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Functional genomics identifies regulators of the phototransduction machinery in the *Drosophila* larval eye and adult ocelli

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Sensory perception of light is mediated by specialized Photoreceptor neurons (PRs) in the eye. During development all PRs are genetically determined to express a specific *Rhodopsin (Rh)* gene and genes mediating a functional phototransduction pathway. While the genetic and molecular mechanisms of PR development is well described in the adult compound eye, it remains unclear how the expression of Rhodopsins and the phototransduction cascade is regulated in other visual organs in *Drosophila*, such as the larval eye and adult ocelli. Using transcriptome analysis of larval PR-subtypes and ocellar PRs we identify and study new regulators required during PR differentiation or necessary for the expression of specific signaling molecules of the functional phototransduction pathway. We found that the transcription factor Krüppel (Kr) is enriched in the larval eye and controls PR differentiation by promoting Rh5 and Rh6 expression. We also identified Camta, Lola, Dve and Hazy as key genes acting during ocellar PR differentiation. Further we show that these transcriptional regulators control gene expression of the phototransduction cascade in both larval eye and adult ocelli. Our results show that PR cell type-specific transcriptome profiling is a powerful tool to identify key transcriptional regulators involved during several aspects of PR development and differentiation. Our findings greatly contribute to the understanding of how combinatorial action of key transcriptional regulators control PR development and the regulation of a functional phototransduction pathway in both larval eye and adult ocelli.

1. Introduction

The perception of light is mediated by photoreceptor neurons (PR) in the eye. These specialized cells transform visual inputs into neuronal information, which can then be transmitted and processed in the brain. Each PR expresses a specific sensory receptor gene that defines to which range of wavelengths of light the PR will be sensitive. *Rhodopsins* are sensory receptor genes expressed in PRs and encode photosensitive G protein-coupled receptors (GPCRs) that initiate the phototransduction cascade and lead to the opening or closing of specific ion channels (Hardie, 2001; Hardie, 2012; Hardie and Raghu, 2001; Hubbell et al., 2003; Okada et al., 2001; Okada and Palczewski, 2001; Palczewski, 2006; Sakmar, 2002). Proteins of the phototransduction pathway include the scaffolding protein InaD (Chevesich et al., 1997; Shieh and Niemeyer, 1995; Shieh and Zhu, 1996), effector enzyme phospholipase C (PLC) (Bloomquist et al., 1988), the heterotrimeric Gq protein Gαq (Lee et al., 1990) which activates PLC (Scott et al., 1995) and two distinct classes of light-sensitive channels: Trp (Hardie and Minke, 1992; Montell and Rubin, 1989) and Trpl (Niemeyer et al., 1996; Phillips et al., 1992).

The *Drosophila* adult consists of seven visual organs, which are formed during different developmental stages (Hofbauer and Buchner, 1989): two compound eyes and three ocelli are formed during metamorphosis, while a pair of extraretinal "eyelets" are derived from the larval eyes (also termed "Bolwig organ"). The adult compound eye has been widely used as a model system to study cell fate determination and phototransduction, however it is still less clear how terminal differentiation and the regulation of the phototransduction machinery is controlled in the larval eye and ocelli.

Drosophila larval eyes are comparably simple and consist only of about 12 PRs. However, certain degree of plasticity is found in the larval eyes and the PR number ranges from 8 to 16 (Green et al., 1993; Sprecher et al., 2007). They are further subdivided into two PR-subtypes. Eight PRs express the green-sensitive Rh6 and the remaining four PRs express the blue-sensitive Rh5 (Mishra et al., 2013; Sprecher et al., 2007). In adult flies the three ocelli are

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arranged in a triangle between the compound eyes at the dorsal vertex of the head. Each ocellus contains about 80–100 PRs and based on Rhodopsin expression all of them represent only one PR cell type expressing UV-blue-sensitive Rh2 (Feiler et al., 1988; Mismer et al., 1988; Pollock and Benzer, 1988).

Here, we performed genome-wide transcriptome profiling to identify transcriptional regulators that are expressed in the larval eye and/or adult ocelli. We used cell type-specific fluorescent protein marker lines in conjunction with fluorescence-activated cell sorting (FACS) to isolate large numbers of larval PRs. DNA microarrays were employed to identify candidate transcription factors (TFs) enriched in particular PRs. To verify the functional roles of identified TFs, we performed in vivo expression analysis and loss-of-function studies. We confirmed that the TF Krüppel (Kr) is required during terminal differentiation in the larval eye. We observed a loss of Rh5 and Rh6 expression in the Kr^{1} null mutant larval PRs. We identified Hazy (Flybase: Pph13 for PvuII-PstI homology 13) as a common transcriptional regulator in both larval eye and adult ocelli. Furthermore, we found that Lola (Longitudinals lacking) and Camta (Calmodulin-binding transcriptional activator) are enriched in both larval eye and ocelli. However, these TFs are only essential during ocellar PRs differentiation and regulate Rh2 expression. Dve (Defective proventriculus) is enriched only in the ocellar PRs and regulate Rh2 expression. We also observed that Kr and Hazy regulate PR function in the larvae whereas Lola and Hazy regulate PR function in the ocelli by regulating expression of specific signaling molecules (InaD, PLC, Gαq, Trpl) of the phototransduction cascade. In summary, our findings show that PR identity is achieved by interplay of both common and cell type specific TFs during terminal differentiation. We also show that cell type-specific transcriptome profiling is therefore an effective technique to identify new TF candidates that regulate diverse aspects of PR development and its functions and may further be used to study various topics related to photoreceptor biology.

2. Materials and methods

2.1. Isolation of PRs and CNS neurons

For GeneChips Microarrays, we dissected the cephalopharyngeal skeleton of third instar larvae, which harbors the eye. Third instar larval CNSs were also dissected as a control. Around 200 larvae were dissected for each biological replicate. Dissected samples were transferred in Schneider's insect medium (Sigma) in separate tubes and kept on ice. They were washed twice with cold 1X PBS. PR and CNS neurons were then dissociated by adding 50:50 mix of 1X collagenase (Sigma): 1X Dispase II (Roche) and incubated for 2 h at 25 °C. It was replaced after 2 h by Schneider's medium with 10% Fetal Bovine Serum (FBS). Cells were dissociated by pipetting and filtered through 35 μm nylon mesh filter. Viability was confirmed by trypan blue exclusion method after dissociation. Dissociated cells were then sorted by FACS directly into Arcturus PicoPure Total RNA extraction buffer (Ruben et al., 2012).

2.2. RNA amplification and GeneChip microarray data analysis

For GeneChips Microarrays, we used NuGen Ovation RNA Amplification System V2 to amplify mRNA. Single-stranded DNA was labeled and hybridized to the Affymetrix *Drosophila* 2.0 GeneChips. We followed the manufacturer's protocol and repeated the procedure 3 times independently for each cell type. Raw CEL Affymetrix files were analyzed using FlexArray 1.6.1 software. Normalization of the data was performed by MAS5.0 algorithm. For analyzing significant gene expression levels in the microarray, we used analysis-of-variance (ANOVA) test and for identifying differentially expressed genes in the larval and ocellar PRs in relation to the entire CNS, we performed local-pooled-error test (LPE). For statistical significance of differentially expressed genes, we applied cutoffs for p-value ≤ 0.05 and fold change ≥ 2 fold for enrichment. For identifying and visualizing enriched GO terms in the list of differentially expressed genes, the *GOrilla* web tool (http://cbl-gorilla.cs.technion.ac.il) was used.

3. Fly stocks

Flies were reared on standard food medium at 25 °C. Wildtype Canton S was used as a control in all the cases. The following mutants or marker strains were used: $sens^{E2}$ (Nolo et al., 2000), $Pph13^{hazy}$ (also called $hazy^{-/-}$) (Zelhof et al., 2003), otd^{uvi} (Vandendries et al., 1996), svp^{E22} (Mlodzik et al., 1990), sal^{16} (Kuhnlein et al., 1994), Kr^{1} (Romani et al., 1996), $Camta^{tes2}$ (Han et al., 2006), $lola^{e76}$ (Crowner et al., 2002), *Rh2-lacZ* (Mismer et al., 1988) and dve^{1}-*lacZ* (Nakagoshi et al., 1998).

The following UAS/Gal4 lines were used: *Rh5*-Gal4, *Rh6*-Gal4, *elav*-Gal4, *GMR*-Gal4, *peb*-Gal4, *Jra*-Gal4, *UAS-mCD8::GFP* (Bloomington *Drosophila* Stock Center). To knock down *Camta*, *lola* and *dve*, *UAS-camta*^{RNAi} (BL40849), *UAS-lola*^{RNAi} (BL35721), *UAS-dve*^{RNAi} (BL26225) (Bloomington *Drosophila* Stock Center) was used. All RNAi experiments were carried out at 29 °C.

3.1. Generation of transgenic flies

Rh2 minimal promoter (−293/+55) was PCR amplified from genomic DNA with the following primers and cloned into pBluescript vector using an endogenous SalI site and the NotI site added to the revers primer:

Rh2 fw: CCTCCGGTGGACTGATGTCC

Rh2 NotI rev: **CGGCGGCC**GCTCAGCTACCCGCAACCC

The Hazy binding site in the RCSI region of *Rh2* minimal promoter construct was mutated by point mutations using the following primers replacing the Hazy binding site with a NcoI restriction site:

Rh2 (RCSI mut) NcoI fw:

gcctcttttGATGAGCGGCT**CCA**TGGGTTAGCAAACatctat

Rh2 (RCSI mut) NcoI rev:

atagatGTTTGCTAACCCA**TGG**AGCCGCTCATCaaaagaggc

After verification of the mutations by restriction digest and sequencing non-mutated and Hazy-binding site mutated *Rh2* promoter sequences were cloned into a GFP reporter plasmid. All reporter constructs were injected into nos-φC31; attP40 flies for integration on the second chromosome using φC31 site-specific integration system (Bischof et al., 2007).

