A lysosomal tetraspanin associated with retinal degeneration identified via a genome-wide screen

Hong Xu¹, Seung-Jae Lee¹, Emiko Suzuki², Katherine D Dugan³, Alexander Stoddard³, Hong-Sheng Li^{1,4}, Lewis A Chodosh³ and Craig Montell^{1,*}

¹Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, MD, USA, ²Department of Basic Medical Sciences, The University of Tokyo, Tokyo, Japan, ³Department of Cancer Biology and Abramson Family Cancer Research Institute, University of Pennsylvania School of Medicine, Philadelphia, PA, USA

The *Drosophila* visual system has provided a model to study phototransduction and retinal degeneration. To identify new candidate proteins that contribute to these processes, we conducted a genome-wide screen for genes expressed predominately in the eye, using DNA microarrays. This screen appeared to be comprehensive as it led to the identification of all 22 eye-enriched genes previously shown to function in phototransduction or implicated in retinal degeneration. In addition, we identified 93 eye-enriched genes whose roles have not been previously defined. One of the eye-enriched genes encoded a member of a large family of transmembrane proteins, referred to as tetraspanins. We created a null mutation in the eye-enriched tetraspanin, Sunglasses (Sun), which resulted in light-induced retinal degeneration. We found that the Sun protein was distributed primarily in lysosomes, and functioned in a long-known but poorly understood phenomenon of light-induced degradation of rhodopsin. We propose that lysosomal tetraspanins in mammalian cells may also function in the downregulation of rhodopsin and other G-protein-coupled receptors, in response to intense or prolonged agonist stimulation.

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Introduction

The *Drosophila* visual system has emerged as a paradigm for characterizing the molecular mechanisms underlying phototransduction and retinal degeneration (reviewed in Pak, 1995; Montell, 1999). The common features between *Drosophila* and mammalian visual transduction include the employment

of a number of key related molecules, such as rhodopsin, arrestin and heterotrimeric G-proteins. Both systems also display a high gain, rapid activation, sensitivity to light intensities that vary over many orders of magnitude and rapid adaptation (reviewed in Montell, 1999). However, in fly phototransduction, the effector of the G-protein is a phospholipase C rather than a phosphodiesterase (Bloomquist *et al*, 1988), and the cascade culminates with the opening of the light-sensitive cation channels, TRP, TRPL (reviewed in Montell, 1999) and TRP γ (Xu *et al*, 2000). Although the latter events in *Drosophila* visual transduction diverge from mammalian vision, they share important features with many other signaling cascades that utilize phosphoinositide signaling.

Many questions remain concerning *Drosophila* phototransduction, which are also relevant to mammalian phototransduction. These include the mechanisms mediating termination of signaling, adaptation and light-dependent targeting of proteins in photoreceptor cells. The molecular mechanisms underlying retinal degeneration are also incompletely understood, although *Drosophila* has provided important insights into the bases of certain types of retinal dystrophies. An additional phenomenon common between mammals and *Drosophila* is downregulation of rhodopsin in response to intense or continuous light (reviewed in Minke and Selinger, 1992; Penn and Anderson, 1992), although the proteins participating in this effect are unknown. Therefore, there remains considerable potential in using *Drosophila* genetics to identify additional genes that function in a variety of processes in photoreceptor cells.

Although many *Drosophila* loci have been characterized that function in phototransduction, or are associated with retinal degeneration, the forward genetic screens have fallen short of saturation. Therefore, in a few cases, mutations have been created in candidate retinal genes through a reverse genetics approach, which involved screening for protein nulls on Western blots (Dolph *et al*, 1993). During the last few years, there have been advances that have improved the ease of performing reverse genetics. These include the establishment of large collections of transposable element insertion lines, which provide the potential for generating mutations in flanking genes. Methods for homologous recombination have been developed (Rong and Golic, 2000) and an approach for effectively using RNAi in *Drosophila* photoreceptor cells has been described (Kalidas and Smith, 2002). Therefore, it is now feasible to perform reverse genetics more rapidly and on a larger scale than formerly possible.

A key problem with conducting large-scale reverse genetics is the establishment of reliable criteria for identifying genes likely to be associated with phototransduction or retinal degeneration without a bias toward genes already implicated to function in these processes. In an effort to do so, we have taken advantage of the observation that nearly all of the known mutations in the fruitfly that impair the visual response or cause age-dependent retinal degeneration (15 out

*Corresponding author. Department of Biological Chemistry, The Johns Hopkins University School of Medicine, 725 N Wolfe St., 408 WBSB, Baltimore, MD 21205, USA.

Tel.: +1 410 955 1199; Fax: +1 410 614 9573;

E-mail: cmontell@jhmi.edu

⁴Present address: Department of Neurobiology, Medical School, University of Massachusetts, Worcester, MA 01655, USA

of 16) disrupt genes whose expression is highly enriched in the eye (Hyde *et al*, 1990; Montell, 1999).

In the current work, we conducted a comprehensive microarray analysis to identify all of the eye-enriched genes among the 13 601 genes annotated by the *Drosophila* Genome Projects (Adams *et al*, 2000). This screen appeared to be effective as all 22 eye-enriched genes, known or strongly implicated to function in phototransduction or that are required to prevent retinal degeneration, emerged in this analysis. Furthermore, the screen led to the identification of 93 eye-enriched genes, which were not known or predicted to participate in retinal function. Among these genes was a locus encoding an eye-enriched tetraspanin, which we refer to as *sunglasses* (*sun*).

