the double mutant prior to eclosion. Scale bar: 1 μm.

wild-type and mutant photoreceptor cells. In all three cases, with the double mutant being the most extreme, there appeared to be a clear disorganization of the initial stages of rhabdomere elaboration. At this point, our analysis cannot distinguish whether the phenotypes we observe represent a delay in the process or whether both transcription factors are necessary for the coordination of these events. Nevertheless, we believe our results indicate that even though both Pph13 and Otd are present before the photoreceptor cell apical membrane begins its transformation into a rhabdomere (Vandendries et al., 1996; Zelhof et al., 2003), neither is required for commencement of this process, but rather that both are crucial in orchestrating the already initiated process of actin and membrane reorganization into the specific structure of a rhabdomere.

By 72 hours APF, there was a profound difference in the otd^{luvi}; $Pph13^{hazy}$ double mutants as compared with the other three genotypes. At this developmental time under wild-type conditions, definable microvilli-like projections are observed. However, in each single mutant, we observed continuing defects in rhabdomere morphogenesis, and in the double mutant there was no indication of a coordinated effort to form microvilli on the apical surface of each photoreceptor cell and the phenotype was more severe than that of each single mutant (Fig. 2E-H). Rather, the apical rhabdomeric membrane appeared to lack any definable shape and organization. Thus, these data further corroborate the idea that both transcription factors work in separate pathways that cooperate for the creation of a rhabdomere. Second, these findings indicate that the phenotypes observed at the earlier time points are not simply a temporal delay in the process, such as in other mutants affecting rhabdomeres morphogenesis (Zelhof and Hardy, 2004). Instead, our TEM analyses demonstrate that there is a clear attempt by the apical membrane to reorganize but that this process lacks direction.

We next analyzed rhabdomeres at 96 hours APF (Fig. 2I-L). In wild-type flies, tightly packed elongated microvilli extend the depth of the retina (~100 μm) (Longley and Ready, 1995). As described previously, all rhabdomeres (R1-R8) of the single mutants are present but are smaller, misshapen and do not extend the entire length of the photoreceptor cell (Vandendries et al., 1996; Zelhof et al., 2003). Regardless of their shape, however, they are still capable of housing phototransduction proteins (Zelhof et al., 2003) (data not shown). In the double mutant, by contrast, no rhabdomere was recognizable. Instead, in distal portions of the retina there was a region of the apical membrane that extends out into the inter-rhabdomeral space but lacks any organized structure resembling wild-type photoreceptor cells (Fig. 2L). Furthermore, these structures are not capable of housing the phototransduction machinery (data not shown) but they are visible along the entire length of the photoreceptor cell. However, we could not detect discernible photoreceptor cell structures beyond ~20 μm from the surface of the retina, compared with the 100 μm for a wild-type photoreceptor cell (see Fig. S1 in the supplementary material; data not shown). Together, our temporal morphological analyses confirm that the phenotype we observe in the adult is a failure of the rhabdomeres to form and that, either indirectly or directly, the loss of *Pph13* and *otd* eventually contributes to the overall degeneration of the photoreceptor cells.

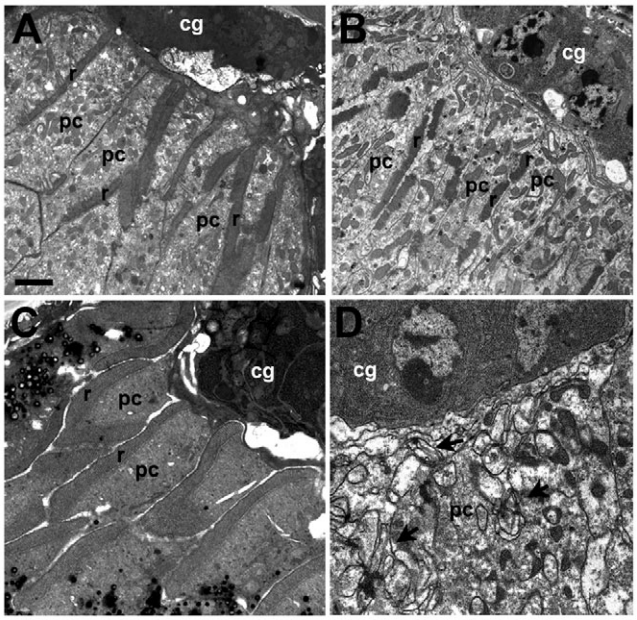

Fig. 3. Pph13 and Otd are required for rhabdomere formation in ocelli photoreceptor cells. (**A-D**) TEM of rhabdomere formation in *cn bw* (A), *Pph13* (B), *Otd* (C) and *otd; Pph13* (D) ocelli. The rhabdomeres are present in each single mutant but there are no apparent rhabdomeres in the double mutant, as compared with wild type; rather, there appear to be superfluous strands of membrane (arrows), and photoreceptor cell boundaries are not discernible. cg, corneagenous cells; pc, photoreceptor cell; r, rhabdomere. Scale bar: 2 μm.