3.2. Generation of Rh2 antibody and immunohistochemistry

For Rh2 antibody production, purified peptide was synthesized against the C-terminus of the protein (amino acid sequence: SDTETTSEADSKA) and used to immunize rabbits (Davids Biotechnologie GmbH, Regensburg, Germany). The anti-serum was affinity purified and used at a concentration of 1:100.

For immunohistochemistry, third instar larval eye, CNS and adult ocelli were dissected in phosphate-buffered-saline (PBS), fixed with 4% formaldehyde prepared in PBS+0.3% triton X-100 (PBST) for 25 min and washed at least 3–4 times with PBST before adding primary antibody. The following primary antibodies were used: Rabbit anti-Rh2 1:100 (this work), Rabbit anti-Hazy 1:500 (Zelhof et al., 2003), Rat anti-Kr 1:200 (Kosman et al., 1998), Guinea pig anti-Kr 1:200 (a gift from J. Jaeger), Rabbit anti-Lola 1:200 (Giniger et al., 1994), Mouse anti-Rh5 1:50 (Chou et al., 1996),

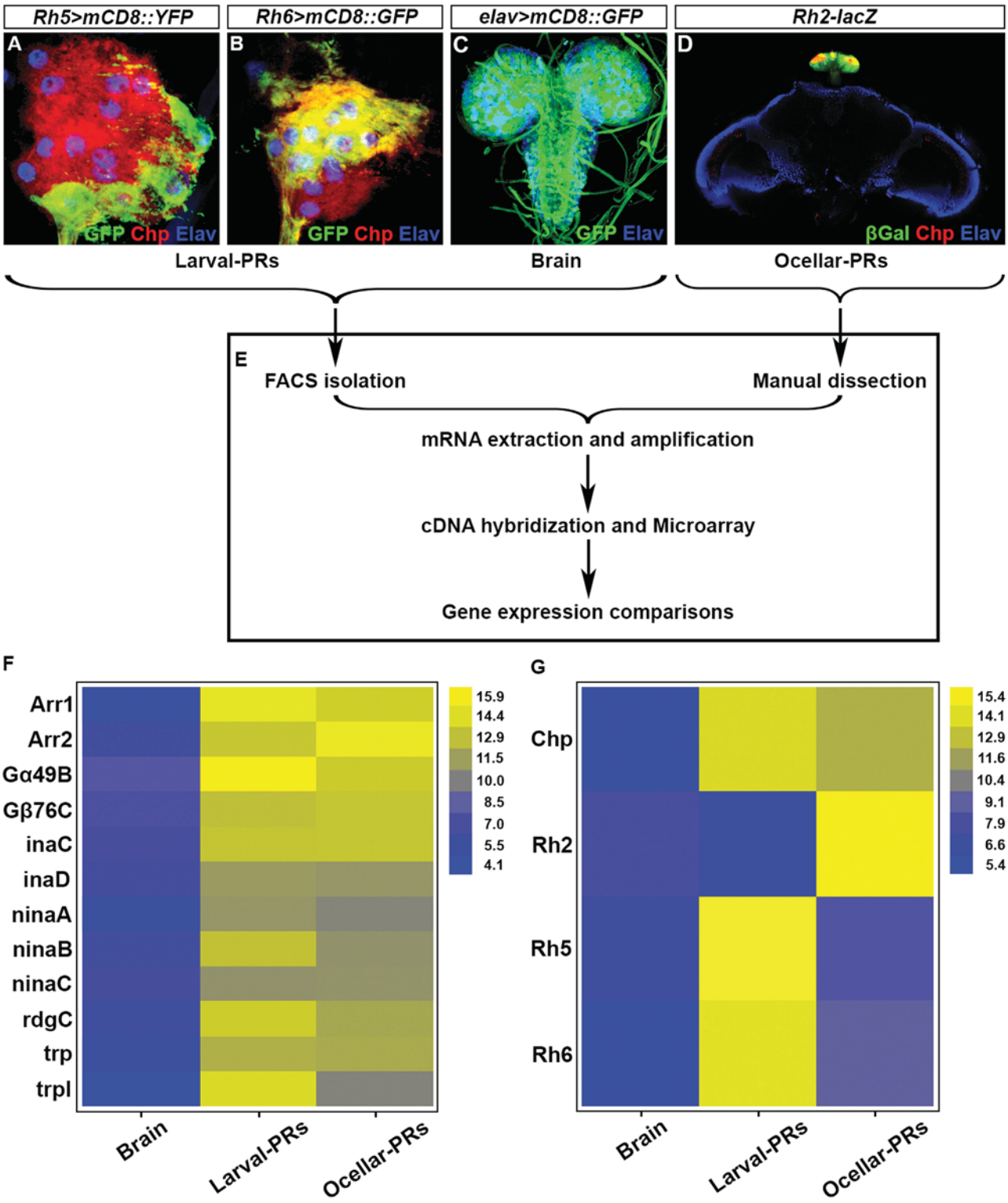

Fig. 1. Schematic representations of FACS, microarray and heat maps to show differential gene expressions. Confocal image representations of cell type-specific larval PRs, larval CNS and adult ocelli stainings show labeling of cells used for FACS isolation or dissection and microarray analysis. (A–B) Third instar larval Rh5 and Rh6 PR-subtypes, represented by *Rh5*-Gal4 X UAS-*mCD8::GFP* and *Rh6*-Gal4 X UAS-*mCD8::GFP* respectively and stained with anti-GFP (green), anti-Chp (red) and anti-Elav (blue) (C) Representation of third instar larval CNS neurons by *elav-Gal4 X UAS-mCD8::GFP* and stained with anti-GFP (green) and anti-Elav (blue) (D) Representation of the adult ocellar-PRs by a tissue-specific *Rh2*-lacZ reporter line and stained with anti-βgal (green), anti-Chp (Red) and anti-Elav (blue); Z-projection of confocal sections. (E) Schematic representation of microarray and gene expression comparisons for both larval and ocellar PRs. (F) Heat map of significant gene expression levels for previously known PR-specific markers by ANOVA test (p-value ≤ 0.05) shows PR-specific genes are enriched in both larval and ocellar PRs in relation to the larval CNS. (G) Heat map of significant gene expression levels for common and larval or ocelli-specific genes by ANOVA test (p-value ≤ 0.05). The common PR-specific gene Chp was enriched in both larval and ocellar PRs whereas cell type specific genes such as Rh2 were specifically enriched in ocellar PRs and Rh5 and Rh6 were specifically enriched in larval PRs.

Table 1
Overrepresented gene ontology (GO) terms in PRs

GO ID	Description	*P*-value	Enrichment (*N*, *B*, *n*, *b*)
GO:0006602	Phototransduction	1.47E-14	27.31 (1028, 23, 18, 11)
GO:0009583	Detection of light stimulus	1.47E-14	27.31 (1028, 23, 18, 11)
GO:0009416	Response to light stimulus	6.35E-12	10.16 (1028, 37, 41, 15)
GO:0007600	Sensory perception	5.96E-08	16.32 (1028, 28, 18, 8)
GO:0007165	Signal transduction	2.39E-07	5.71 (1028, 130, 18, 13)
GO:0019722	Calcium-mediated signaling	3.76E-07	51.40 (1028, 5, 16, 4)
GO:0022400	Regulation of Rhodopsin mediated signaling pathway	4.59E-07	5.73 (1028, 11, 163, 10)
GO:0016059	Deactivation of Rhodopsin mediated signaling	4.59E-07	5.73 (1028, 11, 163, 10)
GO:0050953	Sensory perception of light stimulus	7.63E-07	45.69 (1028, 5, 18, 4)
GO:0007601	Visual perception	7.63E-07	45.69 (1028, 5, 18, 4)
GO:0016060	Metarhodopsin inactivation	1.40E-06	114.22 (1028, 3, 9, 3)
GO:0008277	Regulation of GPCR protein signaling pathway	2.28E-06	4.96 (1028, 14, 163, 11)
GO:0007603	Phototransduction, visible light	6.10E-06	17.91 (1028, 7, 41, 5)
GO:0009584	Detection of visible light	6.10E-06	17.91 (1028, 7, 41, 5)
GO:0045494	Photoreceptor cell maintenance	6.39E-05	46.73 (1028, 6, 11, 3)
GO:0071482	Cellular response to light stimulus	4.57E-04	10.49 (1028, 14, 35, 5)

GO term analysis revealed biological processes expected for PRs.
Enrichment (*N*, *B*, *n*, *b*) is defined as follows:
N – is the total number of genes
B – is the total number of genes assiciated with a specific GO term
n – is the number of genes in the top of the user's input list or in the target set when appropriate
b – is the number of genes in the intersection
Enrichment = $(b/n)/(B/N)$

Rabbit anti-Rh6 1:10,000 (Tahayato et al., 2003), Guinea pig anti-Sens 1:800 (Nolo et al., 2000), Rabbit anti-Otd 1:200 (Hirth et al., 2003), Rabbit anti-Sal 1:300 (Kuhnlein et al., 1994), Mouse anti-Svp 1:100 (Kanai et al., 2005), Rabbit anti-InaD, Rabbit anti-PLC, Rabbit anti-Gαq, Rabbit anti-Trpl (all were a gift from Dr. C. Zuker and used at a dilution of 1:100), Chicken anti-GFP 1:2000 (Life technologies), Sheep anti-GFP 1:1000 (Invitrogen), Mouse anti-Chp 1:20, Mouse anti-FasII 1:20, Mouse anti-22C10 1:20, Rat anti-Elav 1:20 (Developmental Studies Hybridoma Bank), Rabbit anti-βGal 1:1000 and Rabbit anti-HRP 1:500 (Sigma). The secondary antibodies used were anti-mouse, anti-rabbit and anti-rat conjugated with Alexa-488, Alexa-568 or Alexa-647, anti-guinea pig Alexa-488, anti-chicken Alexa-488 and anti-sheep DyLight 488 (Jackson Immunoresearch). All secondary antibodies were raised in goat and/or donkey and used at a dilution of 1:200. All the samples were mounted in Vectashield H-1000 (Vector Laboratories).