Tetraspanins consist of four transmembrane segments and comprise a large family in flies and humans, although the functions of most tetraspanins are unknown (Maecker *et al*, 1997; Todres *et al*, 2000). Among the 37 tetraspanins encoded in the *Drosophila* genome, a function has been unambiguously ascribed to only one, *late-bloomer*, which participates in synapse formation (Kopczynski *et al*, 1996). We found that a null mutation in *sun* caused light-dependent retinal degeneration. In contrast to all other proteins known to be associated with retinal degeneration, *Sun* was enriched in the lysosomes, suggesting that it functions in protein degradation. One protein, which could potentially undergo *Sun*-regulated degradation, is rhodopsin as exposure to intense or continuous light results in a decrease in rhodopsin concentration; however, the proteins mediating this effect have not been described. Here, we report that *Sun* is required for efficient light-induced downregulation of rhodopsin. Given that there are mammalian lysosomal associated tetraspanins (Fukuda, 1991), we propose that light-dependent downregulation of mammalian rhodopsin (Penn and Anderson, 1992) may be dependent on a tetraspanin related to *Sun*.

Results

Comprehensive screen for eye-enriched genes

To identify new candidate loci required for the physiology of photoreceptor cells, we conducted a comprehensive, genome-wide screen for eye-enriched genes in *Drosophila* using DNA microarrays. To perform this analysis, we isolated RNA from the heads of wild-type (wt) flies, from the heads of an eyeless mutant (*sine oculis*; *so*) and from wt bodies. Biotinylated cRNAs were probed with microarrays corresponding to the complete set of 13 601 genes cataloged by the *Drosophila* Genome Project.

A total of 128 genes were identified in the microarray analysis (wt head/*so* head ratios ≥ 2.0), 93 of which were not previously known to be eye enriched. In all, 78 of these genes had not been characterized previously either at the molecular or genetic level (Table 1; Supplementary Table 1), and 14 corresponded to known genes that had not been analyzed in the context of retinal degeneration or phototransduction (Table 1, indicated by a). It was likely that most or all of the eye-enriched genes annotated in the *Drosophila* genome were identified since all 22 genes that were previously known to be eye enriched and which have been implicated in phototransduction/retinal degeneration were detected in this analysis (Table 2). Only one gene previously associated with retinal degeneration or phototransduction, *rdgB*, dis-

played a minor level of eye enrichment (wt head/*so* head ratio = 1.3), consistent with prior studies (Vihtelic *et al*, 1991). In addition, 13 genes corresponded to loci known to function in processes in the eye other than phototransduction (Supplementary Table 2). Comparison of the chromosomal map positions of the 128 eye-enriched genes did not reveal significant clustering in the genome. The greatest concentration of eye-enriched genes consisted of 11, which spanned 92B2-94F14 on the third chromosome.

To provide additional evidence to support the GeneChip data, we performed Northern blots and *in situ* hybridizations to adult heads using probes corresponding to selected genes. We found that there was strong agreement between the Northern blot, *in situ* hybridizations and microarray data (Figures 1A and B; Supplementary Table 3). Thus, the current microarray analysis appears to have identified all of the eye-enriched genes annotated by the genome project. This was in contrast to a microarray analysis in which a subset of *Drosophila* genes (29.6%) was surveyed for targets of the transcription factor Eyes-Absent (EYA) (Arbeitman *et al*, 2002). Among those known eye-enriched genes, which were included on their GeneChips, over half were missed in their analysis (for more details, see the legend to Supplementary Table 2). Although this study purports to identify eye-enriched genes, EYA is also abundantly expressed in the ovary and affects female fertility (Bonini *et al*, 1998). As RNA was prepared from whole flies, rather than isolated heads, many of the identified genes (Arbeitman *et al*, 2002) were likely to be regulated by EYA in the ovary rather than in the eye. In fact, four of the EYA targets are body-enriched and only five appear on the list of novel eye-enriched genes identified in the current analysis (see the legend to Supplementary Table 2).

Classifications of eye-enriched genes

The 93 eye-enriched genes identified here encode multiple classes of proteins whose roles in visual physiology were either poorly understood or completely unknown (Table 1). Examples include proteins, which may have roles in detoxification, such as a glutathione transferase-related protein and a methionine sulfoxide reductase. In addition, there were three short-chain dehydrogenase-like proteins, which might function either in detoxification or synthesis of the retinal. At least nine genes encode proteins that appear to function in post-translational modifications ranging from phosphorylation to ubiquitination, removal of N-terminal methionines, glycosylation and lipid modification. Three eye-enriched genes were related to proteins that regulate proteolysis. These included two protease inhibitor-like proteins and one protein related to serine-type proteases. Five novel eye-enriched proteins may function in lipid metabolism, several of which may regulate the production of lipid second messengers. This latter group includes a phosphatidic acid phosphatase, a CDP-DAG-inositol 3-phosphatidyltransferase and a protein related to a phospholipase A2 inhibitor. In addition, there was a protein related to the LDL receptor. One of the most highly eye-enriched genes encoded a protein related to junctophilin, a protein that has been implicated in Ca^{2+} signaling (Takeshima *et al*, 2000). Other examples of novel eye-enriched genes include two uncharacterized Rab-related proteins, which may function in protein or vesicle transport. There were at least seven genes, which were not formerly

Table 1 Classes of genes identified by the microarray analysis that were not previously known to be eye enriched