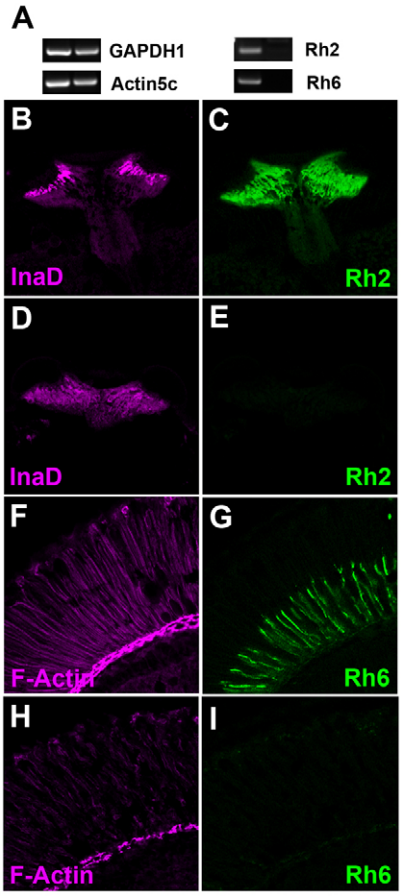

Fig. 4. *Rhodopsin 2* and *Rhodopsin 6* expression is absent in the *Pph13* mutant. (**A**) RT-PCR reactions validating the absence of *Rh2* and *Rh6* in *Pph13* mutants, as compared with wild-type *cn bw*. *Actin 5C* and *Gapdh1* were used as controls and showed no differential expression in our microarray analysis. (**B-E**) InaD (purple) and Rh2 (green) expression in 12 μm sections through *cn bw* (B,C) and *Pph13* (D,E) ocelli. Two of the three ocelli are pictured. Note the complete absence of Rh2 in *Pph13* mutant ocelli photoreceptor cells, whereas InaD expression is maintained, in agreement with our microarray analysis. (**F-I**) F-actin (purple) and Rh6 (green) expression in 12 μm sections through *cn bw* (F,G) and *Pph13* (H,I) eye photoreceptor cells. In the adult eye, Rh6 is expressed in a subset of R8 photoreceptors and localizes to the rhabdomeres of R8 photoreceptors (G) and, like Rh2, Rh6 expression is not detected in *Pph13* mutants (I). All samples are from newly emerged adult *Drosophila*.

In addition to photoreceptors in the adult *Drosophila* compound eye, adult flies have two additional light-sensing tissues composed of rhabdomeric photoreceptors: the eyelet and the ocelli (Helfrich-Forster et al., 2002; Stark et al., 1989). Interestingly, the arrangement of the rhabdomeres in these photoreceptor organs differs from that of retinal photoreceptors in that they do not have an inter-rhabdomeral space separating the rhabdomeres, but are instead juxtaposed to each other. The organization of these photoreceptor cells mimics the organization of photoreceptor cells found in other insects (e.g. *Tribolium castaneum* and *Apis mellifera*), in which the rhabdomere is in a fused/closed orientation. Since Otd and Pph13 are both expressed in ocelli, we next tested whether the same relationship exists between Pph13 and Otd in the elaboration of ocelli-associated rhabdomeres. Surprisingly, the rhabdomeres of the ocelli in otd^{uvi} or *Pph13* single mutants showed little difference from those of wild-type tissues (Fig. 3A-C). However, as with the retinal photoreceptors, loss of both Otd and Pph13 led to a failure in all rhabdomere formation (Fig. 3D) but did not affect cell specification (Fig. 4D; data not shown). Therefore, our results define Pph13 and Otd as two essential transcription factors that cooperate during rhabdomere elaboration in adult *Drosophila* photoreceptor cells.

Pph13 directs the expression of key factors for photoreceptor cell function

Previous work has revealed that both Pph13 and Otd regulate genes responsible not only for rhabdomere formation, but also for photoreceptor cell function. Specifically, phototransduction is eliminated in *Pph13* mutants (Zelhof et al., 2003), although the nature of this defect is unknown. Mutant analysis and genomic survey of transcriptional changes in an *otd* mutant have demonstrated that Otd is crucial for the expression of Arr2, Rh3 and Rh5 (Ranade et al., 2008), but *otd* mutants are still capable of eliciting an ERG response (Vandendries et al., 1996).

