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The cuticular nature of corneal lenses in *Drosophila melanogaster*

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Abstract

The dioptric visual system relies on precisely-focusing lenses that project light onto a neural retina. While the proteins that constitute the lenses of many vertebrates are relatively well-characterized, less is known about the proteins that constitute invertebrate lenses, especially the lens facets in insect compound eyes. To address this question, we used mass spectrophotometry to define the major proteins that comprise the corneal lenses from the adult *Drosophila melanogaster* compound eye. This led to the identification of four cuticular proteins: two previously identified lens proteins, Drosocrystallin and Retinin, and two newly identified proteins Cpr66D and Cpr72Ec. To determine which ommatidial cells contribute each of these proteins to the lens, we conducted *in situ* hybridization at 50% pupal development, a key age for lens secretion. Our results confirm previous reports that *Drosocrystallin* and *retinin* are expressed in the two primary corneagenous cells - cone cells and primary pigment cells. *Cpr72Ec* and *Cpr66D*, on the other hand, are more highly expressed in higher order interommatidial pigment cells. These data suggest that the complementary expression of cuticular proteins give rise to the center vs periphery of the corneal lens facet, possibly facilitating a refractive gradient that is known to reduce spherical aberration. Moreover, these studies provide a framework for future studies aimed at understanding the cuticular basis of corneal lens function in holometabolous insect eyes.

Keywords

cuticular proteins; cone cells; Semper cells; pigment cells; compound eye

Introduction

Precisely crafted lenses are necessary to properly focus light on the retina in image-forming eyes. Such lenses of animals consist of specific proteins that play a key role in light refraction. Other major requirements for lens proteins include transparency, stability over

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large ranges of temperature, and resilience to light exposure, including potentially damaging UV light (de Jong et al. 1989). Currently, all annotated vertebrate lenses utilize at least two major classes of proteins to serves these functions: α and β/γ crystallins (Piatigorsky 1998). The α-crystallins (αA and αB) share evolutionary origins with small heat shock proteins (de Jong et al. 1988). These serve as molecular chaperones to prevent improper folding and aggregation of β and γ crystallins (Horwitz 1992), proteins that represent the structural components of the lens (Bloemendal et al. 2004). All three classes of crystallins play functions outside of the eye, and appear to have been co-opted to the lens through a conserved transcriptional network present in animals ranging from cnidarians to mammals (Blanco et al. 2005; Charlton-Perkins et al. 2011; Kozmik et al. 2008; Tomarev and Piatigorsky 1996; Vopalensky and Kozmik 2009).

Although we are beginning to understand how vertebrate lenses are formed from a variety of different genes (Slingsby and Wistow 2014), it remains less clear what proteins constitute invertebrate lenses. The crystallins from the camera-type eyes of cephalopods is perhaps most well studied. S-crystallin, a protein identified to share sequence homology with the enzyme glutathione S-transferase, is known for its detoxification properties, and was the first cephalopod lens protein discovered (Tomarev and Zinovieva 1988). Along with S-crystallins, three other taxon-specific crystallins were identified within squids, cuttlefishes, and octopi: Ω, L, and O, which again, share ancestries with common housekeeping enzymes (Chiou 1984; Montgomery and McFall-Ngai 1992; Zinovieva et al. 1999). Similarly, of three crystallins (J1-J3) isolated from the camera-type eye of the box jellyfish *Tridpedalia* (Kozmik et al. 2008; Piatigorsky et al. 1993; Piatigorsky et al. 2001), the J3 crystallin shares sequence homologies to proteins with enzymatic and chaperone functions outside of the lens (Castellano et al. 2005; Piatigorsky et al. 2001). This relationship of co-opting “house-keeping” genes to serve a new purpose within the lens (Tomarev and Piatigorsky 1996) demonstrates a convergent evolution pattern between camera eye lenses of both vertebrates and invertebrates.