Gene	Map	Description	Ratio	REL
<i>Redox control/detoxification</i>				
CG6781	66D4	Glutathione transferase	12.1 ± 2.8	209.6 ± 63.3
Eip71CD ^a	71C4	Protein methionine reductase	3.7 ± 0.1	916.2 ± 33.4
CG11765	47A6	Glutathione peroxidase	2.6 ± 0.3	1553.9 ± 356.2
<i>Intermediary metabolism</i>				
CG7077	94A14	Short-chain dehydrogenase	42.8 ± 4.9	873.1 ± 309.4
CG13356	20B1	Short-chain dehydrogenase	23.5 ± 4.9	473.4 ± 86.4
CG9150	26B3	Short-chain dehydrogenase	6.9 ± 1.3	121.5 ± 39.8
CG6870	36C7	Heme-binding protein	9.5 ± 0.8	2650.9 ± 925.6
RPE65 ^a	87F10	B-carotene 15,15'-dioxygenase	2.6 ± 0.3	154.4 ± 11.6
CG7059	94B1	Phosphoglycerate mutase	2.0 ± 0.2	83.0 ± 21.5
<i>Post translational modifications</i>				
Kinase/phosphatase				
CG8889	48E2	Ser/Thr phosphatase	10.7 ± 1.4	192.3 ± 162.8
CG2049 ^b	45C1	Ser/Thr kinase	5.0 ± 1.3	84.9 ± 14.0
Fps85D ^a	85D11	Protein tyrosine kinase	2.3 ± 0.4	102.0 ± 10.4
Ubiquitination				
CG1839	12E1	F-box containing protein	5.6 ± 0.4	149.1 ± 37.1
CG5798	93C1	Ubiquitin carboxyl hydrolase	4.3 ± 0.6	285.5 ± 43.1
CG7220	47B7	Ubiquitin conjugating enzyme	3.1 ± 0.3	1399.7 ± 113.6
Other modifications				
CG9171	26A2	Acetylglucosaminyltransferase	5.3 ± 0.7	72.6 ± 5.8
Und ^a	30D1	Methionyl aminopeptidase	3.2 ± 0.9	245.9 ± 18.8
CG5027	72D10	Disulfide isomerase	2.0 ± 0.2	146.3 ± 11.2
<i>Lipid metabolism</i>				
CG12120	8D4	Weakly related to acyl-trans.	47.3 ± 5.6	783.8 ± 26.3
CG17352	66C8	LDL receptor	38.0 ± 17.3	926.6 ± 318.3
CG11440	79E4	PA-phosphatase	15.7 ± 3.8	241.3 ± 47.5
CG15151	36D3	Insulin like PLA2 inhibitor	3.3 ± 0.8	75.5 ± 11.0
CG9245	13E14	CDP-DAG inositol-3.P. trans.	2.4 ± 0.1	1001.9 ± 49.9
<i>Protease and protease inhibitors</i>				
CG12955	51E9	Serine protease inhibitor	5.3 ± 0.8	92.0 ± 3.5
CG17739	48F8	Trypsin inhibitor	3.2 ± 0.2	775.9 ± 45.1
CG4948	66F4	Serine-type endopeptidase	2.3 ± 0.1	662.1 ± 62.4
<i>Synaptic function</i>				
Slob ^{a,b}	28B1	Slowpoke-binding protein	8.8 ± 0.8	776.8 ± 129.0
CG6656	93F9	Histidine acid phosphatase	7.8 ± 0.5	152.8 ± 9.8
CG14723	86F9	Histamine-gated Cl ⁻ channel	4.4 ± 1.5	86.2 ± 9.2
CG9935	102D	Glutamate receptor	5.1 ± 0.5	70.6 ± 2.1
Hk ^a	9B7	Voltage-gated K ⁺ channel	2.3 ± 0.4	313.5 ± 46.0
Eaat2 ^a	21D1	Na ⁺ /glutamate cotransporter	2.2 ± 0.1	649.6 ± 121.8
CG7411	92B1	Histamine-gated Cl ⁻ channel	2.0 ± 0.6	68.7 ± 20.7
<i>Vesicle transport</i>				
RabRP1 ^{a,b}	45B3	Small GTPase	4.3 ± 0.1	1119.7 ± 39.4
Rab14 ^a	35A1	Small GTPase	2.1 ± 0.1	805.7 ± 140.5
<i>Signaling</i>				
CG10233	83A1	Related to junctophilin	146.7 ± 12.0	2506.2 ± 221.0
Chp53E ^a	53E4	Ca ²⁺ -binding protein	2.8 ± 1.2	345.2 ± 79.6
CG7823	76D5	Rho GDI	2.1 ± 0.4	470.3 ± 139.4
<i>Apoptosis</i>				
CG10257	51D7	Anti-FAS apoptosis inhibitor	3.7 ± 1.4	61.4 ± 8.2
<i>Transporters</i>				
CG15891	5E1	Related H ⁺ /cation antiporter	9.0 ± 2.5	201.3 ± 51.5
CG3790	49D3	Organic cation transporter	8.0 ± 1.3	152.8 ± 25.7
<i>Structural/morphological maintenance</i>				
CG18066	57A8	Insect cuticle protein	25.1 ± 0.5	419.8 ± 51.4
CG12143	42E1	Tetraspanin	13.2 ± 2.4	1143.7 ± 108.4
CG4784 ^b	72F1	Cuticle protein	8.0 ± 1.8	401.4 ± 35.6
GH11322	24E4	C-type immunoglobulin	2.8 ± 0.1	646.4 ± 63.3
CG8511	49A3	Cuticle protein	4.5 ± 0.6	64.2 ± 19.5
CG7106	28D2	C-type lectin	2.1 ± 0.3	73.6 ± 10.9
<i>Transcription factors/nucleic acid-binding proteins</i>				
CG2016	82E7	Takeout like protein	25.7 ± 1.6	432.0 ± 68.1
CG11820	96B16	Transcription factor	11.2 ± 1.8	176.4 ± 33.2
Msta ^a	2E2	BOP zinc finger protein	10.6 ± 1.5	896.6 ± 27.4
Pph13	21C6	Pol. II transcription factor	9.8 ± 1.5	148.0 ± 11.7
CG10446	37B10	Transcription factor	7.3 ± 3.0	122.5 ± 21.4
CG2650	3B4	Circadian clock gene	7.8 ± 2.3	131.2 ± 59.7
SP2523	66A8	Related to RNA helicase	4.6 ± 0.6	541.5 ± 151.5
mre11 ^a	32E2	DNA repair protein	3.7 ± 0.5	130.4 ± 17.1
Rbp9 ^a	23C1	RNA-binding protein	3.3 ± 0.6	365.0 ± 20.2
CG6866	34B9	RNA-binding protein	2.3 ± 0.3	156.6 ± 22.2

