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

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ABSTRACT

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),