While this pattern appears to be common in camera-type eyes, little is known about lens composition in the other major eye type: the compound eyes of arthropods. In insects, lenses have long been known to contain cuticular material. The involvement of specific cuticular proteins has been demonstrated in studies of mosquitos (*Anopheles gambiae*) and flies (*Drosophila melanogaster*). In mosquitoes, cuticle-encoding proteins are expressed in several cell types in the eye, including those situated below the lens (Vannini et al. 2014; Zhou et al. 2016). In *Drosophila melanogaster*, the adult eye consists of approximately 800 individual ommatidia, visualized externally by a biconvex corneal lens facet. In these eyes, the refractive properties of the lens allows images to be focused through a vitreous-like substance (the pseudocone) onto the underlying light-gathering apical surfaces of the photoreceptors (Franceschini 1972). Developmental studies have shown that *Drosophila* lens secretion begins at ~50% pupation, with contributions by cone cells (aka Semper cells), primary pigment cells, and interommatidial pigment cells (Cagan and Ready 1989; Perry 1968; Waddington and Perry 1960). Previous biochemical studies of isolated *Drosophila* corneal lenses revealed the presence of 3 major proteins with molecular weights of 52 kDa, 47 kDa and 45 kDa (Komori et al. 1992). Of these, only the 52 kDa protein has been identified, and was called Drosocrystallin (Crys). Crys is a member of the RR-2 subfamily

of cuticular proteins (Karouzou et al. 2007), is expressed in both cone cell and primary pigment cells (Charlton-Perkins et al. 2011; Janssens and Gehring 1999; Komori et al. 1992) and is activated by the transcription factor dPax2/sv. (Dziedzic et al. 2009). Retinin, a second cornea-enriched protein, was originally identified in a screen for eye-enriched genes (Hyde et al. 1990), and subsequently shown to be restricted in expression to the corneagenous cell layer (Kim et al. 2008).

Here, to gain a more complete understanding of *Drosophila* lens composition, we performed mass spectrometry from manually dissected adult corneal lenses to identify major lens-associated proteins. We then performed *in situ* hybridization studies to define the cell types in the developing eye that express the lens protein-encoding genes during lens secretion. The proteomic studies confirmed Crys and Retinin as major lens proteins and led to the identification of two additional major proteins, both of cuticular origin: Cpr66D and Cpr72Ec. Our *in situ* studies revealed overlapping but distinct patterns of expression for these 4 genes in both the major corneagenous cells (cone cells and primary pigment cells) as well as higher order interommatidial pigment cells. Combined, these studies add to our understanding of the molecular and cellular mechanisms underlying *Drosophila* lens facet formation and provide the necessary framework for future studies aimed at testing the role of cuticular protein in light focusing and other possible support roles.

Materials and methods

Proteomic analysis

Lens proteins were isolated from 20 acetone-fixed wild-type *Drosophila* heads as previously described Komori et al (1992). Hand-dissected corneal lenses were solubilized in Laemmli buffer (BioRad), separated on a 10% SDS-PAGE gel, and stained with Coomassie Blue. Two major and eight minor bands from a single gel lane (Fig 1A) were excised, reduced and alkylated, digested with trypsin, and identified by MALDI-TOF/TOF and a MASCOT search at the University of Cincinnati Proteomic Core.

In situ hybridization

Total RNA from 50 adult w^{1118} fly heads was isolated using the RNeasy Lipid Tissue Kit (Qiagen; Valencia, CA) per manufacturer's instructions. cDNA was synthesized from 1μg total RNA using oligo (dT) primers and Omniscript reverse transcriptase (Qiagen, Valencia, CA). Gene-specific cDNAs were generated in a PCR amplification using primers listed in Table 1 (35 cycles of 94°C, 1 min; 60°C, 1 min; 72°C, 1 min). PCR products of correct sizes were purified using the Qiaquick PCR purification kit (Qiagen, Valencia CA). RNA probes were generated following manufacturer's instructions using the SP6/T7 DIG RNA Labeling Kit (Roche Applied Sciences, IN, USA). Antisense probes were generated for each gene of interest using T7-tagged antisense primers, and a control sense probe against *Crys* was generated using a T7 sense primer. Probes were precipitated with 9.5 μM lithium chloride and 3 volumes ethanol overnight at −80°C, resuspended in 10 μL RNase-free ddH_2O and quantified by spectrophotometry using a Nanodrop-1000 (ThermoFisher Scientific, MA).