Table 1 (Continued)

<i>Others with predictable functions</i>				
CG13409	94A1	Biotin synthetase		
CG8483	87E2	Related to venom allergen5	3.4 ± 0.1	195.8 ± 22.8
CHIP ^a	31E2	Tetrapeptide repeat	2.1 ± 0.2	206.1 ± 38.6
CG12020	62B4	Heat-shock protein	2.0 ± 0.2	48.7 ± 10.7
<i>No recognizable domains (homologs in a diversity of organisms)</i>				
CG13057	72E4	D, M	491.5 ± 67.5	8834.3 ± 2357.0
CG9427	85E1	C, D, M	9.9 ± 2.9	300.1 ± 53.4
CG6983	66D1	D, M, H	7.3 ± 1.4	107.0 ± 21.5
CG4686	92B6	A, C, D, M, H	2.9 ± 0.3	734.9 ± 85.3
CG14464	41A1	H	2.3 ± 0.4	243.9 ± 94.5
CG4306	75C6	M, H	2.2 ± 0.2	53.4 ± 8.2
<i>No recognizable domains (invertebrate homologs only)</i>				
CG1561	10C5	C, D	93.1 ± 30.6	1596.7 ± 63.0
CG7135	16F7	C, D	3.6 ± 1.0	100.9 ± 13.1

Minimum wt head/so head ratio is 2.0. The relative expression levels (RELs) were obtained by subtracting the expression level in *so* heads from that in wt (Canton S) heads. The standard errors (SEs) are shown ($n = 3$).

^aGenes that have been previously described, but not previously reported to be eye enriched or known to function in visual transduction.

^bGenes that are regulated during the circadian cycle (Claridge-Chang *et al*, 2001; McDonald and Rosbash, 2001).

A: *A. thaliana*; C: *C. elegans*; D: *D. melanogaster*; H: *H. sapiens*; M: *M. musculus*.

No recognizable domains and no homologs—24 genes (Supplementary Table 1).

Table 2 Ratios of genes known or implicated in retinal degeneration or phototransduction

Gene	Affymetrix ratio		REL
	wt head/so head	wt head/wt body	
<i>trp</i>	172.2 ± 31.3	232.0 ± 67.6	2941.4 ± 463.8
<i>Gβe</i>	137.6 ± 9.9	182.5 ± 33.6	2346.0 ± 198.9
<i>Rh4</i>	135.2 ± 18.5	140.0 ± 10.5	2261.9 ± 86.3
<i>trpl</i>	116.3 ± 20.2	209.7 ± 39.5	2996.0 ± 280.4
<i>ninaC</i> (myosin III)	86.5 ± 2.9	131.6 ± 32.7	1712.5 ± 87.0
<i>arrestin1</i>	84.9 ± 28.7	112.5 ± 9.6	11269.8 ± 2103.9
<i>inaD</i> (PDZ protein)	80.6 ± 12.4	104.4 ± 26.5	1335.5 ± 31.2
<i>arrestin2</i>	68.7 ± 22.8	130.0 ± 24.3	7823.6 ± 1568.7
<i>ninaE</i> (Rh1)	67.2 ± 31.3	42.3 ± 3.17	14592.7 ± 3191.9
<i>Rh3</i>	54.7 ± 11.7	53.5 ± 11.0	3125.1 ± 389.0
<i>Rh2</i>	52.3 ± 4.4	65.7 ± 10.7	880.6 ± 59.3
<i>Rh5</i>	49.7 ± 11.5	23.6 ± 2.3	1935.4 ± 36.7
<i>inaF</i>	46.0 ± 1.8	57.6 ± 8.8	819.0 ± 106.8
<i>Gqα</i>	27.5 ± 5.7	41.4 ± 9.6	577.6 ± 47.9
<i>ninaA</i> (Rh chaperone)	24.9 ± 8.4	26.9 ± 3.2	754.4 ± 126.0
<i>inaC</i> (PKC)	23.6 ± 3.9	33.0 ± 11.1	393.3 ± 32.8
<i>Rh6</i>	23.2 ± 7.3	78.6 ± 33.5	2120.2 ± 152.8
<i>norpA</i> (PLC)	12.1 ± 2.3	36.4 ± 10.0	426.0 ± 46.7
<i>Gγ30A</i>	12.0 ± 2.7	48.3 ± 15.1	2041.8 ± 941.2
<i>rdgC</i> (Rh phosphatase)	6.2 ± 1.7	14.1 ± 6.4	134.5 ± 27.9
<i>rdgA</i> (DAG kinase)	2.8 ± 0.5	3.9 ± 0.5	107.7 ± 46.2
<i>cds</i>	2.6 ± 0.2	7.5 ± 0.9	250.0 ± 42.5

The expression ratios and RELs are presented along with the SEs.

known to be eye enriched, that encoded proteins likely to have roles in synaptic function. In addition, there were two eye-enriched genes encoding cation transporters.

Retinal degeneration in *sun*¹ flies

Included among the eye-enriched genes was a member of a large family of tetraspanins (Todres *et al*, 2000). Given that the functions of many such proteins are poorly characterized, we set out to determine the phenotype of disrupting the eye-enriched tetraspanin, which we refer to as *sunglasses* (*sun*). As with other tetraspanins, Sun is characterized by four predicted transmembrane segments, a large extracellular domain between the third and fourth transmembrane segments and key residues conserved among most tetraspanins (Figure 2A) (Maecker *et al*, 1997). The tetraspanin most

similar to Sun was a mammalian lysosomal protein, CD63/LAMP3/LIMP1 (Fukuda, 1991) (Figure 2A). Consistent with the microarray data, we found, on the basis of Northern blots (Figure 1B; Supplementary Table 3; CG12143) and *in situ* hybridizations (Figure 1A; CG12143), that the *sun* RNA was concentrated in the retina.