3.3. Confocal microscopy and image analysis

Samples were imaged with a confocal microscope Leica TCS SP5 or with a Leica TCS SPE-II. Image resolution is 1024×1024 pixels and optical sections ranged from 1–2 μm depending on sample size. The images were further processed and analyzed using ImageJ/Fiji software and Adobe Photoshop CS6.

4. Results

4.1. FACS based transcriptome profiling by microarray identifies PR enriched genes in the larval eye and adult ocelli

In order to identify transcriptional regulators expressed during PR differentiation in the larval eye and adult ocelli, we compared transcriptome profiles of different PR cell types using Affymetrix microarrays. Larval PR-subtypes were first isolated using FACS (Fluorescence Activated Cell Sorting). This technique has been previously used in *Drosophila* to isolate undifferentiated and differentiating cells in the larval eye-antennal imaginal disc (Potier et al., 2014), CNS midline cells (Fontana and Crews, 2012), larval neuroblasts (Berger et al., 2012), stem cells in the adult ovaries (Kai et al., 2005), and cells of the wing imaginal disc (Neufeld et al., 1998). The process requires expression of fluorescent proteins (FPs) in a tissue- or cell type-specific manner. In order to label the two larval PR-subtypes, we used a Rh5-specific driver (*Rh5*-Gal4) and a Rh6-specific driver (*Rh6*-Gal4) to drive expression of GFP in a PR-subtype specific fashion (Fig. 1A and B). To distinguish between pan-neuronal and PR-specific transcripts, we compared genes expressed in PRs with those expressed in all neurons of the central nervous system (CNS) (by means of an *elav-Gal4* driver) (Fig. 1C). Lastly, ocellar PRs were labeled by *Rh2*-lacZ and isolated by manually dissecting adult ocelli (represented in Fig. 1D). We compared the transcriptome profiles of these different PR cell types using Affymetrix microarrays (See Section 2). To compare gene expression levels between different cell types in the microarray, we used the analysis-of-variance (ANOVA) test, which is commonly used to analyze differential gene expression in microarray experiments (Kerr et al., 2000) (See Section 2). When applying a p-value cutoff of $p \leq 0.05$ for statistical significance, we found 2122 genes that are either specifically enriched or depleted in larval and/or ocellar PRs. To validate PR-enriched transcripts, we tested if known PR-specific genes are indeed highly enriched. A heat map (of sample mean) of larval and ocellar PRs in comparison to the entire CNS shows that genes of the phototransduction cascade, such as the trp family channels (*trp, trpl*), G-proteins (*Gα49B, Gβ76C*) and other components of the phototransduction pathway *(Arr1, Arr2, inaC, inaD, ninaA, ninaB, ninaC and rdgC)* were enriched in both larval and ocellar PRs as compared to the entire CNS (Fig. 1F). Similarly, the common PR marker Chaoptin (Chp) (Reinke et al., 1988) is enriched in all PR cell types (Fig. 1G). We could also confirm that the well-characterized *Rhodopsin* genes are specifically enriched in the corresponding PR-types of the larval eye (Rh5 and Rh6) and ocelli (Rh2) (Fig. 1G).

Next we analyzed to which functional gene classes PR-enriched genes belong. For this we used gene ontology (GO) terms analysis using the *GOrilla* online tool (http://cbl-gorilla.cs.technion.ac.il). As expected GO terms in PR-enriched genes cover processes such as phototransduction (GO:0006602, $p = 1.47e^{-14}$), detection of light stimulus (GO:0009583, $p = 1.47^{-14}$), response to light stimulus (GO:0009416, $p = 6.35e^{-12}$), sensory perception (GO:0007600, $p = 5.96e^{-8}$), regulation of Rhodopsin mediated signaling pathway (GO:0022400, $p = 4.59e^{-7}$), sensory perception of light stimulus

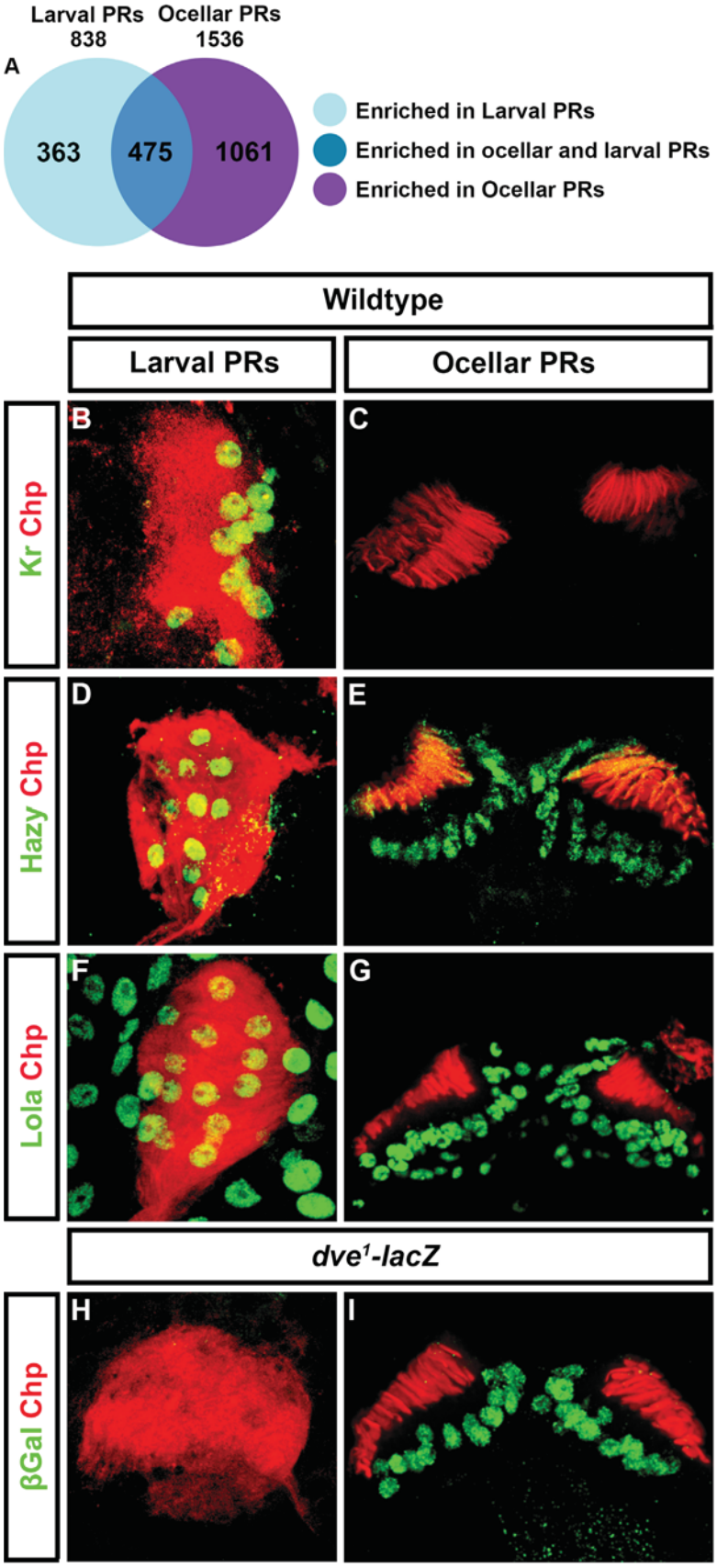

Fig. 2. Venn diagram of enriched genes in the larval and ocellar PRs and their expression studies. (A) Venn diagram showing the number of genes, which are either common or specifically enriched in the larval and ocellar PRs. 838 genes are enriched in the larval PRs and 1536 genes are enriched in ocellar PRs in comparison to the larval CNS. Of these, 363 genes are larval PRs specific, 1061 genes are ocellar PRs specific and 475 genes are common to both ocellar and larval PRs. (B, C) Kr expression (green) in the wildtype third instar larval PRs and adult ocellar PRs marked by a general PR marker Chp (in red); z-projection of confocal sections. Kr is specifically expressed in the differentiated larval PRs and absent from PRs of the adult ocelli. (D–G) Hazy and Lola expression (green) in the wildtype third instar larval and adult ocellar PRs marked by Chp (in red); z-projection of confocal sections (D, F); single confocal sections (E, G). Hazy and Lola are common TFs and expressed in both larval and ocellar PRs. Lola is also expressed in other cells surrounding the larval PRs. (H, I) Dve expression was monitored in the larval and ocellar PRs by an enhancer-trap allele *dve*^{1} (*dve*^{1}*-lacZ)* and stained with anti-βGal (green) and anti-Chp (red); z-projection of confocal sections (H); single confocal section (I). The *dve*-reporter is specifically expressed in the ocellar PRs and absent from PRs of the larval eye.

(GO:0050953, $p=7.63e^{-7}$), visual perception (GO:0007601, $p=7.63e^{-7}$) and metarhodopsin inactivation (GO:0016060, $p=1.4e^{-6}$) (Table 1).

In order to gain insight into the differences between larval and ocellar PRs, we compared each microarray dataset separately with the transcriptome data obtained from the entire CNS using a local-pooled-error (LPE) test (Jain et al., 2003). We used a *p*-value cutoff of $p \leq 0.05$ and a fold change cutoff of FC ≥ 2 in order to stringently select for genes that are enriched in either ocellar or larval PRs. We found 838 genes in the larval PRs and 1536 genes in the ocellar PRs that were enriched when compared to the entire CNS. Within larval and ocellar PR cell type we found that 363 genes were only expressed in the larval PRs and 1061 genes were only expressed in the ocellar PRs whereas 475 genes were common to both PR cell types (Fig. 2A).