In situ hybridizations were performed as previously described by Sakamoto et al., (1996). For tissue, w^{118} flies were reared at 25°C at a 12:12 day night cycle and 50% pupal retinas (50 hr after puparium formation) were dissected and fixed 15 minutes at room temperature (RT) with 4% paraformaldehyde diluted in phosphate buffered saline (PBS, 7.4) (Gibco). Samples were washed 3 × 10 min in PBS, and pre-hybridized with hybridization solution for 1 hr at 60°C. RNA probes were added to fresh hybridization buffer at a final concentration of 1.0 μgml^{-1} solution, denatured at 90°C for 3 minutes, and placed directly on ice. The denatured RNA probe/hybridization buffer was added to the tissue and incubated overnight (~16 hours) at 60°C. Tissue was then washed once in 5× saline sodium citrate (SSC) (ThermoFisher Scientific, MA) for 5 minutes at 55°C, once with 2× SSC for 30 minutes, and twice with 0.2× SSC with the final SSC rinse done at RT. All subsequent procedures were performed at RT. Tissue was permeabilized in PBS with 0.1% Triton X-100 (PBX) for 10 minutes, and blocked for 1 hr with PBX containing 10% normal sheep serum. Alkaline phosphatase-conjugated Anti-Dig Fab antibody (Roche Applied Sciences, IN, USA), diluted 1:500 in blocking solution, was added for 3 hours, followed by 3×10 minute washes with PBX. Signal was detected using the Vector Red Alkaline Phosphatase Substrate Kit (Vector Laboratories, CA, USA) per manufacturer's suggested, followed by DAPI (Roche Applied Sciences, IN, USA) staining for 30 minutes. Samples were rinsed with dH_2O and mounted in Fluoromount G (Southern Biotech, AL, USA), preserving the 3-D structure of the tissue using small coverslip pieces as a bridge. Z-stack images were acquired using a Nikon Eclipse Ti motorized inverted microscope equipped with Nikon A1R deconvolution software (Cincinnati Children's Confocal Imaging Core) and processed using NIS Element (Nikon). All staining was reproduced in biological triplicates to confirm expression analyses.

Results and discussion

The *Drosophila* corneal lens is composed of 4 major proteins

Corneal lenses from wild-type *Drosophila melanogaster* were dissected, and proteins were separated by SDS-PAGE. This revealed 2 major bands with molecular weights of ~50 and ~60kDa, and several bands of weaker intensity with molecular weights ranging from 20 to 150kDa (Fig 1A). Individual bands were excised and identified by MALDI-TOF/TOF mass spectrophotometry. The advantage of this method is that it allowed us to establish the identity, relative concentration, and molecular weight of the main proteins that constitute the lenses. Indeed, to achieve functionally relevant levels of refraction, high concentrations of proteins are necessary (Sweeney et al. 2007).

Amino acid sequence analysis of the 10 excised bands (Fig 1A) revealed that the larger of the two major lens proteins (Band 5, 60kDa) corresponds to the Drosocrystallin (Crys) protein (Komori et al. 1992), which has a predicted MW of 56 kDa. The second major lens protein (Band 6, 50 kDa) we identified corresponds to Cpr72Ec (predicted MW = 51 kDa). This likely corresponds to the 47 kDa protein isolated by Komori et al. (1992) based on abundance, molecular weight and amino acid composition. Consistent with cross-linking of cuticular proteins during chitin formation (Zhu et al. 2016), the larger, less-abundant complexes in Bands 1–4 were comprised of both Crys and Cpr72Ec. Crys was also identified in three lower molecular complexes (Bands 7–9), two of which were solely Crys

(possibly degradation products). Band 9 also included Cpr66d, a 31 kDa cuticular protein, identifying this protein as new relative low-abundance lens proteins. Finally, the lowest molecular weight protein in this analysis (Band 10) corresponded to the previously identified 20 kDa Retinin protein (Kim et al. 2008). Combined, this analysis revealed the presence of 4 major cuticular proteins within the *Drosophila* corneal lens.