To obtain a mutation in *sun*, we mobilized a P-element, which inserted near the 5' end of *sun* (Figure 2B), since the original element did not exhibit any visual physiology defect (data not shown). To identify a deletion of *sun*, we screened pools of genomic DNA by PCR (see Materials and methods and Figure 2B). One excision line (*sun*¹) contained a 1.8 kb deletion, which removed nearly half of the region encoding Sun (Figure 2B). We raised anti-Sun antibodies and found that expression of the Sun protein was eliminated in *sun*¹ and

Figure 1 Expression patterns of *sun* and novel eye-enriched genes. (A) Spatial distributions of selected eye-enriched mRNAs in adult heads. *In situ* hybridizations to frontal sections were performed with antisense RNAs, except for the *trp* sense control (sense). The Flybase ID numbers are indicated. B, brain; L, lamina; M, medulla; R, retina. (B) Expression of *sun* and selected eye-enriched mRNAs by Northern blot analysis. Lanes contained mRNAs prepared from wt fly heads (WH), *so* heads (SH) and wt bodies (WB). CG15406 was predicted to be body enriched, based on the GeneChip data (Supplementary Table 3). *The order of the SH and WB samples probed with the CG15406 DNA was reversed relative to the other samples. The filters were re-probed with *rp49*, demonstrating that the RNA loading in each lane was similar (data not shown). (C) The Sun protein was enriched in photoreceptor cells. Anti-Sun antibodies were used to probe a Western blot containing Triton X-100 soluble proteins extracted from the indicated 1-day-old flies (except for 7-day-old *rdgA*). The lower 15 kDa protein of unknown identity cross-reacted with the anti-Sun antibodies. This band provided an internal loading control.

restored in mutant flies containing the *sun* genomic DNA transgene, P[g-*sun*] (Figure 1C).

We examined whether *sun*⁻ flies exhibited retinal degeneration using an assay on intact fly heads, the optical neutralization technique (Figure 2C) (Franceschini and Kirschfeld, 1971). The fly compound eye consists of ~800 ommatidial units, each of which contains eight photoreceptor cells, although only seven are present in any given plane of section. The photoreceptor cells include a microvillar structure, the rhabdomere, which is the site of phototransduction.

Flies were either maintained under a normal light/dark cycle or kept in the dark, and the average number of rhabdomeres per ommatidium was assessed. In wt ommatidia, seven rhabdomeres were detected regardless of the age of the flies or light conditions (Figures 2C and D).

We found that *sun*⁻ flies exhibited light- and age-dependent retinal degeneration. In mutant flies that were incubated in the dark, there was relatively little reduction in rhabdomere number as the flies aged (Figure 2D). However, *sun*⁻ flies maintained under a light/dark cycle exhibited a gradual loss of rhabdomeres (Figure 2D). To confirm these data, we used transmission EM. While sections of 4-week-old wt ommatidia displayed seven rhabdomeres, few (0–4 rhabdomeres/ommatidia) were detected in *sun*⁻ exposed to a light/dark cycle (Figure 2E). In contrast to most known retinal degeneration mutants in *Drosophila*, which display the initial defects in the rhabdomeres, the degeneration in *sun* photoreceptor cells was first evident in the cell bodies (data not shown). The full complement of rhabdomeres was restored in *sun*⁻ flies containing the *sun* genomic or cDNA transgenes (Figure 2E). The retinal degeneration was significantly suppressed either in a genetic background (*ninaE*^{P332}), which greatly reduced the concentration of the major rhodopsin, or upon introduction of a transgene that expressed Baculovirus P35, a broad-spectrum caspase inhibitor (Figure 2D).

Sun is a lysosomal-enriched protein

To address the function of Sun, we examined the expression pattern of the protein, initially using a biochemical approach. The Sun protein appeared to be expressed predominantly in photoreceptor cells, as it was eliminated in a mutant, *glass*, which does not form photoreceptor cells (Figure 1C). Sun was present in *rdgA* flies, which were devoid of rhabdomeres due to degeneration (Figure 1C), indicating that a significant proportion of the Sun pool was present in the cell bodies. However, Sun did not appear to be specific to the cell bodies as there was a small but reproducible decrease in Sun levels in *rdgA*. The anti-Sun antibodies did not recognize Sun in sections of compound eyes. Therefore, to examine the subcellular distribution of Sun, we generated transgenic flies expressing a Myc-tagged version of Sun (P[w⁺;c-*sun*]). The Myc-Sun protein was functional *in vivo* as it largely rescued the Sun mutant phenotype (Figure 2E). Consistent with the Western blot data, the anti-Myc staining was detected primarily in a punctate pattern in the cell bodies (Figures 3A–C). A small amount of labeling was detected near the base of some rhabdomeres (Figure 3C). Only a low level of background staining resulted from using anti-Myc antibodies on sections of nontransgenic flies and this staining was primarily associated with the extracellular central matrix (Figures 3D–F).

