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Eye proteome of *Drosophila melanogaster*

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Abstract

Drosophila melanogaster is a popular model organism to elucidate the molecular mechanisms that underlie the structure and function of the eye as well as the causes of retinopathies, aging, light-induced damage, or dietary deficiencies. Large-scale screens have isolated genes whose mutation causes morphological and functional ocular defects, which led to the discovery of key components of the phototransduction cascade. However, the proteome of the *Drosophila* eye is poorly characterized. Here, we used GeLC-MS/MS to quantify 3516 proteins, including the absolute (molar) quantities of 43 proteins in the eye of adult male *Drosophila melanogaster* reared on standard laboratory food. This work provides a generic and expandable resource for further genetic, pharmacological, and dietary studies.

Introduction

Drosophila melanogaster is an established model organism to elucidate the molecular mechanisms that underlie the structure and function of the eye and how their disruption causes retinopathies (Colley, 2012; Cook et al., 2011; Gaspar et al., 2019; Kumar, 2012; Treisman, 2013; Wang & Montell, 2007). The adult compound eye is a highly repetitive structure consisting of about 800 units that are called ommatidia. Each ommatidium contains eight photoreceptor neurons (R1-R8) that express different light-sensing Rhodopsin's and phototransduction proteins in specialized light-sensing compartments (Hardie & Juusola, 2015; Poupault et al., 2021; Rister et al., 2013). The expression of these proteins depends on vitamin A (Dewett, Lam-Kamath, et al., 2021; Kumar et al., 2022). The photoreceptors are surrounded by accessory cells that include cone, pigment, and mechanosensory bristle cells (Ready, 1989).

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The powerful genetic toolkit of *Drosophila* allows the generation of loss-of-function clones specifically in the eye (Newsome et al., 2000; Stowers & Schwarz, 1999; Weasner et al., 2017) and large-scale screens in *Drosophila* identified genes whose mutation causes morphological and functional ocular defects (Harvey et al., 2003; Karim et al., 1996; Pak, 2010; Senturk & Bellen, 2018). This approach led to the discovery of signaling pathways that mediate major developmental processes and phototransduction (Yau & Hardie, 2009). For instance, a crucial discovery was the identification of light-activated Transient Receptor Potential (TRP) channels (Montell & Rubin, 1989) that founded a conserved superfamily of cation channels with diverse functions ranging from mediating the responses to various sensory stimuli (Montell, 2011) to immune responses (Khalil et al., 2018). Moreover, TRP channels have been implicated in various human diseases including cancer and neurodegenerative disorders (Minke, 2002; Yue & Xu, 2021). Lastly, the *Drosophila* eye has also been used as a model to investigate the impacts of ageing (Hall et al., 2017) and environmental stresses such as light-induced damage (Hall et al., 2018; Moehlman et al., 2018; Stark & Carlson, 1984) or nutrient-deficient diets (Kumar et al., 2022; Lee et al., 1996; Randall et al., 2015).

Despite these important discoveries, the *Drosophila* eye proteome remains poorly characterized. Several resources for gene expression in the adult eye are available, such as transcriptomes of whole eyes, isolated photoreceptor nuclei, or single cells (Hall et al., 2017; Huang & Ryoo, 2021; Leader et al., 2018; Stegeman et al., 2018; Yeung et al., 2022), but global transcript levels often poorly correlate with protein expression levels (Bonaldi et al., 2008; Buccitelli & Selbach, 2020; Liu et al., 2016). Yet, there are very few resources for the ocular proteome or the molar abundances of proteins that are expressed in the eye (Hall et al., 2021; Kumar et al., 2022; Raghuraman et al., 2020).

Hall et al. (Hall et al., 2021) employed tandem mass tag (TMT) quantification for proteomics analysis of male *Drosophila* flies expressing a nuclear membrane-localized GFP tag (Rh1-Gal4>GFP^{KASH}) in R1–R6 photoreceptors. In other studies, by the same group, this genotype has been used for transcriptome and chromatin profiling of photoreceptors during aging and under environmental stress conditions (Hall et al., 2018; Hall et al., 2017; Jauregui-Lozano et al., 2021).