Since the nature of the defects in phototransduction is poorly understood for *Pph13* mutants, we performed a series of microarray analyses comparing *Pph13 cn bw* mutant heads with those of the *cn bw* isogenic line from which the *Pph13* mutant line was derived (Koundakjian et al., 2004). Heads were chosen because *Pph13* is exclusively expressed in photoreceptor cells, and this approach has proved successful for identifying Otd-dependent target genes (Ranade et al., 2008). We chose three developmental time points for analysis: 48 hours and 72 hours APF and less than

1-day-old adult flies. These time points correspond to the distinct late stages of photoreceptor cell development: initiation of rhabdomere biogenesis, the process of rhabdomere elongation/first detection of genes involved in phototransduction, and the mature functional photoreceptor cell, respectively.

In agreement with our morphological studies, even though Pph13 protein is detected in photoreceptors as early as 36 hours APF, we did not find any significant differences between control and *Pph13* mutants at 48 hours APF (data not shown). However, we identified 12 genes that are significantly downregulated in *Pph13* mutant heads at 72 hours APF (see Table S1 in the supplementary material). Two of these were previously identified as misregulated in *Pph13* mutants, confirming the validity of this approach (Zelhof et al., 2003). In addition, 11 of the 12 genes we identified by microarray analysis were confirmed as being downregulated in *Pph13* mutants by RT-PCR (see Fig. S2 in the supplementary material). Importantly, of these 11 targets, four are known components of phototransduction: *Gβ* (*Gβ5* – FlyBase), *Arr2*, *trp* and *ninaC*, and when mutated, all four of these have previously been shown to affect photoreceptor cell function and/or influence the localization and function of other phototransduction proteins (reviewed by Wang and Montell, 2007). Of the remaining downregulated genes in *Pph13* mutants, their in vivo function and role in photoreceptor cell development remain unknown. Interestingly, the inclusion of more than one phototransduction gene suggests that the lack of phototransduction in *Pph13* mutants might be a cumulative effect of multiple missing components and that Pph13 represents a key transcriptional regulator of photoreceptor cell function.

Like Crx and Otd, Pph13 directs Rhodopsin expression

Knowing that Pph13 function is essential for phototransduction and knowing the identity of the phototransduction genes that are expressed at 72 hours APF, we hypothesized that the microarray analysis comparing the transcriptional differences between control and *Pph13* mutant newly eclosed flies would identify additional factors required for the response to light or maintenance of the photoreceptor cell. Indeed, there was an increase (compared with 72 hours APF) in the number of genes showing a dependency on Pph13 for expression, including, more importantly, the identification of other phototransduction factors, specifically Rhodopsins (see Table S2 in the supplementary material). Like Otd, Pph13 appears to activate the transcription of two Rhodopsin genes. Otd is essential and activates *Rh3* and *Rh5*, and here we found that Pph13 is necessary for *Rh2* and *Rh6* expression. Rh2 expression is limited to the photoreceptors of the ocelli (Mismer et al., 1988; Pollock and Benzer, 1988). Rh6, by contrast, is expressed in a subset of adult R8 photoreceptor cells, a subset of photoreceptor cells in the larval eye (Bolwig's organ), and in photoreceptor cells of the adult eyelet (a derivative of Bolwig's organ) (Huber et al., 1997; Sprecher and Desplan, 2008; Sprecher et al., 2007; Yasuyama and Meinertzhagen, 1999). As mentioned above, consistent with the ability of Pph13 to regulate *Rh2* and *Rh6* expression, Pph13 is indeed expressed in all photoreceptor cells of the adult eye, the adult eyelet, ocelli (Goriely et al., 1999; Zelhof et al., 2003) and in Bolwig's organ (data not shown). RT-PCR confirmed an absence of mRNA for *Rh2* and *Rh6* (Fig. 4A) and immunofluorescent staining showed that no Rh2 or Rh6 protein is detected in ocelli or retinal photoreceptor cells in a *Pph13* mutant (Fig. 4B-I). Together, these results demonstrate that of the six Rhodopsins expressed in *Drosophila*, two (*Rh3* and *Rh5*) are dependent on *otd* and two (*Rh2* and *Rh6*) are dependent on *Pph13*, further supporting the idea that Pph13 is a key transcriptional regulator of photoreceptor cell function.