Immunolocalization at the EM level confirmed that the anti-Myc staining was within vesicles in the cell bodies. No immunoreactivity was detected in the nucleus or mitochondria (Figures 3M and N). Given that the tetraspanin most related to Sun, CD63, is a lysosomal protein, we considered whether Sun is also present in lysosomes. However, it is not possible to unequivocally identify lysosomes in these preparations due to an absence of multilamellar structures after the fixation and staining protocol. Nevertheless, the Myc-Sun positive vesicles appeared as dense bodies, reminiscent of structures previously described as lysosome-like and

Figure 2 *sun* gene and *sun*¹ retinal degeneration phenotype. (A) Alignment of Sun with other tetraspanins. The putative transmembrane segments are underlined. *Residues that are $\geq 80\%$ identical among all tetraspanins. Invariant residues are boxed. (B) *sun* gene. The two open triangles at the left and right ends indicate the locations of the primers used to screen for deletions generated by imprecise excision of the P-element, EP(2)2425. The 1.8 kb deletion in *sun*¹ is indicated. (C) Rhabdomeres from 4-week-old wt and *sun*¹ flies observed using the optical neutralization technique (Franceschini and Kirschfeld, 1971). The flies were reared at 25°C under a 12 h light/12 h dark cycle. (D) Mean number of rhabdomeres/ommatidium as a function of age. The flies were reared at 25°C under a 12 h light/12 h dark cycle, except for the dark-reared *sun*¹ flies. The number of rhabdomeres was determined by the optical neutralization technique. Each data point was based on examination of ≥ 90 ommatidia from ≥ 8 flies. Error bars represent the SDs. (E) Retinal morphology of 4-week-old flies examined by transmission EM. Tangential sections were obtained from the distal region of the compound eye. Sections from wt, *sun*¹, the genomic transgene in a *sun*¹ background (*sun*¹;P[g-*sun*]) and the *myc-sun* cDNA transgene in a *sun*¹ background (*sun*¹;P[c-*sun*]) are shown.

Figure 3 Immunolocalization of Sun. (A–F) Fly heads were embedded in LR White and 0.5 μ m cross-sections of compound eyes were stained with rabbit anti-Myc and a rhabdomeral marker, rat anti-INAD. (A) Section from *myc-sun* transgenic fly, P[w⁺;c-*sun*];*cn bw*, stained with rabbit anti-Myc polyclonal antibodies. (B) Same section as in (A) stained with rat anti-INAD antibodies. (C) Merged images from (A, B). (D) Section from a white-eyed (*cn bw*) nontransgenic fly stained with anti-Myc antibodies. (E) Same section as in (D) stained with anti-INAD. (F) Merged images from (D, E). (G–I) 293 cells transfected with a plasmid encoding a YFP-Sun fusion protein. (G) Localization of YFP. (H) Same cells as in (J) stained with LysoTracker. (I) Merged images from (G, H). (J–L) 293 cells transfected with a plasmid encoding YFP. (J) Localization of YFP. (K) Same cells as (G) stained with LysoTracker. (L) Merged images from (J, K). (M, N) Immuno-EM localization of Myc-Sun and Rh1 in the cell bodies of P[c-*sun*];*cn bw* photoreceptor cells. Sections were stained with anti-Myc rabbit polyclonal antibodies (15 nm gold particles; several are indicated by arrows) and anti-Rh1 mouse monoclonal antibodies (5 nm gold particles, several are indicated by arrowheads). The dashed and solid boxes show lower and higher magnification views of Myc-Sun and Rh1 positive lysosomal-like vesicles (M) and MVB-like vesicles (N). The small (inset) and large-scale bars represent 0.1 and 1.0 μ m, respectively. Mt, mitochondrion; Nu, nucleus; Rha, rhabdomere.

multivesicular body (MVB)-like (Suzuki and Hirose, 1991). To address whether Sun was localized in lysosomes, we expressed a YFP-tagged version of Sun in tissue culture cells. In contrast to the wide distribution of YFP alone (Figures 3J–L), YFP-Sun displayed a punctate pattern that colocalized with a lysosomal/late endosomal marker,

LysoTracker (Figures 3G–I). There was also significant colocalization of Sun with the lysosomal protein LAMP1 (Supplementary Figures 1A–C). A small proportion of the YFP-Sun pool colocalized with an endosomal marker, RhoB (Supplementary Figures 1D–F). Additional evidence that Sun is primarily a lysosomal protein is presented below.

Defects in light-dependent turnover of rhodopsin in *sun*¹

The spatial distribution of Sun suggested that it might be involved in the turnover of a photoreceptor cell protein. Therefore, we compared the concentrations of a variety of proteins in wt and *sun*¹ heads. Young flies were used in these experiments, which did not exhibit any significant age- and light-dependent degeneration due to the *sun*¹ mutation. The flies were either maintained in ambient light or exposed for 6 h to bright blue light, which stably converts rhodopsin to the light-activated metarhodopsin. We found that the levels of the photoreceptor cell enriched proteins, TRP, INAD and the rhodopsin binding protein Arrestin2, were similar in wt and *sun*¹ flies, regardless of the light conditions (Figure 4A).

The major rhodopsin (Rh1) was an excellent candidate for degradation through a Sun-dependent pathway since it is the only photoreceptor cell protein reported to undergo down-regulation in response to continuous bright light (reviewed in Minke and Selinger, 1992). In wt photoreceptor cells challenged for 6 h with blue light, the level of rhodopsin was greatly reduced (Figures 4A and B; $31 \pm 5\%$ of wt levels). This reduction in Rh1 levels was similar in the *arrestin2* mutant (data not shown), which was unexpected given that down-regulation of G-protein-coupled receptors (GPCRs) typically occurs through an arrestin-dependent mechanism (Claing *et al*, 2002). This suggested that there was a mechanism for bright light-dependent downregulation of Rh1, in addition to

clathrin-mediated endocytosis of Rh1/arrestin complexes (Alloway *et al*, 2000; Kiselev *et al*, 2000). Of primary significance here, the reduction in Rh1 concentration resulting from exposure to continuous bright blue light was suppressed to a significant extent in *sun*¹ (Figures 4A and B; $82 \pm 18\%$). The combination of these data suggests that Sun participates in the turnover of Rh1 through promoting its degradation in lysosomes.