In two previous studies, the targeted absolute (molar) quantification of selected components of the *Drosophila* phototransduction machinery was performed with the MS Western method in combination with full-proteome profiling by GeLC-MS/MS: Raghuraman et al. (Raghuraman et al., 2020) applied MS Western to study the impacts of specific *crumbs* (*crb*) mutations damaging the light-sensing compartments of the photoreceptors on the molar abundances of several photoreceptor proteins in the *Drosophila* eye. The values determined by MS Western corroborated the expected molar abundances of mutant Crb proteins and reflected the difference in severity of the photoreceptor defects. In another study, Kumar et al. (Kumar et al., 2022), used MS Western and GeLC-MS/MS to quantify the proteome-wide response of the *Drosophila* eye to vitamin A deprivation using semi-synthetic lipid-depleted food media. These two studies established MS Westen as a robust and reproducible absolute quantification method, while GeLC-MS/MS delivered good proteome coverage including a large number of membrane proteins. Building upon the previous two studies, the current

manuscript provides a complete catalogue of the total eye proteome of wild type *Drosophila* flies raised on standard lab food. The new study also provides the absolute quantities of selected proteins that are important for maintaining the structure and function of the normal eye.

Here, we used GeLC-MS/MS to quantify 3516 proteins in the adult *Drosophila* eye; approximately 30% of them are membrane-related proteins and 16% have at least one transmembrane domain. We also quantified the absolute (molar) abundances of a set of proteins that is critical for phototransduction and photoreceptor morphology. Taken together, we provide a quantitative and expandable resource for further genetic, pharmacological, and dietary studies in the *Drosophila* eye.

Results and Discussion

For cataloging the proteome of the *Drosophila melanogaster*, we followed the experimental workflow that is shown in Figure 1. *Drosophila melanogaster* was raised from the embryonic to the adult stage on ‘standard’ lab food (SF) under a 12h light/12h dark cycle at 25°C (for details, see Materials and Methods). Three to four days-old male flies were collected and used for all the experiments described below. Raising *Drosophila melanogaster* on nutrient-rich SF resulted in a normal morphology of the compound eye (Figure 2A) and wild type ommatidia with six rhabdomeres of the rod-equivalent outer rhabdomeres arranged in a trapezoid shape around the rhabdomeres of the cone-equivalent inner photoreceptors (Figure 2B). We also detected a wild-type expression pattern of the major Rhodopsin Rh1 in the rhabdomeres of outer photoreceptors (Figure 2B), which suggests that SF supports wild type visual pigment formation and is vitamin A-sufficient (Dewett, Labaf, et al., 2021; Kumar et al., 2022).

To characterize the adult eye proteome, we analyzed protein extracts by label-free GeLC-MS/MS proteomics. The analysis provides relative abundance of a total of 3516 proteins (3017 of them were identified with at least two peptides) (Figure 2C); the complete list of quantified proteins is provided in Supplementary Dataset S1. According to FlyBase (FB2022_02, released March 29, 2022), 2872 proteins had not been previously attributed to eye tissue (Figure 2D and Supplementary Dataset S2). Approximately 30% of all quantified proteins (910 from 3017) were membrane-related and 16% of them (502 from 3017) had at least one transmembrane domain (Figure 2C). To rank the proteins by their abundance , we used intensity-based absolute quantification (iBAQ) (Schwanhausser et al., 2011); based on the iBAQ value, we performed functional annotation and classification by Gene Ontology (GO) enrichment analysis. We obtained 34 GO terms, of which cellular metabolic, multicellular organismal, and biosynthetic process represented the major biological processes (Figure 2E). A subsequent GO enrichment analysis of the membrane proteins assigned them to 20 cellular components (Figure 2E).

Next, we used MS Western method, which allows the multiplexed, antibody-free and label-free molar quantification of user-selected proteins (Kumar et al., 2017), to quantify the absolute (molar) abundances of proteins that play crucial roles in photoreceptor structure and function (Figure 2F and Supplementary Dataset S3). We found that the visual pigment

Rhodopsin Rh1 (NinaE) was the most abundant phototransduction-related protein (~409 fmoles/eye). The next three most abundant proteins were all involved in the termination of the light response (Figure 2F): the major visual arrestin Arr2 (~350 fmoles/eye), the unconventional myosin NinaC (210 fmoles/eye), and the other visual arrestin Arr1 (~185 fmoles/eye). Phototransduction proteins that are activated by Rh1 (Yau & Hardie, 2009) were about 10-fold less abundant than Rh1 (Figure 2F): the G protein alpha subunit Galphaq (~76 fmoles/eye), the phospholipase C NorpA (~45 fmoles/eye), and the major light-sensitive cation channel Trp (~36 fmoles/eye).