Given the cooperation between Pph13 and Otd for rhabdomere elaboration and Rhodopsin expression, we also performed microarray profiling of otd^{1svi}; $Pph13^{hazy}$ double mutants at 72 hours APF and in newly eclosed flies. These studies not only identified genes that were previously identified from otd^{1svi} or $Pph13^{hazy}$ mutants (Ranade et al., 2008) (see above), but also a large number of other genes not identified by single mutant analysis (see Table S4 in the supplementary material). For instance, not only were *Rh2*, *Rh3*, *Rh5* and *Rh6* transcripts missing, but now *Rh1* (*ninaE* – FlyBase) and *Rh4* transcripts were also reduced (see Fig. S3 in the supplementary material). Immunofluorescent staining suggested, however, that although Rh4 was not detected, some residual Rh1 was present (see Fig. S4 in the supplementary material). There are a few possible explanations for this result. First, the reduction or absence of Rh4 and Rh1 might be indirect. As mentioned, the double-mutant photoreceptors are unhealthy and thus the stability of Rhodopsin gene expression in general is likely to be severely impacted by the degenerating photoreceptor cells and the absence of rhabdomeres. Furthermore, the detection of some Rh1 is probably due to the fact that there are at least 6-fold more photoreceptor cells that express *Rh1* than *Rh4*. The second possibility is that both of these Rhodopsins are downregulated in the double mutant and that the residual amount of Rh1 present is related to the nature of the *otd* allele used, as otd^{1svi} is not a null allele. Thus, Pph13 and Otd cooperate for maximal expression. However, as there does not appear to be an Otd binding site in the *Rh1* minimal promoter, this latter scenario is unlikely. The third option, based on our binding results (see below), derives from the very nature of the bipartite structure of Rhodopsin promoters (Fortini and Rubin, 1990): a combination of promoter elements and transcription factors are required for both maximal and cell-specific expression. In this scenario, Pph13 binds to the *Rh1* promoter but is not necessary for expression; rather, a second transcription factor provides this function (see Discussion).

The Pph13 consensus binding site resembles an RCSI element

Given that *Pph13* mutant photoreceptors are not functional and that the rhabdomeres are already disorganized by 72 hours APF, we needed to determine whether our downregulated targets, especially our phototransduction factors, represent direct or indirect transcriptional targets of Pph13. As such, we asked whether a consensus binding site could be identified within any of the targets. Pph13 contains a paired-like homeodomain that includes a glutamine at position 50, and previous studies supported the hypothesis that a Pph13 binding site would include a core sequence of TAATTG (CAATTA) (Wilson et al., 1993; Wilson and Desplan, 1995; Wilson et al., 1996). Using this sequence as a reference, we asked whether a consensus motif for Pph13 could be established from the target genes that we identified in our Pph13 microarray studies. For this, we focused on the downregulated genes identified at 72 hours APF to enrich for potentially direct targets. By comparing the upstream regions (500 bp) of these genes, we found that four share a consensus motif of fourteen nucleotides that includes the predicted binding site CAATTA.

Analysis of the consensus motif identified above reveals that it is a degenerative palindrome of TAAT spaced by three nucleotides (Fig. 5). This is similar to putative Pph13 binding sites previously shown to be required for Pph13-dependent activation of the *Gβ* promoter (Zelhof et al., 2003), and suggests that Pph13 has the

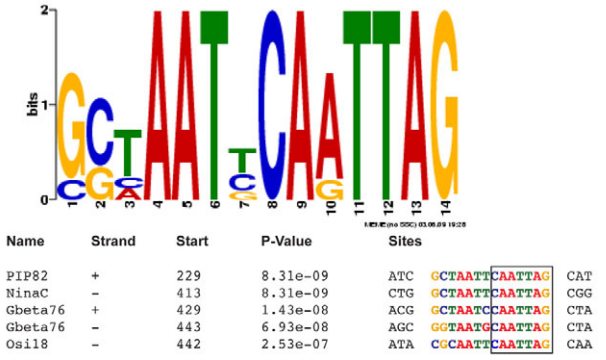

Fig. 5. Identification of a Pph13 binding motif. Representation of conserved sequences found in the promoter regions of putative Pph13-dependent transcriptional targets using MEME (Bailey and Elkan, 1994). The consensus sequence is boxed.

potential to bind as a homodimer or interact with another homeodomain protein to promote gene transcription. Strikingly, the consensus site also resembles a perfect Pax6 homeodomain palindromic binding site and the RCSI element found in all *Drosophila* Rhodopsin promoters (Fig. 6) (Fortini and Rubin, 1990; Mismer et al., 1988; Papatsenko et al., 2001).