We note that none of the proteins identified in the current study showed a good match to the 45kDa corneal protein identified by Komori et al. (1992). It is possible that, since our analysis is based on excision of specific bands (rather than analysis of the entire lens extract) and did not involve 2D electrophoresis, as was the case in the Komori study, additional minor lens components remain unidentified. Consistent with this possibility, a single peptide from alcohol dehydrogenase was identified from the faint band 10. Based on its low abundance, it was not further analyzed.

Cell-selective expression patterns of *Drosophila* lens protein-encoding genes

The midpupal stage of *Drosophila* development is when lens formation if first observed (Cagan and Ready 1989; Perry 1968; Waddington and Perry 1960). Consistent with previous data (Janssens and Gehring 1999), we found that the *Crys* gene is strongly expressed at this stage of development. Also in agreement with previous immunohistochemical and enhancer analyses (Charlton-Perkins et al. 2011; Janssens and Gehring 1999), our *in situ* studies suggest that *Crys* mRNA expression is restricted to cone cells and primary pigment cells. These findings are together in line with the hypothesis that Crys is a particularly important refractive component of *Drosophila* lenses (Komori et al. 1992).

Like *Crys*, we found *retinin* to be expressed in both cone cells and primary pigment cells, and not in photoreceptors or high order interommatidial pigment cells. This is consistent with previous expression studies suggesting that *retinin* has distally-restricted expression in the eye (Kim et al. 2008). Also, although *retinin* mRNA was not detected by Northern blot analysis until the end of pupation (Kim et al. 2008), our analysis indicates that its expression has been initiated during early stages of lens formation.

Of the newly defined lens protein-encoding genes, *Cpr72Ec* shows a much broader pattern of expression. Specifically, it is expressed in all cell types of the eye, with particularly high expression in higher order interommatidial pigment cells, and weaker expression in cone cells, primary pigment cells and photoreceptors (Fig. 3). Based on its distribution, this protein could be an important peripheral lens component, possibly allowing lenses to have a graded refractive index, which is a common strategy to reduce spherical aberration (Land and Nilsson 2012). It also could play a role in the establishment of the general raster of the compound eye array. Importantly, this gene was previously identified as an eye-specific gene product (Xu et al. 2004), with the current study suggesting that this major eye protein plays an important role in lens formation.

The fourth protein that was revealed through this proteomic approach is Cpr66D. This protein is likely to be a relatively minor contributor to the lens, as it was detected in only one of the 10 protein bands (Fig 1). However, like *Cpr72Ec*, its expression is particularly notable in higher order interommatidial pigment cells and may be weakly expressed within

photoreceptors. In contrast to the eye-enriched nature of *Cpr72Ec*, Cpr66d has also been identified in several embryonic tissues, the wing, and the trachea (Chandran et al. 2014; Haussmann et al. 2008; Ren et al. 2005), suggesting a more generalized role for this protein in cuticle formation.

Combined, these *in situ* studies suggest that the previously identified Crys and Retinin proteins contribute to the central core of the lens through expression in cone and primary pigment cells, while Cpr72Ec and Cpr66d contribute to its periphery through expression in interommatidial cells. These findings support earlier TEM studies noting the differential contribution of lens materials by core vs interommatidial cells (Cagan and Ready 1989) and provide a possible mechanistic basis for the establishment of a refractive gradient. Gradated lenses typically reduce spherical aberration through lower refractive indices in the periphery compared to the center, and are common in both invertebrates (Blest and Land 1977; Sweeney et al. 2007) and vertebrates (Pumphrey 1961).

The cuticular basis and evolution of corneal proteins

Our data suggest that the proteomic architecture underlying the *Drosophila* corneal lens consists largely, if not exclusively, of members from the cuticular family of proteins. Cuticular proteins are classified into thirteen subfamilies (Zhou et al. 2016). Two of these share highly conserved residues, named after the classic Rebers and Riddiford (RR) studies: RR-1 and RR-2 (Rebers and Riddiford 1988). Crys has previously been identified as a member of the RR-2 family of cuticular proteins (Karouzou et al. 2007), and based on the CutProtFam predictor (Ioannidou et al. 2014), Cpr72Ec and Cpr66D are also members of this family. The fourth protein, Retinin, is a Cuticular Protein of Low complexity, a family of proteins identified by a conserved Retinin domain (Cornman and Willis 2009).