We also used MS Western to compare the molar abundances of proteins that are required for photoreceptor morphology and maintenance (Gurudev et al., 2014). The molar abundances of the structural proteins were consistent with their spatial expression pattern (broad or restricted) in the eye: the cell adhesion protein Chaoptin (Chp) (~212 fmoles/eye), which is required for the adhesion of the rhabdomeric microvilli and is broadly expressed throughout their perimeter (Reinke et al., 1988; Zelhof et al., 2006), was the most abundant (Figure 2F). Much less abundant were two structural proteins with more restricted expression patterns that are critical for rhabdomere separation (Nie et al., 2012), the secreted glycoprotein Eyes shut (~31 fmoles/eye) that is localized to the interrhabdomeral space (Eys, also called Spacemaker / Spam) (Zelhof et al., 2006) and the transmembrane protein Prominin (Prom) (~10 fmoles/eye) that is spatially restricted to the stalk membrane of the rhabdomeres as well as the tips of the microvilli (Zelhof et al., 2006). The transmembrane protein Crumbs (Crb) (~2 fmoles/eye) that is essential for rhabdomere morphology was the least abundant morphology related protein, which is consistent with its even more restricted expression in the stalk membrane (Tepass et al., 1990).

Lastly, we quantified the molar abundances of the three Actins Act5C, Act87E, and Act57B that are expressed in the adult eye (Raghuraman et al., 2020). Act5C was the most abundant actin (~573 fmoles/eye) and expressed at even higher levels than Rh1. The molar abundances of Act87E and Act57B were about half of those of Act5C, ~260 and ~262 fmoles/eye, respectively (Figure 2F). Lastly, the scaffolding protein and Crumbs complex member Veli/Lin-7 (~4 fmoles/eye) (Bachmann et al., 2008) and the actin depolymerization factor Twinstar (a homolog of Cofilin/ADF) (~73 fmoles/eye), showed significantly lower absolute abundances (Figure 2F).

Conclusion

Our study provides a comprehensive catalogue of the proteome of the adult *Drosophila melanogaster* eye. We envision that these data will be a useful resource for the scientific community that uses the *Drosophila* eye as a model to study visual signaling or the genetic and environmental stresses that cause various retinopathies.

Material and Methods

Drosophila stock keeping

The *Drosophila melanogaster* wild-type strain Oregon R was reared under a 12h light/12h dark cycle at 25°C. The flies were raised on ‘standard’ lab food (SF), which contained per

liter: 8g agar, 18g brewer's yeast, 10g soybean, 22g molasses, 80g cornmeal, 80g malt, 6.3ml propionic acid, and 1.5g Nipagin.

Drosophila compound eye images

Adult male *Drosophila melanogaster* was immobilized with CO_2 and embedded in a liquid agarose gel as previously described (Kumar et al., 2022). After solidification of the gel, the petri dish was placed under a Stemi 508 Trinoc microscope (Zeiss model #4350649030), and the fly head was adjusted with a forcep such that one compound eye faced the lens of the microscope. Imaging was performed with an Axiocam 208 HD/4k color camera (Zeiss model #4265709000) that was set to auto exposure and auto white balance. Pictures were processed with Fiji, Adobe Photoshop 2020, and Adobe Illustrator 2020 software.

Confocal microscopy and immunohistochemistry of *Drosophila* photoreceptors

We dissected retinas of four days old male flies as previously described (Hsiao et al., 2012). Briefly, we fixed the retinas in 3.8% formaldehyde solution before removing brain tissue and head cuticle. Retinas were incubated overnight in the primary antibody (mouse anti-Rh1 4C5, from Developmental Studies Hybridoma Bank, University of Iowa) diluted 1:10 in PBST (PBS with added 0.3% Triton-X, Sigma) and then washed three times with PBST. Retinas were then incubated overnight in secondary antibody diluted 1:800 in PBST (Alexa Fluor 647-conjugated raised in donkey, Invitrogen) and Alexa Fluor 488-conjugated Phalloidin (1:100, Invitrogen) and then washed three times with PBST. Retinas were mounted on a bridge slide using SlowFade (Molecular Probes) and imaged with a Zeiss LSM 8 confocal microscope. Raw images were processed with Fiji (https://imagej.net/software/fiji/) and then further processed with Adobe Photoshop and Adobe Illustrator software.