Pph13 binds the RCSI element

RCSI is essential for the expression of all Rhodopsins, and previous studies suggested that Pax6 was necessary for activating transcription through this element, although the data are conflicting (Papatsenko et al., 2001; Punzo et al., 2001; Sheng et al., 1997; Tahayato et al., 2003). Nevertheless, our data suggest the possibility that Pph13 represents the paired-like homeodomain protein important for directing the expression of key genes required for late photoreceptor cell development and function, especially Rhodopsins, in rhabdomeric photoreceptor cells.

If Pph13 is a key transcription factor for late photoreceptor development and function, our data raise a series of testable predictions. First, Pph13 should bind an RCSI element and a predicted Pax6 homeodomain binding site. In addition, given the nature of these sites as a palindromic sequence of TAAT, Pph13 has the potential to bind as a homodimer. To test these predictions, we performed a series of electrophoretic mobility shift assays (EMSAs). As shown in Fig. 6, Pph13 can bind a Pax6 site. Furthermore, using two differently sized forms of Pph13, we demonstrated that Pph13 can bind as a homodimer on this element. When the different RCSI sequences were used as probes, a perfect correlation was found between the sites that contain a consensus binding sequence for Pph13 (CAATTA) and the ability of Pph13 to bind the RCSI sites of *Rh1*, *Rh2*, *Rh4* and *Rh6* (Fig. 6). However, upon longer exposures, we observed that Pph13 does have a lower affinity for the RCSI sites of *Rh3* and *Rh5* (see Fig. S5 in the supplementary material). EMSAs with Otd, by contrast, showed that Otd binds only to RCSI sequences that contain a K50 homeodomain consensus binding site (see Fig. S6 in the supplementary material). When Pph13 and Otd were mixed we did not see any evidence of heterodimers forming on any of the RCSI sequences (data not shown).

Pph13 regulates a subset of Rhodopsin promoters in vitro

Based on our findings that Pph13 binds to a subset of RCSI sites in vitro and that Pph13 is required for the transcription of *Rh2* and *Rh6* in vivo, we tested the possibility that Pph13 is sufficient to

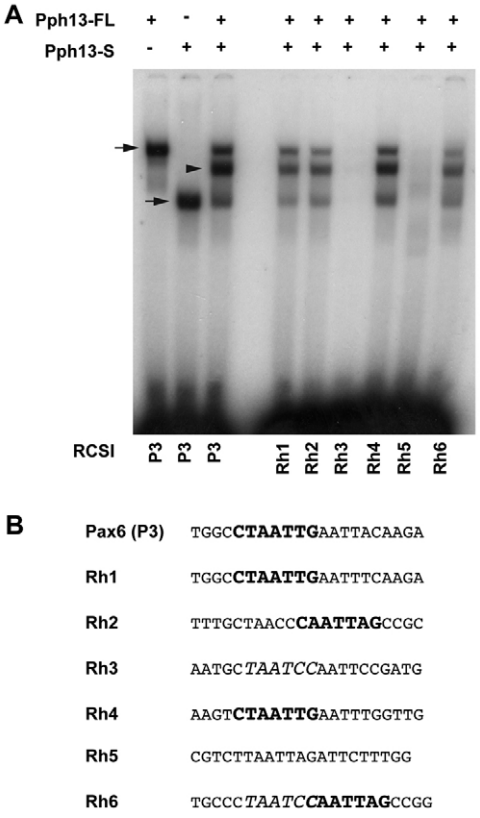

Fig. 6. Pph13 binds to Rhodopsin core sequence I (RCSI). (**A**) Electrophoretic shift mobility assay (EMSA) of Pph13 binding to a Pax6 homeodomain binding site (P3) and the various Rhodopsin gene RCSI elements. Two versions of Pph13 containing the homeodomain were used: full length (Pph13-FL, 358 amino acids) and a smaller version (Pph13-S, 208 amino acids). The mixing of these two forms produces an intermediate band (arrowhead; compare with each one alone, arrows), demonstrating that Pph13 is binding as a homodimer. (**B**) Sequence of the DNA elements used in the EMSA in A. Bold indicates the consensus binding site for Pph13 (P3, Rh1, Rh2, Rh4 and Rh6) and italics indicates the binding site for Otd (Rh3, Rh6).

regulate distinct subsets of Rhodopsin promoters using a reporter-based assay in *Drosophila* S2 cells. In addition, we compared Pph13 activity with that of Otd, which was previously shown to activate *Rh3* and *Rh5* in this system, similarly to its function in vivo (Xie et al., 2007). Our data raise the following predictions: (1) that Pph13 should activate transcription of *Rh6*; and (2) that Otd, and not Pph13, should have the ability to activate transcription from the RCSI of *Rh5*. Indeed, we observed a direct correlation with our predictions (Fig. 7), further demonstrating that Otd and Pph13 are required for a subset of Rhodopsin gene expression and photoreceptor cell function.