In a recent study on *Anopheles*,Zhou et al. (2016) used LC/MS on adult lenses and eye capsules, and identified a large number (89) of cuticular proteins. Whether this marked difference in the number of proteins identified between our study and that in *Anopheles* is not yet clear, as their analysis was geared towards a comprehensive identification strategy, while our analysis focused on specifically identifying the nature and abundance of key proteins that could significantly contribute to lens refraction. Regardless, it is notable that the largest number of cuticular proteins identified from the mosquito lens array consisted of members of the RR-1 family (Vannini et al. 2014; Zhou et al. 2016), while the majority (3 out of 4) of our identified *Drosophila* lens proteins belong to the RR-2 family. It is possible that this difference relates to relative abundance of these protein classes, as the evolution rate of RR-1 members in *Anopheles* has been reported to be nearly twice that of RR-2 members (Cornman et al. 2008). However, it is also possible that this difference could relate to evolutionary differences between the lens properties between flies and mosquitos, as RR-1 are more commonly associated with softer tissues, while RR-2 proteins are associated with harder portions of the insect exoskeleton (Zhou et al. 2016).

From a material-science point of view, our findings indicate that *Drosophila* has evolved a clever way to craft its lenses, with the abundance of cuticular proteins likely to differ between the center and the periphery of the lens. In fact, a recent publication suggests that a relatively dramatic difference between the core and periphery exists in the moth midge

Clogmia albipunctata, which is another dipteran species. In *Drosophila* it is also possible that, through the formation of complexes between proteins such as Crys and Cpr72Ec, the refractive index could be further altered. Together this is a powerful strategy to locally fine-tune refractive indexes as needed, which in many way is far superior to how we craft artificial lenses.

Finally, it is worth noting that a literature review of the main *Drosophila* lens proteins identified here have been identified in screens associated with desiccation, neurodegeneration, and aging (Kang et al. 2016; Napoletano et al. 2011; Robinson et al. 2010; Xun et al. 2008). Thus, like camera eye crystallins, the cuticular "crystallins" of the insect eye are likely to play more than just refractive functions in the eye. Indeed, this is consistent with reports that Crys is not only expressed in fly lenses but is also present in mechanosensory bristle cell types (Dziedzic et al., 2009). Future studies aimed at specifically knocking down the different components of the Drosophila lens will hence be an important step toward dissecting the multi-faceted roles of cuticular proteins and "crystallins" in visual system function and maintenance.