Since our interest lies primarily in the identification of both common and cell type specific transcriptional regulators required during PR differentiation and phototransduction, we selected TFs that were differentially expressed in either larval or ocellar PRs as well as those enriched in both PR cell types. We found 11 larval PR specific TFs, 16 ocellar PR specific TFs and 10 TFs that were common and enriched in both PR cell types (Table 2).

Among these enriched TFs we chose to test genes, for which reagents were available. In agreement with the microarray data we found that the zinc finger TF Krüppel (Kr), which is an early segmentation gene of the *Drosophila* embryo (Preiss et al., 1985) is expressed only in the larval PRs and is absent from the PRs of adult ocelli (Figs. 2B and C). We also found that the homeodomain TF Hazy, which is involved in rhabdomere morphogenesis in the adult compound eye (Zelhof et al., 2003) and the zinc finger TF Longitudinals lacking (Lola), which is involved in axon growth and guidance (Giniger et al., 1994) are acting as common TFs and expressed in both larval and ocellar PRs (Fig. 2D–G). The homeobox TF Defective proventriculus (Dve) is required for the specific repression of Rh3, Rh5 and Rh6 in the adult retina (Johnston et al., 2011). Its expression was assessed by a *lacZ* reporter of the enhancer-trap allele dve^{1} (Nakagoshi et al., 1998). We found that it is expressed only in the ocelli and is absent from the larval PRs (Figs. 2H and I). We further confirmed the expression of two transcription factors Pebbled (Peb) and Jun-related antigen (Jra) in the larval eye using Gal4 lines (Fig. S1D and E). The zinc finger TF Pebbled (Peb) is involved in regulating PR cell morphology, planar polarity and epithelial integrity during compound eye development (Pickup et al., 2002) whereas the TF Jra is involved in the development of all PR neurons in the compound eye (Bohmann et al., 1994). Thus, using microarray based transcriptome analysis of larval and ocellar PRs, we identified TFs, which could potentially play a role during PR differentiation and its associated functions. We further aimed to investigate the role of Kr, Hazy, Lola, and Dve during terminal differentiation in both larval and ocellar PRs.

Table 2
Enriched transcripts in larval PRs and/or ocellar PRs

Larval PRs	Larval and ocellar PRs	Ocellar PRs
br	*Atf-2*	*bbx*
bun	*Camta*	*CG18619*
CG32006	*CTCF*	*clk*
dei	*mamo*	*crc*
dl	*Mef2*	*cwo*
fru	*Nfl*	*dve*
Jra	*lola*	*foxo*
Kr	*Pif1A*	*gl*
peb	*Pph13 (Hazy)*	*maf-s*
salr	*Wek*	*Max*
stc		*Rel*
		sd
		srp
		Stat92E
		Tab2
		Xbp1

4.2. Krüppel acts as a key transcriptional regulator during terminal differentiation of larval PRs

During embryonic development of the larval eye Kr is required for proper targeting of the PR axons to the larval visual system (Schmucker et al., 1992). Since we found that Kr is also maintained in the differentiated larval PRs, we firstly analyzed the temporal expression pattern of Kr in developing and differentiated PRs of the larval eye. In accordance with the previous study, we found that Kr is expressed in all larval PR precursors throughout embryogenesis (Fig. 3A and B). Thereafter, Kr expression is maintained in both PR-subtypes during larval stages (Fig. 3C).

Since Kr is already expressed in larval PR precursors in the embryo we tested whether Kr is required for the specification of a general PR precursor or only for the specification of PR-subtype. We therefore analyzed the genetic interaction of Kr with previously identified transcriptional regulators in the larval eye, such as Senseless (Sens), Spalt (Sal), Seven-up (Svp), Hazy and Orthodenticle (Otd), which are required for PR-subtype specification (Mishra et al., 2013; Sprecher et al., 2007). We analyzed Kr expression in $sens^{E2}$, $hazy^{-/-}$, otd^{ovi}, sal^{16} and svp^{E22} mutants and vice-versa. Interestingly Kr expression remains unaffected in all those mutant conditions (Fig. 3D–H). Conversely, the expression of Sens, Otd, Sal, Hazy and Svp remains unaffected in Kr^{1} mutants (Fig. 3J, L, N and P).

Thus, the initial specification of larval PR precursors and the specification into the two precursor subtypes do not require Kr. We next addressed if Kr functions during terminal differentiation by analyzing the expression of Rh5 and Rh6. Since Kr^{1} null mutants are late embryonic lethal we addressed expression of Rhodopsins at embryonic stage 17, just prior to larval hatching. Interestingly, we found that while larval PRs are present in Kr^{1} mutants they fail to express Rh5 and Rh6 (Fig. 3R and T). However the expression of the specific PR marker Chp and Hazy remain unaffected (Fig. 3R and T). Thus while early specification of photoreceptor precursors and a general neuronal identity occur normal in Kr^{1} null mutant embryos, these cells subsequently fail to express Rhodopsins during larval stages.

We have previously shown that the TF Hazy controls the expression of both *Rhodopsins* in the larval eye (Mishra et al., 2013). Since in $hazy^{-/-}$ null mutants, initial PR-subtype specification occurs normally, the defects observed in Kr^{1} mutants resemble the $hazy^{-/-}$ mutant phenotype. This raises the question if Kr and Hazy genetically interact to mediate the expression of Rh5 and Rh6 in larval PRs. However, Hazy expression does not change in Kr^{1} mutants (Fig. 3P and T) and Kr expression does not change in $hazy^{-/-}$ mutants (Fig. 3E). Hence we propose that Kr and Hazy do not cross regulate each other's expression and mediate Rhodopsin expression in the larval eye.

Terminal differentiation of PRs is defined by the genetic network of developmental control genes, which ensures the proper expression of Rhodopsins as well as phototransduction cascade genes. Since in Kr^{1} mutants Rhodopsin expression is lost, we tested if phototransduction cascade genes are also affected. In wildtype larvae InaD, PLC, Gαq and Trpl are expressed in all PRs starting from late embryonic stage 17 (Fig. 4A–D) to the late third instar larvae (Fig. 4I–L). Next, we investigated if these markers are also expressed in Kr^{1} and $hazy^{-/-}$ null mutants. We found that InaD (Fig. 4E), PLC (Fig. 4F) and Trpl (Fig. 4H) are normally expressed in Kr^{1} mutants. However, Gαq expression is lost (Fig. 4G). In $hazy^{-/-}$ mutants we found that PLC expression is not altered (Fig. 4N) while the expression of InaD is reduced (Fig. 4M) and Gαq (Fig. 4O) and Trpl (Fig. 4P) expression are lost.