Pph13 is necessary and sufficient to regulate RCSI-dependent transcription in vivo

Our binding data implicate Pph13 as essential for activating transcription via an RCSI element and thus as the key factor for late *Drosophila* photoreceptor cell development. However, Pax6, or a Pax6-like factor, has been previously proposed to function in this same role (Papatsenko et al., 2001; Sheng et al., 1997; Tahayato et al., 2003). The idea that Pax6 is essential for late *Drosophila* photoreceptor cell function was in part determined by

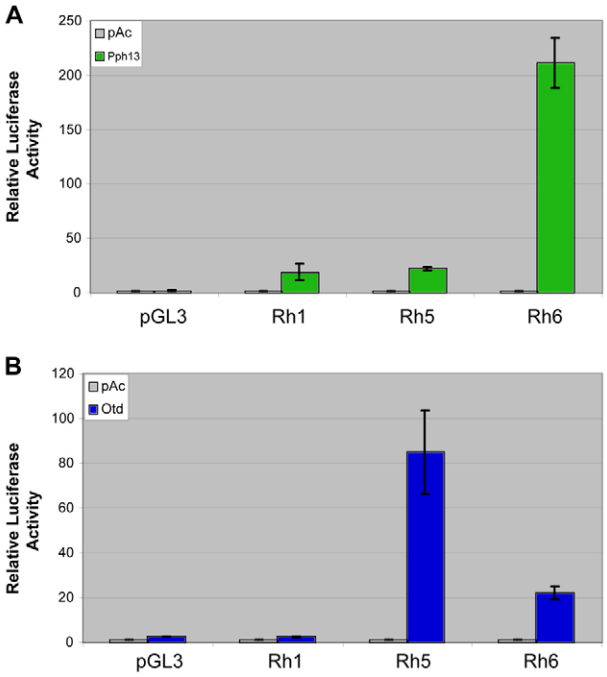

Fig. 7. Pph13 can activate transcription through the RCSI. Relative luciferase activity of *Rh1*, *Rh5* and *Rh6* minimal promoters with (**A**) Pph13 or (**B**) Otd, as compared with the pAc vector alone. Pph13 can activate transcription via the *Rh6* promoter and Otd can activate transcription via the *Rh5* promoter.

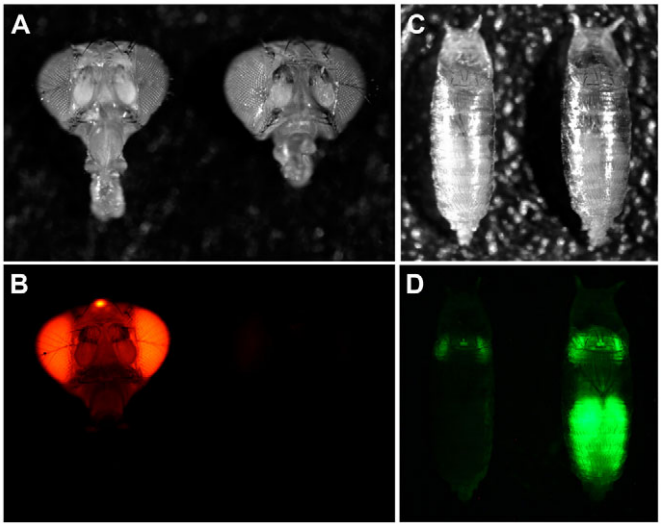

Fig. 8. Pph13 is essential and sufficient for activation of the 3XP3 reporter. (**A**,**B**) 3XP3-RFP expression in a *Pph13*/+ heterozygote (left) compared with a homozygous *Pph13* mutant (right). Note the complete loss of expression in the eyes and ocelli in the *Pph13* mutant. (**C**,**D**) 3XP3-GFP expression in wild-type (left) and *hs-Pph13* (right) *Drosophila* pupae. Both genotypes were subject to two heat shocks (37°C) twice a day for 40 minutes, commencing at 24 hours APF. Note that the ectopic expression of Pph13 is sufficient to drive expression of GFP outside of photoreceptor cells.