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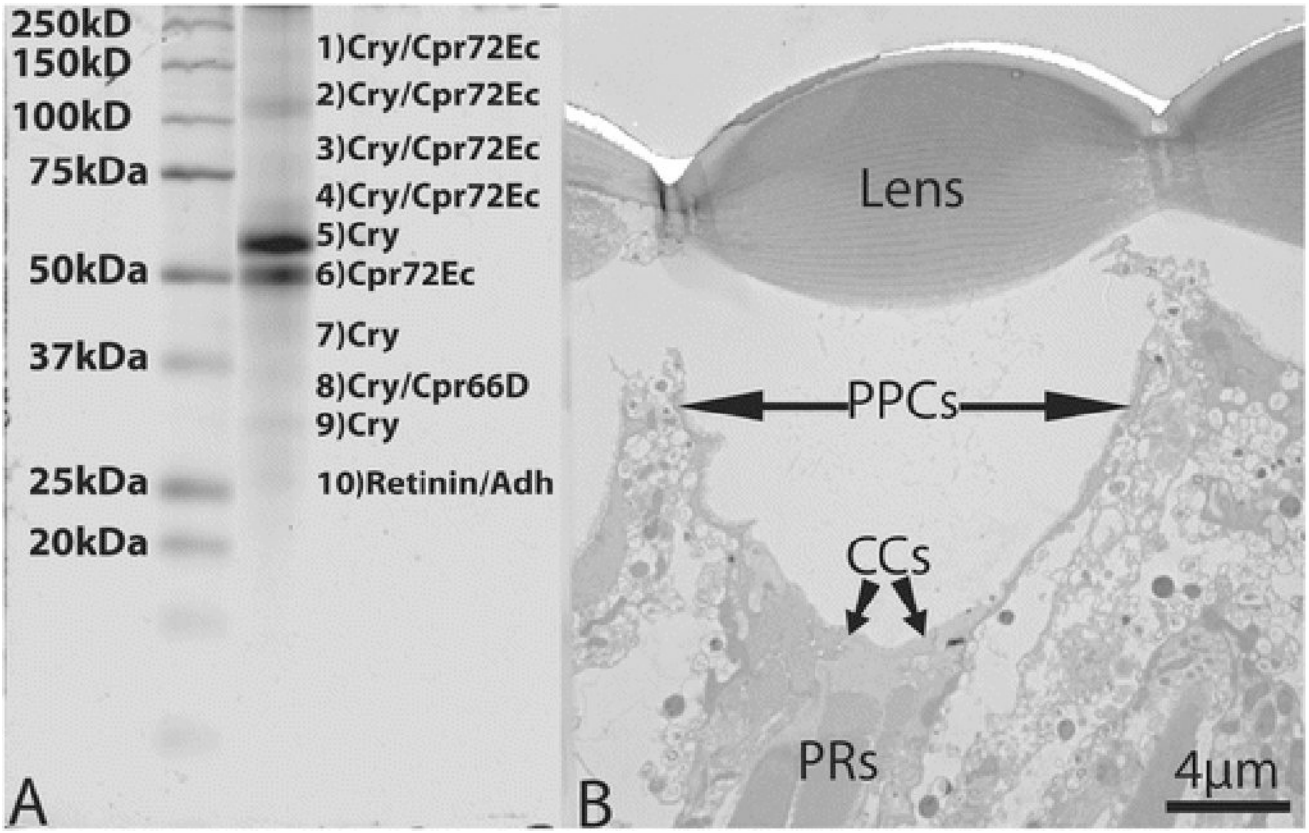

Figure 1.

Proteomic analysis of *D. melanogaster* lenses revealed the presence of four major proteins within ten bands that were subjected to a MALDI-TOF/TOF analysis (A). Scanning electron micrograph of an adult ommatidium demonstrates the anatomical structure of an individual lens facet region with specific cell types labeled: photoreceptors (PRs), cone cells (CCs), and primary pigment cells (PPCs) (B).