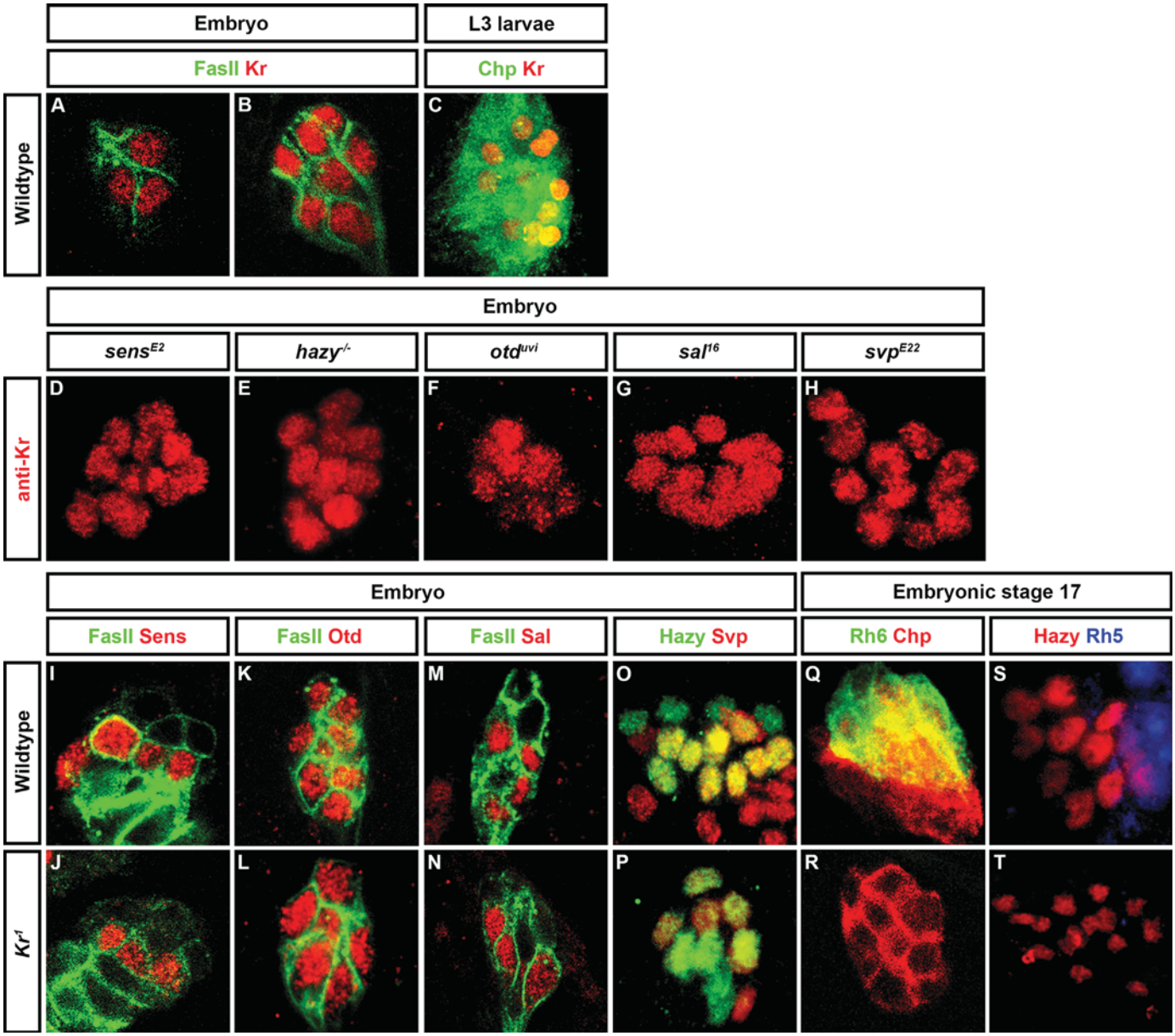

Fig. 3. Krüppel is involved in larval PRs differentiation. (A–C) Differential expression of Kr in PRs during different developmental stages starting from embryonic stage 12 (A, B) in all PR precursors (marked in red) and co-stained with anti-FasII (green); single confocal sections (A and B) to show all PRs. Kr continues to be expressed throughout embryogenesis. (C) Kr expression is maintained in the differentiated larval PRs, which are marked by Chp (green); z-projection of confocal sections. (D–H) Kr expression in all PR precursors in the embryo in mutants that affect PR-subtype specification. Kr expression was unchanged in $sens^{E2}$ (D), $hazy^{-/-}$ (E), otd^{uvi} (F), sal^{16} (G) and svp^{E22} (H); z-projections of confocal sections. Similarly, compared to the wildtype, expression of Sens (I, J), Otd (K, L), Sal (M, N), Hazy and Svp (O, P) were unchanged in Kr^1 mutant embryos; single confocal sections. Expression of Rh6 (green) and Rh5 (blue) in the wildtype (Q, S) and Kr^1 mutant (R, T) PRs at embryonic stage 17 marked by either Chp (Q, R) or Hazy (S, T) (in red); z-projection of confocal sections. Expression of both Rh5 and Rh6 was lost in Kr^1 mutants showing that Kr is involved in the terminal differentiation of larval PRs.

Taken together, these results provide evidence that Kr is required for controlling expression of Rhodopsins during terminal differentiation of larval PRs. We show that both Kr and Hazy are involved in phototransduction by separately regulating expression of specific phototransduction cascade genes whereas combinatorial action of Kr and Hazy is also required for regulating the expression of a specific phototransduction molecule Gαq (Fig. 9A).

4.3. Transcriptome profiling identifies transcriptional regulators during terminal differentiation of ocellar PRs

In order to gain insight into terminal differentiation of ocelli, we analyzed the candidate genes that we had found to be expressed in ocellar PRs: Hazy, Lola, and Dve (Fig. 2E, G and I; see above). We also tested Camta as a candidate since it was differentially expressed in the ocelli by microarray and also shown to be relevant for Rhodopsin biochemistry in the adult retina (Han et al., 2006). To assess if these genes are required for ocellar PR differentiation and its associated function, we analyzed the expression of Rh2 as well as phototransduction cascade genes.

While in wildtype animals all ocellar PRs express Rh2 (Fig. 5A) we found that in $Camta^{tes2}$ mutants Rh2 expression is lost (Fig. 5B). We confirmed the loss of Rh2 expression by knocking down *Camta* by *RNAi* in ocellar PRs using the pan-photoreceptor *lGMR*-Gal4 driver together with *Dicer-2* (Fig. 5D). Similarly, the knockdown of *lola* and *dve* by *RNAi* resulted in a loss of Rh2 expression (Fig. 5E and F). Rh2 expression was lost in $hazy^{-/-}$ null mutants (Fig. 5C) in accordance with previous observations (Mishra et al., 2010). In

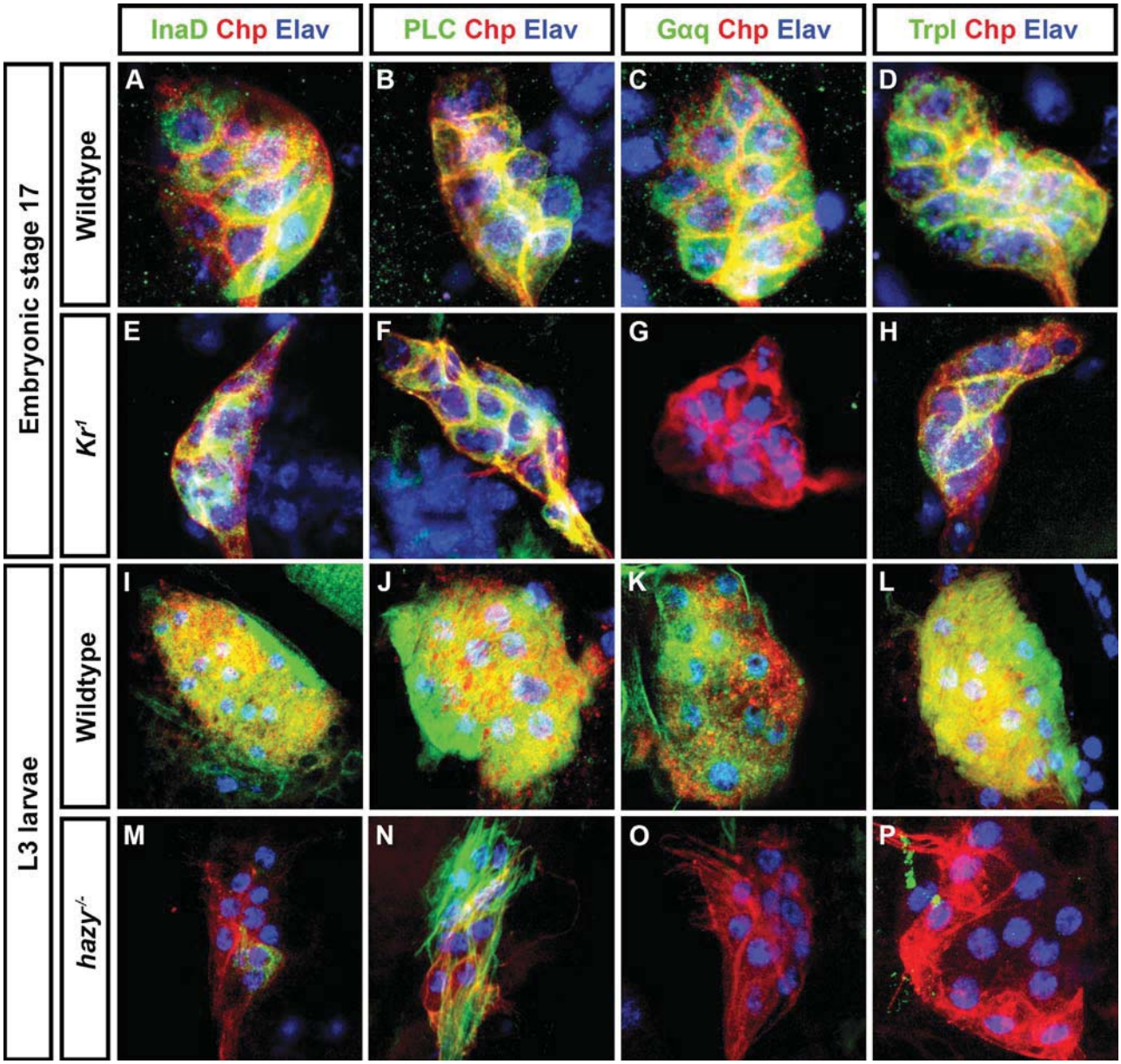

Fig. 4. Expression of specific signaling molecules of phototransduction pathway in wildtype, Kr^1 and $hazy^{-/-}$ mutant embryonic and larval PRs. Expression of key phototransduction molecules such as InaD, PLC, Gαq and Trpl in wildtype (A–D) and Kr^1 mutant (E–H) PRs at embryonic stage 17. All of them are expressed in the wildtype embryonic PRs whereas Gαq expression (G) is specifically lost in Kr^1 mutant embryonic PRs. Expression of InaD, PLC, Gαq and Trpl in wildtype (I–L) and $hazy^{-/-}$ mutant (M–P) third instar larval PRs. All of them are expressed in wildtype larval PRs whereas in $hazy^{-/-}$ mutants InaD expression (M) is reduced and there is a specific loss of Gαq and Trpl expression (O, P). This shows that phototransduction machinery is affected in Kr^1 and $hazy^{-/-}$ mutants.

all cases the expression of the pan-neuronal marker Elav showed that differentiation of PR precursors towards a general neural cell fate is not affected. Interestingly, even though Camta and Lola are also enriched in larval PRs in microarray profiles (see also Fig. 2F), we did not observe defects in the expression of Rh5 and Rh6 in their respective mutants in the larval eye (Fig. S1C, G and I).

Next we investigated if Camta, Lola, Hazy and Dve are necessary for the expression of phototransduction cascade genes in ocellar PRs. We therefore tested the expression of the phototransduction proteins InaD, PLC, Gαq and Trpl as well as the general neuronal markers Elav, anti-horseradish peroxidase (HRP) and Futsch (22C10) and found that all of them are expressed in wildtype ocellar PRs (Fig. 6A–E).

In $Camta^{tes2}$ mutants and in *dve* knockdown we did not observe any change in any of the investigated markers compared to the wildtype control animals (Fig. 6F–J and P–T). Conversely, in $hazy^{-/-}$ mutants, we observed a reduction of InaD (Fig. 6K) and Gαq (Fig. 6M) expression and a loss of Trpl expression (Fig. 6N). In *lola* knockdown we found a reduction in the expression of InaD, PLC, Trpl and anti-HRP epitopes (Fig. 6U, V, X and Y) and a loss of Gαq expression (Fig. 6W). Knockdown of *lola* also disrupts rhabdomere morphology (marked by abnormal chp expression), while in all other investigated mutants rhabdomere morphology was not affected.