the fact that three copies of a perfect Pax6 homeodomain palindromic binding site, a P3 RCSI site, is capable of driving expression of reporter genes only in photoreceptor cells (Sheng et al., 1997). Unfortunately, given the importance of Pax6 in early eye development, testing the possibility that Pax6 is directly necessary for late photoreceptor cell function has been hampered, and thus the possibility of Pax6 or other factors acting through the RCSI element cannot be eliminated. Nevertheless, we examined whether Pph13 is necessary and/or sufficient for 3XP3 (multimerized RCSI) expression in vivo. We compared 3XP3-RFP expression in the photoreceptors of *Pph13* mutants and their heterozygous siblings. As shown in Fig. 8, all photoreceptor cell expression of the 3XP3 reporter was lost in the absence of Pph13 (Fig. 8A,B). To address whether Pph13 is sufficient for reporter expression, we ectopically expressed Pph13 in other tissues in the reporter strain. Expressing Pph13 under the control of a heat shock-inducible promoter resulted in expanded expression of the 3XP3 reporter beyond photoreceptor cells (Fig. 8C,D). The ectopic expansion was observed at all stages and did not appear to be tissue specific (data not shown); expansion was dependent on the presence of Pph13. Together, these results consolidate the idea that Pph13 is a fundamental factor not only for photoreceptor cell morphogenesis, but also for photoreceptor cell function, in particular Rhodopsin expression.

DISCUSSION

Pph13 and Otd act in concert to create a rhabdomere

To date, little is known about the transcriptional network required for establishing a rhabdomere. Significantly, our findings emphasize that there are at least two homeodomain transcription factors, Pph13 and Otd, that are required for photoreceptor cell morphogenesis and function. First and foremost, Pph13 and Otd cooperate for rhabdomere elaboration. The loss of either results in poorly formed rhabdomeres, although the rhabdomeres are still present and phototransduction proteins still accumulate within (Zelhof et al., 2003) (data not shown). Only upon the removal of both factors do the rhabdomeres fail to materialize. Moreover, our morphological analyses demonstrate that the failure of rhabdomeres to develop is due neither to degeneration nor to an inability to initiate the process. Our data suggest that the roles of Pph13 and Otd are to coordinate and direct the morphological changes of the actin cytoskeleton and apical membrane into the specific and stereotypic structure of a rhabdomere. This dependency on two homeodomain transcription factors is in contrast to vertebrate photoreceptor cells, in which the identical process of expanding the membrane to house the phototransduction machinery is relegated to one protein, Crx, a vertebrate homolog of Otd.

Another intriguing observation from our studies is the difference between the morphological role of Otd and Pph13 in ocelli versus eye photoreceptor cells. The loss of either factor in ocelli does not result in noticeable defects in rhabdomere formation, in stark contrast with the situation for the eye. Why the difference? One possibility is that the lack of an inter-rhabdomeral space decreases the pressure on the microvilli to create a cohesive structure. For example, a defined target of Otd is *chaoptin*, which encodes a protein that is crucial for proper microvilli adhesion and which is essential to keep the microvilli together during the formation of the inter-rhabdomeral space (Krantz and Zipursky, 1990; Reinke et al., 1988; Van Vactor et al., 1988; Zelhof et al., 2006) (our unpublished data). As a result, the lack of an extracellular matrix does not interfere with the ability of the apical membrane to form microvilli. Nevertheless, determining exactly how Pph13 and Otd coordinate

rhabdomere morphogenesis or why expansion of the photoreceptor membrane in rhabdomeres has been partitioned to two homeodomain transcription factors will require further characterization of many of the Pph13-, Otd- and Pph13-Otd-dependent transcription targets.