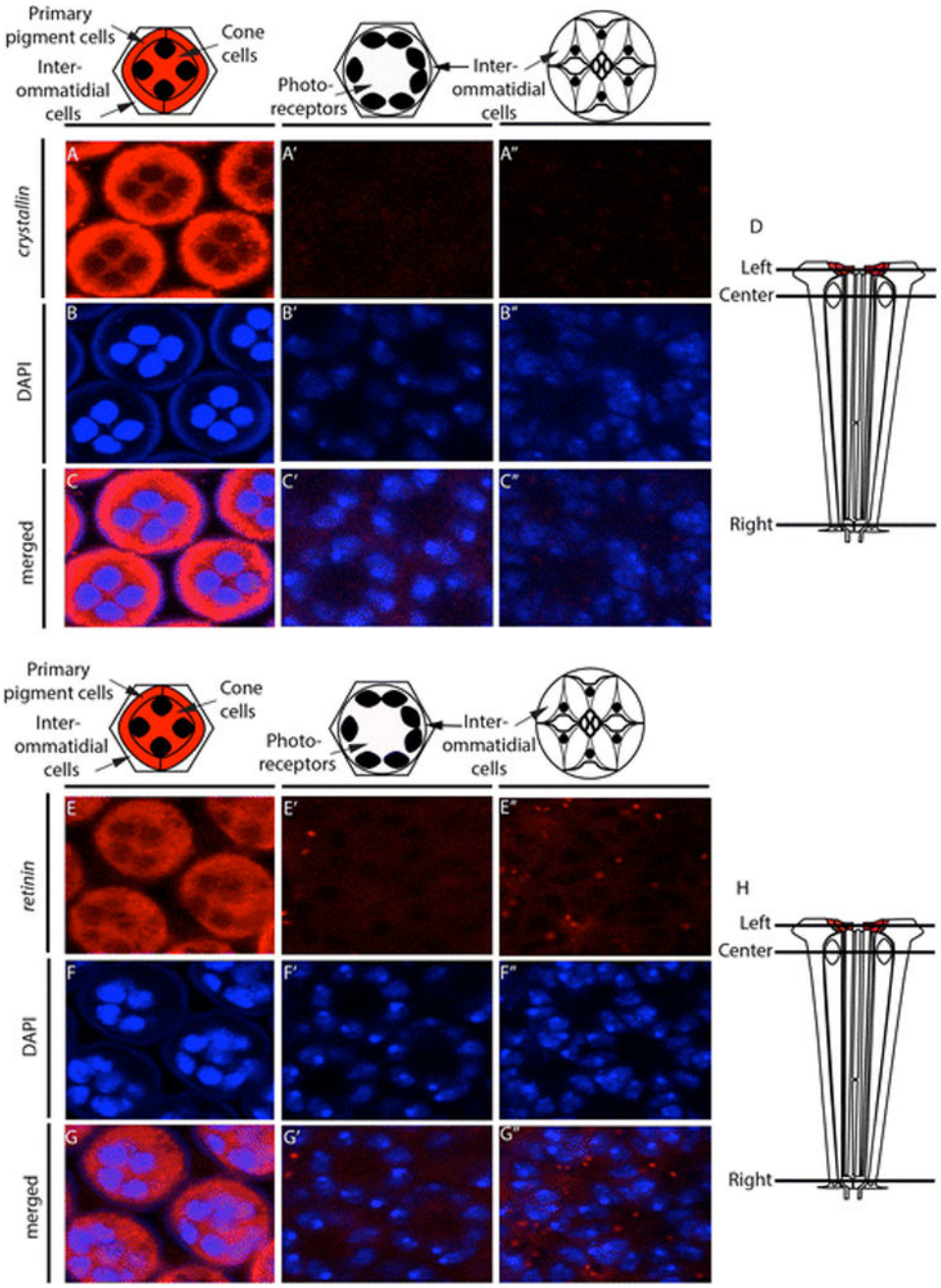

Figure 2.

Profiles of *Crys* and *retinin* gene expression at 50% pupation, when lens formation begins. The cellular identity of expression was identified in confocal Z-stacks based on the known position of these cell's nuclei. Representative expression profiles are illustrated in schematics. Those on top of each column summarize expression patterns at approximate section planes as indicated in the ommatidial schematics D & H. Vector red alkaline phosphatase staining (A-A", C-C", E-E", G-G") counterstained with DAPI (blue, B-B",C-C", F-F", G-G") illustrates expression of *Crys* (A,C,D) and *retinin* (E,G,H) within the PPC (primary pigment cells) and CC (cone cells).