We found that most of the Rh1 pool in the cell bodies colocalized with the Sun-containing vesicles in photoreceptor cells (Figures 3M and N). Evidence that the Rh1-containing vesicles in photoreceptor cells are lysosomes is presented below. Rh1 appeared to associate with Sun *in vivo* as Rh1 was detected in immune complexes after performing immunoprecipitations (IPs) with anti-Sun antibodies (Figure 4C). Another abundant membrane protein, TRP, did not co-IP with Sun (data not shown). We did not detect Rh1 after performing IPs with anti-Sun from *sun*¹ extracts or after using preimmune serum and wt extracts (Figure 4C).

Light-dependent lysosomal localization of Rh1 in isolated ommatidia

To characterize the mechanism through which Sun contributes to light-dependent degradation of Rh1 in greater detail, we attempted to recapitulate the phenomenon in isolated ommatidia. To facilitate this analysis, we examined the relative concentrations and localization of GFP-Rh1 in

Figure 4 Defect in blue light-induced lysosomal degradation of Rh1 in *sun*¹. (A) Rh1 levels in wt and *sun*¹ flies. Three-day-old flies were either kept under ambient light (A) or stimulated for 6 h with blue light (L). Head extracts were prepared and a Western blot was probed with anti-Rh1 antibodies and ¹²⁵I-labeled-anti-mouse IgG. Parallel Western blots, using the same samples, were probed with anti-TRP, anti-INAD and anti-Arr2 antibodies. (B) Quantitation of relative Rh1 levels from (A). Band intensities were determined using a phosphorimager. (C) Rh1 co-immunoprecipitated (co-IPed) with Sun *in vivo*. Head extracts were prepared from wt or *sun*¹. IPs were performed with anti-Sun antibodies or preimmune serum and a Western blot was probed with anti-Rh1 antibodies. A 1% input in lanes 1–3 corresponds to lanes 4–6, respectively. Lanes: 4, IP with anti-Sun and *sun*¹ extracts; 5, IP with preimmune serum and wt extracts; 6, IP with anti-Sun and wt extracts. In some but not all experiments with nonimmune serum, an ~35 kDa band was detected. This band was not detected with anti-Sun antibodies. (D) A lysosomal inhibitor suppressed blue light-induced degradation of Rh1. Isolated wt or *sun*¹ ommatidia were maintained in the dark (D) or exposed to blue light for 4 h (L). Prior to the light treatment, some of the ommatidia were transferred to media containing either a lysosomal inhibitor (LI; 10 μ g/ μ l leupeptin) or a proteasomal inhibitor (PI; 10 μ M lactacystin). The Western blot was probed with anti-Rh1 antibodies and ¹²⁵I-labeled-anti-mouse IgG. Parallel Western blots containing the same samples were probed with antibodies that recognize the retinal-enriched proteins INAD and TRP. (E) Quantitation of the relative Rh1 levels from (D). The relative intensities of the bands were determined using a phosphorimager.

young wt and *sun*¹ photoreceptor cells, before and after exposure of the isolated ommatidia to bright blue light. After exposing isolated ommatidia to blue light for 4 h, the concentration of rhodopsin was decreased (Figures 4D and E).

To examine the effects of light on the distribution of GFP-Rh1, we examined GFP staining in ommatidia after a 1.5 h exposure to blue light, prior to when there is a dramatic decrease in the overall concentration of GFP-Rh1. In ommatidia incubated in the dark, the GFP-Rh1 was present almost exclusively in the rhabdomeres (Figure 5A). After the blue light exposure, GFP-Rh1 was also detected in many puncta in the cytoplasm (Figure 5B). Many of these puncta corresponded to lysosomes as there was significant overlap between GFP-Rh1 and LysoTracker (Figures 5B–E). Since after exposure to bright light GFP-Rh1 is detected in the cell bodies, primarily in lysosomes, and since Sun and Rh1 colocalize in vesicles (Figures 3M and N), these data further support the conclusion that Sun is a lysosomal protein. Additional evidence that lysosomes participated in the bright light-induced degradation of Rh1 was that the light-dependent degradation of Rh1 was significantly suppressed by a lysosomal inhibitor, but unaffected by a proteasomal inhibitor (Figures 4D and E).

To determine whether there was a decrease in the localization of Rh1 in *sun*¹ lysosomes, we examined the distribution of LysoTracker and GFP-Rh1 in ommatidia isolated from young *sun*¹ flies, prior to any apparent retinal degeneration. The *sun*¹ and wt photoreceptor cells contained a similar number of lysosomes, indicating that Sun was not required for lysosomal biogenesis (Supplementary Figure 2). Of significance here, we found that the overlap between LysoTracker and GFP-Rh1 was significantly reduced in *sun*¹ (Figures 5G–J), consistent with the conclusion that Sun facilitates targeting or entry of Rh1 into lysosomes. In *sun*¹ photoreceptor cells, the GFP-Rh1 staining was also detected in an extensive array of large loops emanating from the rhabdomeres (Figures 5G, I and J). Such loops, which were fewer in number and much smaller in wt (Figure 5E), appeared to correspond to invaginations of the rhabdomeral

membrane, which are dynamically produced in wt in a light-dependent manner (Kosloff *et al*, 2003).

Discussion

Comparison of eye-enriched genes in flies and mammals

The prime motivation for performing the microarray study was to identify candidate loci that participate in phototransduction or which would provide new insights into retinal dystrophies. In support of the efficacy of the microarray analysis, all 22 of the known eye-enriched genes, which function or have been implicated to function in retinal degeneration or phototransduction, emerged in this study. Furthermore, there was excellent concordance between the GeneChip, Northern blot and *in situ* hybridization data.