Protein extraction and GeLC-MS/MS analysis

The compound eyes (n=40) were dissected (including the lamina neuropil) from three to four days old male flies raised on SF and placed in lysis buffer containing 150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl (pH7.5), 1 tablet Roche protease inhibitors, 0.2% w/v CHAPS, 0.1% w/v OGP (Octyl β --D-glucopyranoside), 0.7% v/v triton X-100, 0.25 μg/mL DNase, and RNase. The samples were immediately snap frozen using liquid nitrogen and stored at −80°C or further processed. The eyes were homogenized and to the supernatant an equal volume of 2x SDS Laemmli sample buffer (SERVA Electrophoresis GmbH, Heidelberg, Germany) was added. The samples were heated at 80°C for 15 minutes and loaded on 4-20% gradient one dimensional SDS polyacrylamide gel. The gel slab was stained with Coomassie Brilliant Blue. Each gel lane was cut into six gel slices, and to enable the quantification by MS Western, each gel slice was co-digested with heavy isotope labeled chimeric protein standard (CP) and gel band containing 1pmol of Bovine Serum Albumin (BSA) standard. The MS Western method was used as described previously (Kumar et al., 2017). Briefly, the sequence of 265 kDa chimeric protein (CP) standard was designed *in-silico* by concatenating sequences of 197 unique peptides from 43 fly proteins together with reference peptides from glycogen phosphorylase and from BSA. The corresponding DNA sequence was synthetized, cloned into the expression vector, and expressed in a ΔLysΔArg-dual auxotrophic strain of E. coli in media supplemented with 15N7, 13C6-Arginine, and

13C6 –Lysine. An aliquot of E. coli extract was subjected to 1D SDS PAGE and the protein band corresponding to the full molecular weight of the CP was excised and co-digested with gels slabs containing the targeted Drosophila proteins. The list of target proteins and the corresponding peptides contained in the CP are provided in Supplementary Table 1. In-gel digestion was carried out as previously described (Shevchenko et al., 2006). Briefly, upon electrophoresis the gel was rinsed with water, stained with Coomassie Brilliant Blue R-250 for 10 minutes at RT, and then destained with destaining solution (Water: Methanol: Acetic acid, 50:40:10 v/v/v). The gel slices were further cut into small pieces (~1 mm size) and transferred into 1.5 ml LoBind Eppendorf tubes. They were destained by acetonitrile (ACN) / water, and proteins reduced with 10 mM dithiothreitol at 56°C for 45 min and alkylated with 55mM Iodoacetamide for 30 minutes in dark at room temperature. The reduced and alkylated gel pieces were washed with water / ACN and finally shrunk with ACN, ice-cold trypsin (10ng/µl) was added to cover the shrunk gel pieces and after 1hr of incubation on ice, excess trypsin (if any) was discarded. The gel pieces were then covered with 10mM NH_4HCO_3 and incubated overnight at 37°C. The tryptic peptides were extracted using water/ACN/ formic acid (FA), dried in a vacuum centrifuge, and stored at −20°C until use. The tryptic peptides were recovered in 5% aqueous FA and 5 µl were injected using an autosampler into a Dionex Ultimate 3000 nano-HPLC system, equipped with a 300 µm i.d. × 5 mm trap column and a 75 µm × 15 cm Acclaim PepMap100 C18 separation column. 0.1% FA in water and ACN were used as solvent A and B, respectively. The samples were loaded on the trap column for 5 min with solvent A at a flow of 20 µL/min. The trap column was then switched online to the separation column, and the flow rate was set to 200nL/min. The peptides were fractionated using 180 min elution program: a linear gradient of 0% to 30% B delivered in 145 min and then B% was increased to 100% within 10 min and maintained for another 5 min, dropped to 0% in 10 min, and maintained for another 10 min. Mass spectra were acquired using a Q Exactive HF mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The Data Dependent Acquisition (DDA) settings used for are provided in Supplementary Table S2.