Since both Hazy and Lola showed defects in the expression of phototransduction cascade genes we next investigated the genetic interactions between Hazy and Lola. Expression of Hazy and Lola remains unchanged in $Camta^{tes2}$ mutants (Fig. 7A and B) or in the *dve* knockdown (Fig. 7C and D). In $hazy^{-/-}$ mutants no change in Lola expression was observed (Fig. 7F). However, knockdown of *lola* causes a loss of Hazy expression (Fig. 7E). Thus Lola seems to act genetically upstream of Hazy and may regulate aspects of

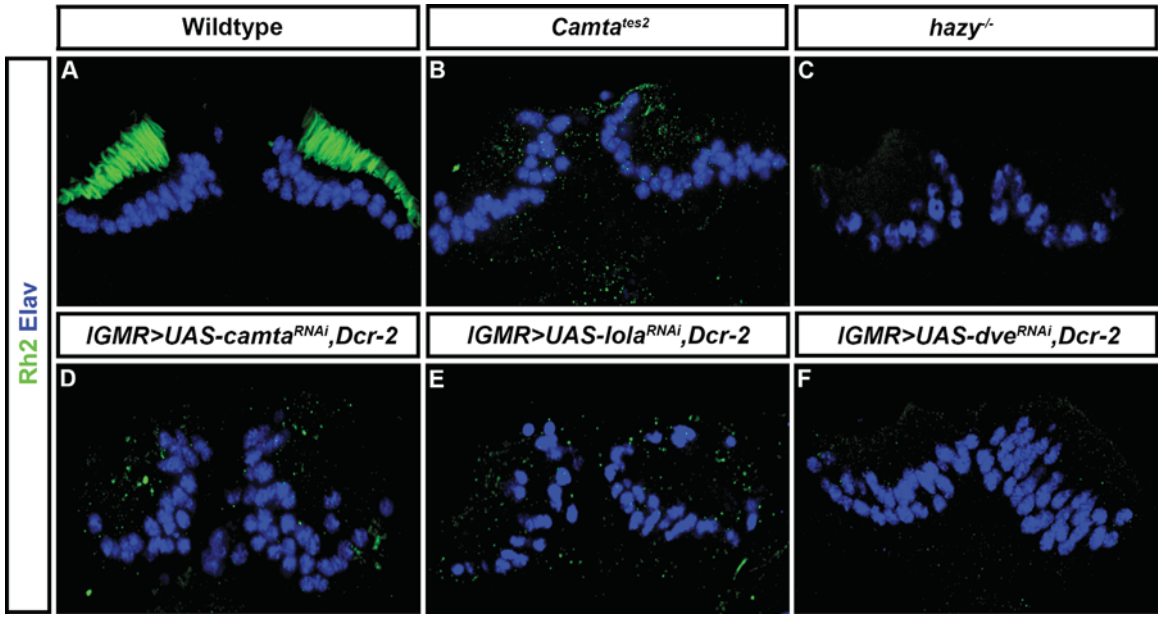

Fig. 5. Role of differentially enriched transcriptional regulators during ocellar PR differentiation. (A) Wildtype expression of Rh2 (green) in adult ocelli, which are also marked by elav (blue); four confocal sections. Rh2 is expressed in wildtype adult ocellar PRs. A Loss of Rh2 expression (green) in the *Camta*^{tes2} (B) and *hazy*$^{-/-}$ (C) mutant ocelli as well as in the knockdown of *Camta* (D), *lola* (E) and *dve* (F) using *RNAi* specifically driven in the ocellar PRs by *lGMR*-Gal4*; UAS-Dcr2*. This suggests that all these TFs are involved in ocellar PR differentiation.

ocellar PRs differentiation and phototransduction potentially in a Hazy-dependent manner.

Hazy directly binds to the promoter of *Rh2* in vitro (Mishra et al., 2010) suggesting that binding of Hazy to the promoter of *Rh2* directly activates its transcription. In order to test this, we used an *Rh2-lacZ* reporter line and made a recombinant of *Rh2-lacZ* in *hazy*$^{-/-}$ mutant background *(hazy*$^{-/-}$*; Rh2-lacZ).* Compared to the control where β-gal activity is seen specifically in the ocellar PRs (Fig. 8A and A'), it is lost in *hazy*$^{-/-}$ mutants (Fig. 8B and B'). This suggests that Hazy transcriptionally regulates Rh2 expression.

It has also been shown previously that Hazy regulates Rhodopsin expression through *Rhodopsin core sequence I* (RCSI) which is found in the proximal promoters of all *Rhodopsin* genes (Mishra et al., 2010; Papatsenko et al., 2001; Sheng et al., 1997). We next investigated if binding of Hazy to the RCSI site in the proximal promoter region of Rh2 is indeed necessary for its expression. We made GFP reporter lines of Rh2 by cloning its minimal promoter region (see Section 2 for details) and changed the Hazy binding site in the RCSI region by altering three nucleotides in the core sequence (Fig. 8C). Expression of an unmutated control construct (*Rh2(wt)-GFP)* shows specific GFP expression in the ocellar PRs (Fig. 8D and D'). In constrast, no GFP expression in the ocelli is detectable with the construct bearing a mutation of the Hazy-binding site in the RCSI region (Fig. 8E and E') demonstrating that Hazy was no longer able to regulate GFP reporter expression by binding to the RCSI element of the Rh2 minimal promoter.

Taken together, these results show that terminal differentiation of ocellar PRs requires the combinatorial action of common transcriptional regulators such as Hazy, Camta and Lola as well as some cell type specific transcriptional regulators such as Dve to properly mediate rhabdomere morphology, expression of Rh2 and a functional phototransduction pathway (Fig. 9B). We further extended into the mechanistic insights of how ocellar PRs are determined. We showed that Hazy transcriptionally regulates Rh2 expression by binding to the RCSI region in the proximal promoter of Rh2 and that Hazy is necessary for Rh2 expression in the ocellar PRs.

5. Discussion

5.1. Transcriptome profiling of PRs identified new regulators of the phototransduction pathway in the larval eye and adult ocelli

Full genome transcriptome profiling by DNA microarray allows the identification of candidates involved in transcriptional regulation of gene expression. It has been previously used in *Drosophila* to identify genes involved in compound eye development (Fichelson et al., 2012; Michaut et al., 2003; Yang et al., 2005), CNS development (Carney et al., 2012), muscle development (Elgar et al., 2008) as well as hearing (Senthilan et al., 2012) and molecular clock oscillations (Ruben et al., 2012). To identify regulators of the phototransduction cascade in the larval eye and adult ocelli, we performed comparative transcriptome profiling for both larval and ocellar PRs. In our analysis, we identified genes in the PRs that showed typical PR processes and functions, illustrating the authenticity of the microarray. Expression and knockdown analysis of the identified genes revealed mechanistic insight into PR terminal differentiation and phototransduction. The comparative analysis of two visual organs allowed us to determine common TFs that are likely to function during terminal differentiation and phototransduction in all PRs (Hazy, Lola) as well as TFs that are restricted to specific PR-subtype (Kr in larval eye and Dve in adult ocelli). Moreover, the employed method of cell type-specific transcriptome profiling by microarrays allows a rapid way to identify regulators that are linked with various aspects of PR development and function. In addition, the transcriptome data provided here may also be employed as a resource to study other questions related to the biology of PR neurons from developmental, physiological and functional perspectives.

5.2. Regulation of phototransduction cascade in the larval eye

The genetic program of TFs and signaling pathways that acts during embryogenesis triggers terminal differentiation of larval PRs and specification of larval PR-subtypes. We identified Krüppel (Kr) as a transcriptional regulator that acts during embryogenesis and interestingly re-deploys during terminal PR differentiation