Pph13 ensures the expression of factors required for photoreceptor cell function

The expression of Rhodopsin or other eye-specific phototransduction proteins is a key indicator of when a ciliated or rhabdomeric cell has been designated to act as a photoreceptor cell. With respect to *Drosophila*, the characterization of Rhodopsin promoters has indicated a bipartite structure (Fortini and Rubin, 1990) for directing photoreceptor cell expression and subtype specificity. To ensure photoreceptor cell expression there is a common essential element that is found in all *Drosophila* Rhodopsin promoters: RCSI. RCSI represents one half of the bipartite structure, and mutation of this element eliminates photoreceptor cell expression. Although this element alone is not sufficient for photoreceptor cell expression (Fortini and Rubin, 1990; Mismer and Rubin, 1987; Mismer and Rubin, 1989; Sheng et al., 1997), when multimerized, such as in the 3XP3 reporter, it is sufficient to drive and limit expression to all photoreceptor cells (Sheng et al., 1997). Thus, the presence of the RCSI suggests that there is a common factor(s) required in all photoreceptor cells that ensures eye-specific expression of Rhodopsin genes (Papatsenko et al., 2001). Based on these observations, we would expect that an RCSI regulatory factor would have the following characteristics: (1) it should be expressed in all *Drosophila* photoreceptor cells; (2) it would be a homeodomain transcription factor that is able to form a hetero- or homodimer due to the presence of the palindromic sequence TAAT; and (3) it should be capable of binding the various RCSI elements and, most importantly, be sufficient and necessary for the expression of 3XP3 in vivo. The data presented here indicate that Pph13 satisfies all the above criteria. Furthermore, our microarray profiling has identified other known, and yet to be characterized, photoreceptor proteins (Rhodopsins, *G*β, NinaC, Arr2, Osi18, PIP82) that also share this RCSI sequence in their promoter region and are dependent on Pph13 for expression. Overall, Pph13 is not merely a factor ensuring Rhodopsin expression, but has a greater role in photoreceptor cell function.

One important and conflicting question is, if Pph13 is the general transcription factor binding to the RCSI elements then why is there selective downregulation of specific Rhodopsin promoters in a *Pph13* mutant? We propose a model in which the dependency for expression of Rhodopsins on either the RCSI site or additional elements responsible for subtype-specific expression has shifted between the different Rhodopsin promoters. In other words, Pph13 does bind to every RCSI site but, owing to the bipartite structure of the Rhodopsin promoters, sequence differences in the individual RCSI elements and resultant differences in Pph13 affinity contribute to a situation in which the presence of Pph13 alone is not a limiting factor for expression. There are several observations that support such a model. First, whereas Pph13 is necessary and sufficient for expression of 3XP3, we do not observe any ectopic expression of any Rhodopsin promoter reporters in tissues outside of photoreceptor cells (our unpublished data), confirming the idea that Rhodopsin promoters are coordinately regulated by other factors. Second, each RCSI element is not created equally. Swapping of RCSI domains between the different promoters does not dramatically affect spatial or temporal specificity (Papatsenko et al., 2001), but does reflect predictable changes in the level of expression based on Pph13 affinities that we observe here. For example, when the *Rh6* RCSI site is placed into an *Rh3* or *Rh5* minimal promoter, there is an increase in expression level, and a reciprocal downregulation of expression is observed when the *Rh6* RCSI is replaced by the RCSI of *Rh3*. These results directly correlate with the relative affinity of Pph13 for these RCSI elements, as described here. Furthermore, the lower affinity of Pph13 for the *Rh3* or *Rh5* RCSI element would predict a greater dependency on other elements in these promoters for expression – hence the dependency on Otd. Indeed, the expression of both *Rh3* and *Rh5* is dependent on Otd binding to, and activating transcription outside the region of, their respective RCSI sequences (Tahayato et al., 2003). By contrast, as observed in *Drosophila*, the perfect palindrome/higher affinity binding site contained within the *Rh6* RCSI element requires promoter regions outside of the RCSI element to repress expression in every photoreceptor cell (Tahayato et al., 2003).

Altogether, these data correlate well with our model in which there is a shifting of dependency between the RCSI element and other upstream photoreceptor subtype-specific elements among the different Rhodopsin promoters. As for the residual Rh1 expression in the *otd*; *Pph13* double mutant, our model predicts that a second element outside of the RCSI site contributes to the expression of *Rh1*. There are two binding sites for Glass, one inside the minimal promoter of *Rh1* and one outside (Mismer and Rubin, 1989; Moses and Rubin, 1991). Glass is expressed in all photoreceptor cells and is required for *Rh1* expression (Moses et al., 1989; Moses and Rubin, 1991). Moreover, like the RCSI element, Glass binding sites when multimerized are sufficient to drive and limit expression to photoreceptor cells (Moses and Rubin, 1991).

Lastly, our results form the basis for a better understanding of all rhabdomeric photoreceptor cell development and function. Interestingly, the activity of the 3XP3 reporter is a common marker for transgenic constructs in many invertebrate species and thus its activity is not limited to *Drosophila* (Berghammer et al., 1999). Given the relationship between Pph13 and Otd in determining rhabdomere morphogenesis and the ability of both factors to ensure photoreceptor cell function, it will be crucial to determine whether these same relationships exists in other invertebrate rhabdomeric photoreceptor cells.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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