Data processing for protein identification and quantification

Mascot v2.2.04 (Matrix Science, London, UK) was used for peptide identifications against the custom-made database containing the sequence of the target protein, to which sequences of human keratins and porcine trypsin were added. For eye proteome analysis, the *Drosophila* reference proteome database from UniProt was used. The database searches were performed with the following mascot settings: precursor mass tolerance of 5 ppm; fragment mass tolerance 0.03 Da; fixed modification: carbamidomethyl (C); variable modifications: acetyl (protein N-terminus), oxidation (M); Label: 13C (6) (K), Label: 13C (6) 15N (4) (R), 2 missed cleavages were allowed. Progenesis LC-MS v4.1 (Nonlinear dynamics, UK) was used for the peptide feature extraction and the raw abundance of identified peptide was used for absolute quantification. MaxQuant v1.5.5.1 and Perseus v1.5.5.3 were used for label-free quantification and subsequent statistical analysis. MaxQuant analysis was done with default settings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability statement

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Perez-Riverol et al., 2022) with the dataset identifier PXD044845".

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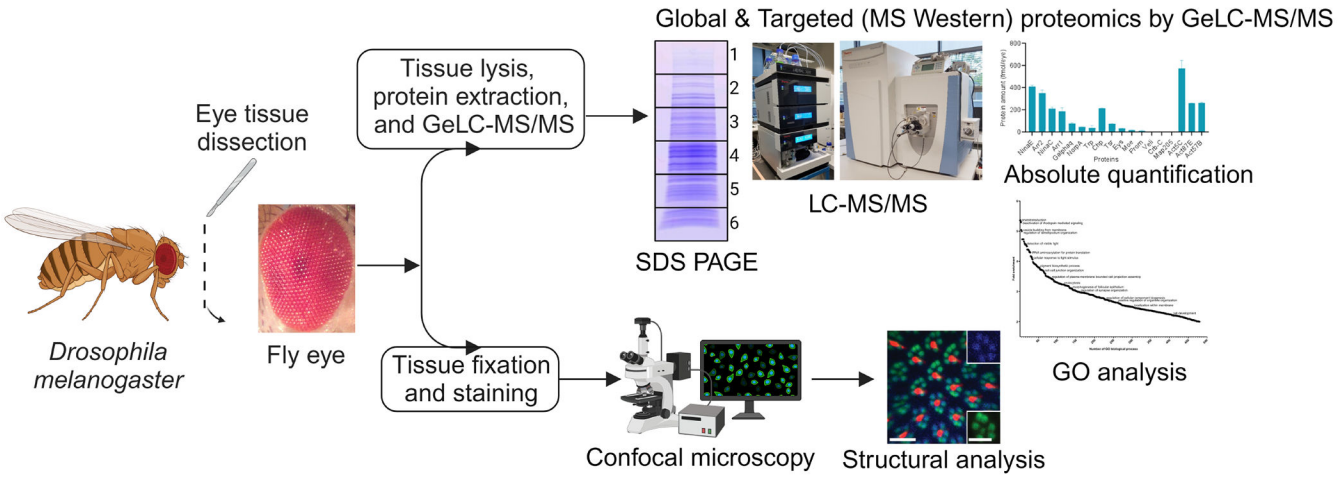

Figure 1: Experimental workflow for cataloging the proteome of the *Drosophila melanogaster* compound eye.

Schematics were created with BioRender.