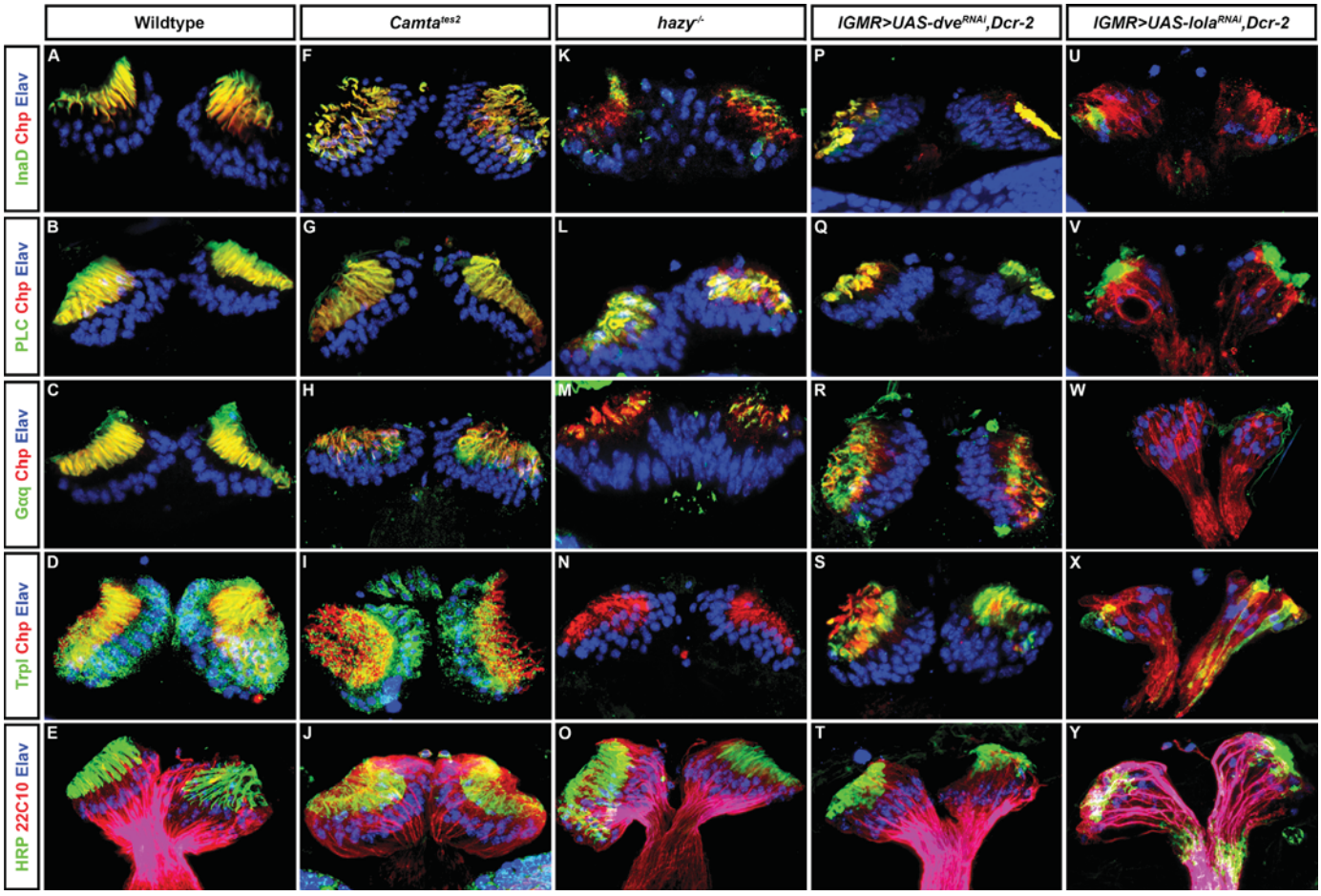

Fig. 6. Expression of phototransduction specific molecules in the ocellar PRs in wildtype and in mutants or *RNAi* knockdown of enriched TF candidates. Expression of phototransduction specific markers such as InaD, PLC, Gαq, Trpl and neuronal-specific markers such as anti-HRP, 22C10 and Elav in wildtype, $Camta^{tes2}$ and $hazy^{-/-}$ mutants as well as in *dve* and *lola* knockdown by *RNAi* specifically in the ocellar PRs. (A–E) Wildtype expression of PR-specific markers (A–D) and neuronal-specific markers (E) in the ocellar PRs. All of them are expressed in adult ocelli. These markers are normally expressed in $Camta^{tes2}$ mutants (F–J). While expression of PLC (L) and neuronal markers (O) are normal in $hazy^{-/-}$ mutants, InaD (K) and Gαq (M) are reduced and expression of Trpl (N) is lost. Expression of these markers in *dve* (P–T) and *lola* (U–Y) knockdown by *RNAi* specifically in ocellar PRs. They are normally expressed in *dve* knockdown whereas *lola* knockdown results in disrupted rhabdomere morphology (marked by Chp expression) and reduction in the expression of InaD (U), PLC (V), Trpl (X), anti-HRP (Y) as well as loss of Gαq expression (W); z-projection of confocal sections.

and regulates Rhodopsin expression in the larval eye. Similarly, during compound eye development the homeodomain TF Homothorax (Hth) acts early and promotes proliferation of the undifferentiated eye tissue before specification of PR neuron (Bessa et al., 2002). It later re-deploys during terminal differentiation and gets specifically expressed in both R7 and R8 of DRA ommatidia and regulates Rh3 expression (Wernet and Desplan, 2014; Wernet et al., 2003). The zinc finger TF spalt (Sal) is found as a complex and acts early during pupation for the induction of inner PR cell fate and critical for the distinction between 'outer' and 'inner' PRs (Domingos et al., 2004; Mollereau et al., 2001). It re-deploys in the adults and gets expressed in the inner PRs where it represses Dve and acts together with Otd to induce Rh3 expression. Repression of Dve in the inner PRs by Sal is also important for the derepression of Rh5 and Rh6 expression (Johnston et al., 2011). Kr has mainly been studied for its function during early embryogenesis as a segmentation gene (Preiss et al., 1985; Wieschaus et al., 1984) as well as required for axon path finding of the developing larval eye (Schmucker et al., 1992). It also acts as a temporal identity factor in neuroblast cell-lineage (Isshiki et al., 2001). While Kr is activated during embryogenesis and is maintained in the differentiated larval PRs, it is however not expressed in the adult ocellar PRs. Interestingly, PR defects are restricted to terminal differentiation and early developmental processes such as the determination of neural identity or PR-subtype specification are not affected in Kr^1 mutants. While the terminal differentiation deficits in Kr^1 null mutant embryos resemble the phenotype observed in $hazy^{-/-}$ null mutants, they do not appear to cross regulate each other's expression. They also regulate the expression of different signaling molecules involved in the phototransduction pathway. This raises the question of how the genetic network consisting Kr and Hazy converge on the regulation of *Rhodopsin* genes and the proper regulation of genes belonging to the phototransduction cascade.

For Rh5 and Rh6 expression we previously showed that Hazy acts genetically through a conserved regulatory sequence termed RCSI (Rhodopsin Core Sequence I) (Mishra et al., 2013; Papatsenko et al., 2001), which is present in all *Rhodopsin* genes. Since Hazy is still present in Kr^1 mutants, it appears likely that at the molecular level Kr does not act through the RCSI. Sequence analysis of the Rh5 and Rh6 regulatory regions (also used to make *Rh5*- and *Rh6*-Gal4 lines) do not show defined Kr binding sites. However, they have defined Hazy binding site in the RCSI region upstream of the proximal promoter of both Rh5 and Rh6 further suggesting that Kr may act indirectly on the regulation of larval Rhodopsins. Future investigations on the molecular defect of Kr^1 mutants will be

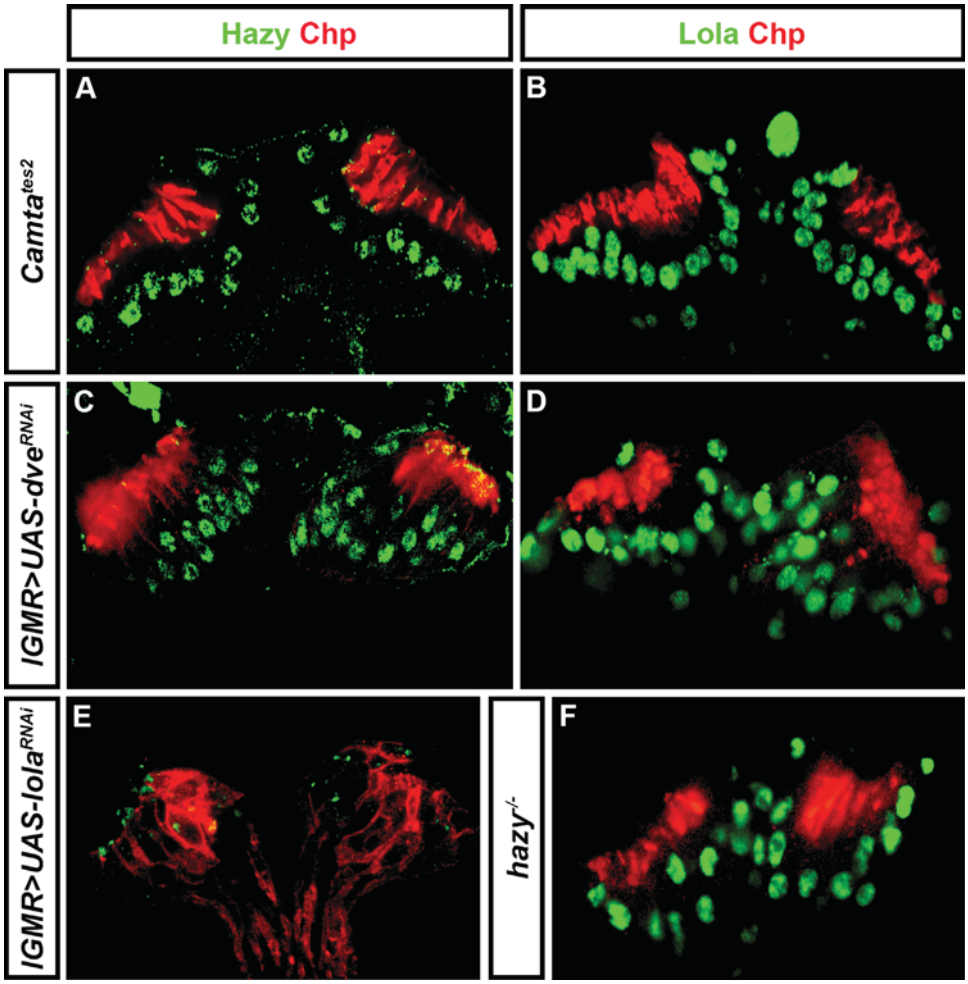

Fig. 7. Epistatic relationship of Hazy and Lola during ocellar PR differentiation. Hazy and Lola expression was analyzed in *Camta*^{tes2} (A, B) and in *dve* knockdown by *RNAi* (C, D) specifically in the ocellar PRs. Hazy and Lola were found to be normally expressed. (E) Hazy expression was analyzed in *lola* knockdown using *RNAi* specifically in the ocelli and (F) Lola expression was monitored in *hazy*$^{-/-}$ mutant ocellar PRs. Lola was normally expressed in the ocellar PRs in *hazy*$^{-/-}$ mutants. However, Hazy expression was absent from the ocellar PRs specifically in the *lola* knockdown showing a probable epistatic relationship between Lola and Hazy during ocellar PRs differentiation. Additionally, ocellar PR morphology was abnormal in *lola* knockdown (E) shown by Chp (Red) expression.

required to characterize how Kr and Hazy work together during terminal differentiation of larval PRs and Rhodopsin expression.