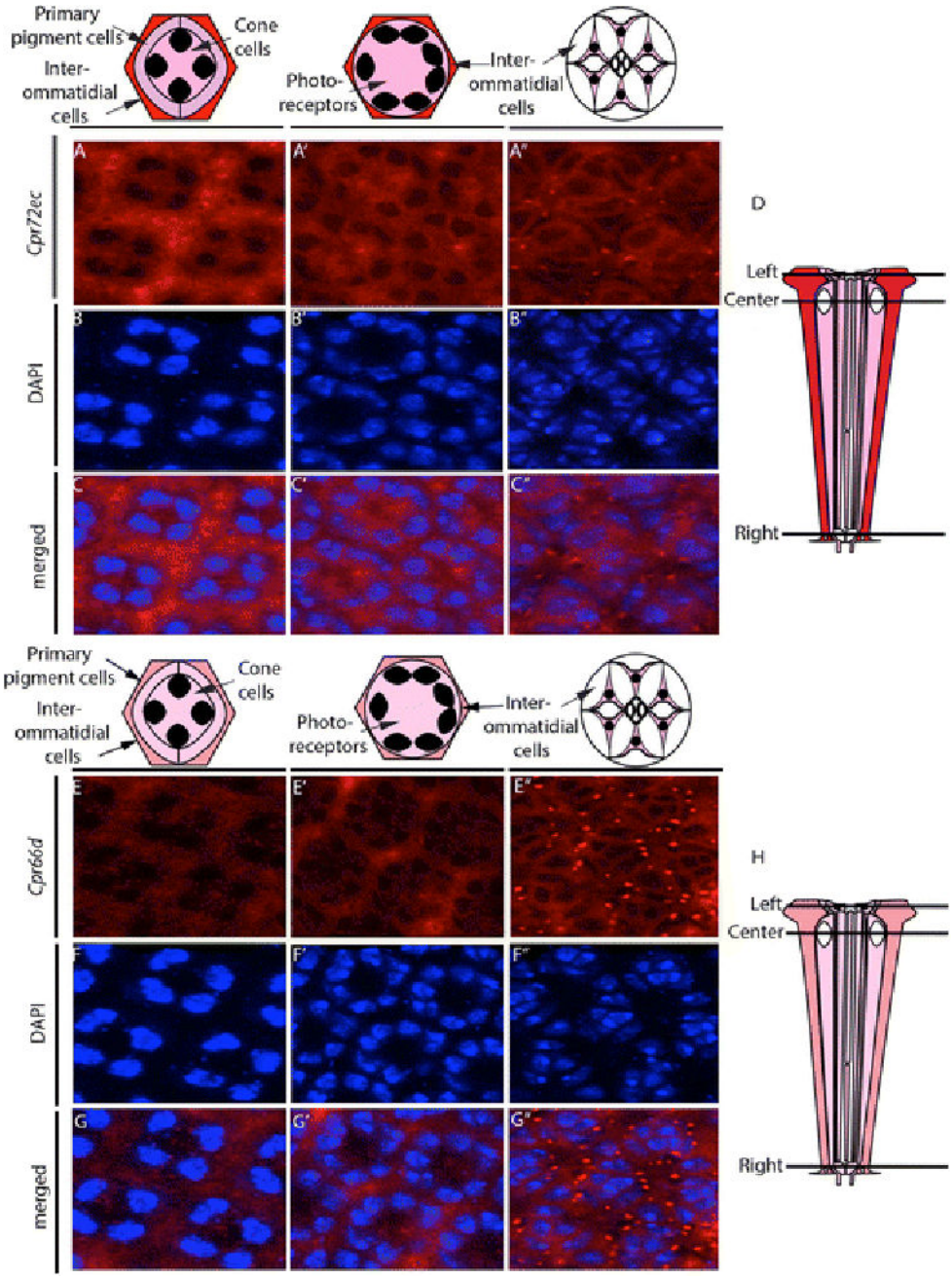

Figure 3.

Profiles of *Cpr72Ec* and *Cpr66D* gene expression at 50% pupation. Schematics on top of each column summarize expression patterns at approximate section planes as indicated in the ommatidial schematics D & H. *In situ* hybridization of *Cpr72ec* (A-A”, C-C”) with DAPI counterstain (B- B”, C- C”) illustrates particularly strong expression within the interommatidial cells (IOCs), and weaker expression within the PPC, CC, and photoreceptors (D). Similarly, *Cpr66D* expression (E- E”, G- G”) is highest within the

interommatidial cells (H). All cells were identified based on nucleus positions visualized with DAPI counter staining (F- F", G- G").

Table 1

Primer sequences used to amplify lens protein-encoding genes.

Primer	**Sequence 5’-3’**
Antisense probe primers	
crystallin Fw	CTTAACGTCAGCAGCCATGA
crystallin Rv T7	TAATACGACTCACTATAGGGCTGGGGACGAGTGTCGTAAT
retinin Fw	CCGTATTGACCATCCAGGAG
retinin Rv T7	TAATACGACTCACTATAGGGGGATCCTTAGTTGCGGATGA
Cpr72Ec Fw	GGAAATCCGACAGGATCAGA
Cpr72Ec Rv T7	TAATACGACTCACTATAGGGTCTTGACGGGATTCTTGGTC
Cpr66D Fw	CAGCAATATCAGCCTCAGCA
Cpr66D Rv T7	TAATACGACTCACTATAGGGCGAGGAGTTTTGGTCATCGT
Sense probe primers	
crystallin Fw T7	CAGCAATATCAGCCTCAGCA
crystallin Rv	TAATACGACTCACTATAGGGCGAGGAGTTTTGGTCATCGT