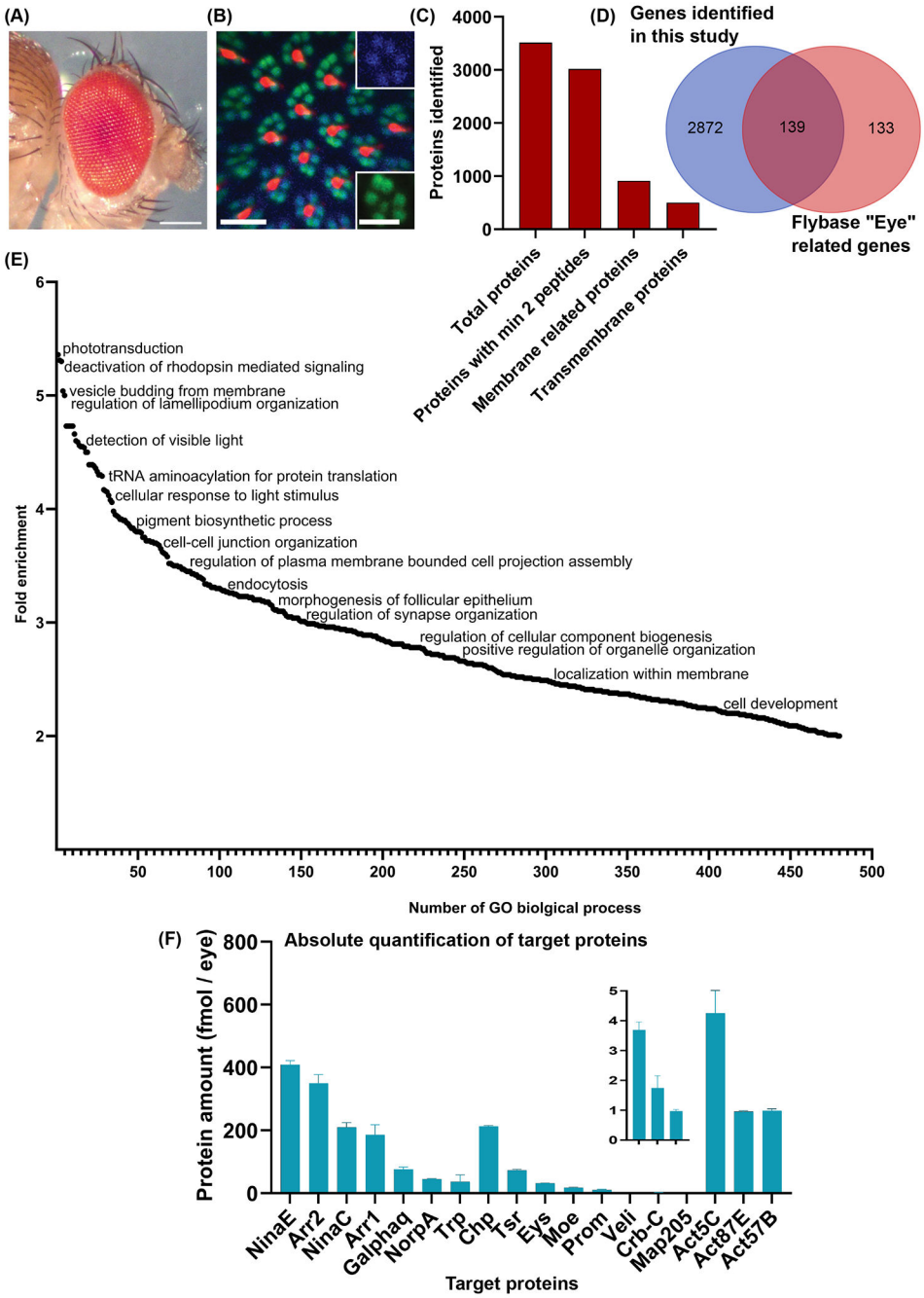

Figure 2: Eye structure and proteome of wild-type *Drosophila melanogaster*.

(A) Side view of the compound eye of a male wild-type fly that was raised on ‘standard’ lab food (SF). Scale bar, 10 μm. **(B)** Adult retina confocal cross-sections of male wild type fly raised on SF. Seven F actin rich (Phalloidin, green) rhabdomeres are visible in each unit eye; Rh1 (blue inset) is expressed in the rhabdomeres of ‘outer’ photoreceptors and Rh6 (red) in the rhabdomeres of ‘inner’ photoreceptors. Scale bars, 10 μm (insets, 5 μm). **(C)** Features of eye proteome composition. (**D**) Protein-coding genes identified in this study (blue) compared with previously annotated ‘eye’-related genes in FlyBase (red;

from www.flybase.org). (**E**) GO enrichment analysis of ocular proteins. (**F**) MS Western quantification of the absolute (molar) abundances of proteins that play a major role in photoreceptor morphology and phototransduction.