5.3. Developmental control genes mediating ocellar PRs differentiation and regulation of the phototransduction machinery

While it was shown over two decades ago that the *Drosophila* ocelli express Rh2, the genetic mechanism of how ocellar PRs differentiate and express Rh2 remain largely unexplored. The candidate gene approach based on transcriptome profiling helped us to identify transcriptional regulators such as Camta, Hazy, Lola and Dve that function in ocellar PR differentiation. As in the case of the PRs of adult retina and larval eye, Hazy is required for proper terminal differentiation and Rhodopsin expression. Interestingly, in ocelli Lola is required for maintaining rhabdomere morphology and regulates Hazy expression, thus providing an important step in the differentiation of PR precursors to mature PRs. Lola provides a similar function as Otd in the retina which is required for rhabdomere biogenesis and regulates photoreceptor specific genes including the activation of Rh3 and Rh5 (Tahayato et al., 2003; Vandendries et al., 1996). Otd acts together with Hazy they maintain rhabdomere morphology and regulate PR cell function (Mishra et al., 2010). During CNS development, Lola is required to maintain neurons in their differentiated state. Loss of Lola results in the dedifferentiation of neurons and the formation of tumors (Southall et al., 2014). While we did not observe any tumor formation in the ocellar PRs, it seems likely that Lola provides a similar role in the visual system and in the CNS. In the adult retina, Lola is expressed in PRs and cone cells and regulates the binary cell fate decision of R3/R4 and R7/cone cells in the developing eye in a Notch dependent manner (Zheng and Carthew, 2008). While Dve is excluded form larval PRs, it is expressed in the ocelli, where it is required for Rh2 expression in terminal differentiated PRs. Interestingly Dve is also expressed in the adult retina where it regulates PR differentiation by repressing Rh3 in yR7 and Rh3, Rh5 and Rh6 in outer PRs (Johnston et al., 2011). Thus, even though Dve acts in the terminal differentiation of PRs in the retina and ocelli, its role appears to be distinct in different developmental contexts. Furthermore, while Camta, Hazy, Lola and Dve are required during ocellar PRs differentiation, only Hazy and Lola are required to regulate specific signaling molecules of the phototransduction machinery. Future studies will be required to dissect the genetic and molecular mechanisms of how these identified TFs regulate terminal differentiation and the regulation of the phototransduction machinery in larval and adult ocellar PRs in a context dependent manner.

Author contributions

AKM, BORB, MT and SGS designed the experiments. AKM with the help from BORB, CF and SGS performed all the experiments and

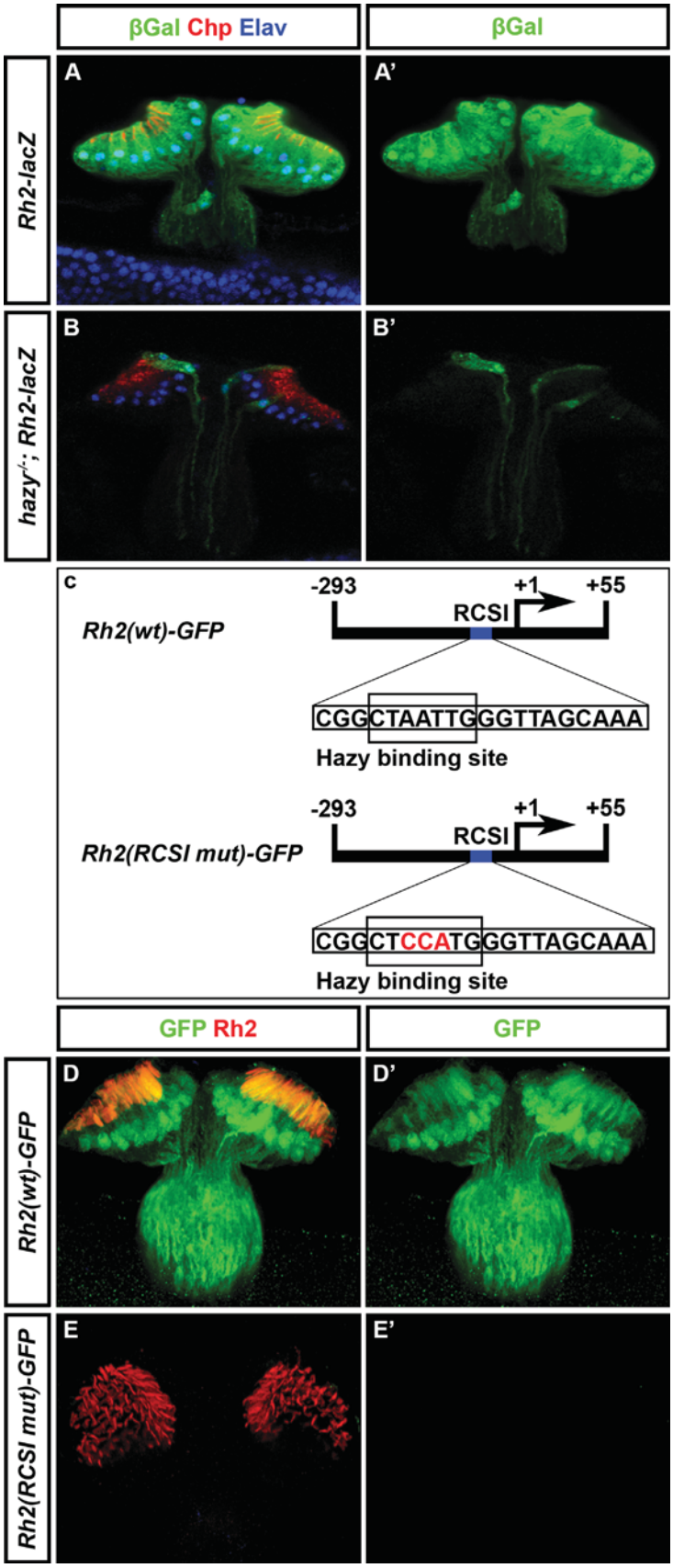

Fig. 8. Molecular mechanism of Rh2 regulation in the adult ocelli by Hazy. (A, A', B, B') Expression of *Rh2-lacZ* in the ocelli in wildtype control and in $hazy^{-/-}$ mutant background stained by β-gal (green), Chp (red) and Elav (blue). β-gal is specifically expressed in the ocelli in wildtype control (A') whereas it is lost in the background of $hazy^{-/-}$ mutants (B') suggesting that Hazy transcriptionally regulates expression of Rh2; single confocal sections. (C) Schematic representations of the Rh2 minimal promoter region ($-293/+55$) showing the RCSI region were Hazy binds. *Rh2(RCSI mut)-GFP* enhancer line was made by mutating the Hazy binding site of the RCSI region (CTAATTG → CT**CCA**TG; See Section 2 for details). (D, D', E, E') Staining of GFP (green) and Rh2 (red) shows that GFP is localized specifically in the ocellar PRs in the case of unmutated *Rh2(wt)-GFP* whereas it is lost completely in the *Rh2(RCSI mut)-GFP* enhancer line suggesting that binding of Hazy to the RCSI region is critical for Rh2 expression.

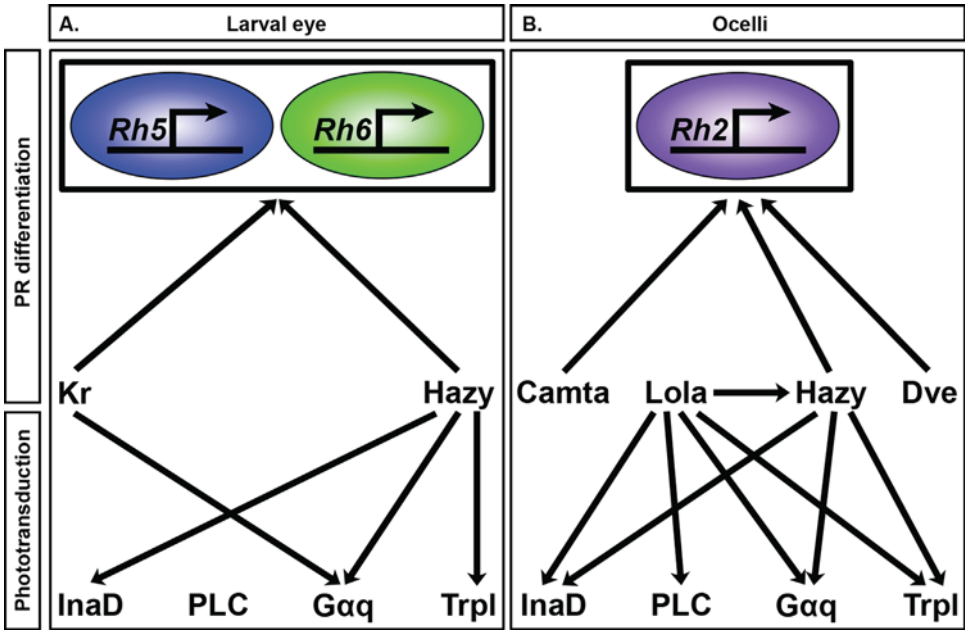

Fig. 9. Schematic representation of the genetic interactions in the larval eye and ocelli. (A, B) A model representing genetic interactions during PRs differentiation and regulation of phototransduction machinery in both larval and ocellar PRs. (A) Kr and Hazy are required for the differentiation of larval PRs and regulate the expression of some specific phototransduction molecules (Kr: Gαq; Hazy: InaD, Gαq and Trpl). Camta and Lola are expressed in the larval PRs but they do not regulate Rh5 and Rh6 expression whereas neither Kr nor Hazy regulates PLC expression in the larval eye. (B) Camta, Lola, Hazy and Dve control ocellar PR differentiation by regulating the expression of Rh2. Lola regulates Rh2 expression in a Hazy dependent manner. Only Lola and Hazy are required for regulating expression of specific phototransduction molecules (Lola: InaD, PLC, Gαq, Trpl; Hazy: InaD, Gαq, Trpl).

analyzed the data. AKM, BORB, MT, CF and SGS contributed reagents/materials/analysis tools. AKM and SGS wrote the manuscript.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.12.026.

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