In addition to the known eye-enriched genes, 93 genes were identified that encode multiple classes of proteins whose roles in visual physiology were either poorly understood or completely unknown. Many of these genes fall into the same classes as those reported in an analysis of rod-enriched genes in the mouse (Blackshaw *et al*, 2001). A large class encodes proteins that function in post-translational modifications, which includes related ubiquitin hydrolases and similar glycosyltransferases. Another group consists of eye-enriched genes that encode proteins that may be involved in redox control and/or detoxification. These proteins may be present to reduce the deleterious effects resulting from oxidative stress in photoreceptor cells. An eye-enriched DNA repair protein, Mre11, may also be present to limit chromosomal damage induced by intense illumination. Similar mechanisms for controlling oxidative stress and light-induced damage may exist in flies and mammals as functionally related glutathione peroxidase and DNA repair proteins are enriched in rod cells (Blackshaw *et al*, 2001).

Several genes encoding proteins that function in proteolysis were eye enriched in both *Drosophila* and the mouse. In addition to light-dependent downregulation of rhodopsin, these proteins might control the levels of photoreceptor cell proteins that oscillate during the circadian cycle. Ten

Figure 5 Light-dependent translocation of Rh1 in wt but not *sun*¹ isolated ommatidia. (A–E) Ommatidia from wt expressing a GFP-Rh1 transgene. (F–J) Ommatidia from *sun*¹ expressing a GFP-Rh1 transgene. (A, F) GFP-Rh1 staining (green) in ommatidia maintained in the dark. (B, G) Ommatidia incubated in media containing LysoTracker and exposed to blue light for 1.5 h. The GFP-Rh1 expression is shown (green). (C, H) LysoTracker staining (red) in the same ommatidia shown in (B) and (G), respectively. (D, I) Merge of staining shown in (B, C) and (G, H), respectively. (E, J) Higher magnifications of regions indicated by boxes in (D, I), respectively.

eye-enriched RNAs oscillate during the circadian cycle (Claridge-Chang *et al*, 2001; McDonald and Rosbash, 2001), including the ubiquitin hydrolase, which is similar to the rod-enriched protein (Blackshaw *et al*, 2001). In addition, protein degradation may be essential for the elimination of damaged proteins generated during metabolic stress (reviewed in Penn and Anderson, 1992). Regulated removal of such damaged proteins could provide a mechanism to limit the accumulation of inactive proteins, which might lead to photoreceptor cell death.

Several other classes of formerly unrecognized eye-enriched genes in flies were also represented among the mammalian rod-enriched genes. These included genes encoding proteins involved in synaptic function, the regulation of cell death and lipid metabolism. This latter category was expected to be included among the *Drosophila* eye-enriched genes as fly phototransduction utilizes phosphoinositide signaling. However, the second messenger in mammalian phototransduction is cGMP. A regulatory role for phosphoinositides, which may be common between mammalian and *Drosophila* photoreceptors, may be the light-dependent movement of proteins, as occurs for *Drosophila* visual arrestin (Lee *et al*, 2003). Thus, despite the striking differences in morphology and second-messenger systems, the similarities in classes of proteins between the fly and mammalian visual systems may reflect similar mechanisms for dealing with light-dependent and oxidative damage, rapid signaling, light-dependent protein translocation and circadian rhythm.

Requirement for Sun for downregulation of rhodopsin and to prevent light-dependent retinal degeneration

In the current work, we focused on *sun*, since it was the only eye-enriched member of a particularly large family of transmembrane proteins, many of which are poorly characterized. We found that mutation of *sun* resulted in light- and age-dependent retinal degeneration. To address the normal function of Sun in wt photoreceptor cells, we first examined the spatial distribution of the protein. We found that Sun was expressed in lysosomes, suggesting that it might play a role in the degradation of one or more photoreceptor proteins. In support of this possibility, we found that the light-induced degradation of Rh1 was reduced in a *sun*¹ background. Moreover, Rh1 and Sun appeared to colocalize in lysosomes and formed a complex *in vivo*. Therefore, an attractive model is that Sun is required for light-dependent downregulation of rhodopsin by promoting its degradation in lysosomes.

The model presented here is consistent with findings that other GPCRs are internalized and then targeted either to lysosomes or recycling endosomes in response to strong or prolonged exposure to their agonists (reviewed in Ferguson, 2001). Rh1 may normally be internalized and degraded in lysosomes to downregulate the concentration of the receptor in the presence of intense or constant light, in a manner similar to agonist-induced downregulation of most GPCRs. Upon exposure to bright continuous light, there were large invaginations of the rhabdomeral membrane, which were detected in *sun* but not wt ommatidia. These large invaginations might accumulate in *sun* photoreceptor cells due to a requirement for Sun for efficient transfer of Rh1 from these rhabdomeral invaginations to MVBs and subsequently to lysosomes. In the absence of transfer of Rh1-containing membranes to the MVB/lysosomal pathway, the invagina-

tions may increase in size and accumulate. These data raise the possibility that a defect in the downregulation of activated receptors and accumulation of membrane invaginations may underlie the light-dependent retinal degeneration that occurs in *sun*¹ mutants.

The requirement for Sun is distinct from that of two other tetraspanins that are expressed in the disk rims of mammalian photoreceptor cells, peripherin/rds and rom-1, and appear to be structural proteins (Travis *et al*, 1991; Bascom *et al*, 1992). Additional tetraspanins may be situated in intracellular compartments of a variety of cell types, including mammalian photoreceptor cells, and function in agonist-dependent protein degradation. Given that the tetraspanin that is among the most related to Sun (CD63) is present in late endosomal and lysosomal membranes (Fukuda, 1991), we propose that other tetraspanins may interact with and participate in the downregulation of other GPCRs.

Materials and methods

RNA preparation, hybridization to *Drosophila* genome arrays, scanning and staining

Detailed descriptions of the methods that we used to prepare the RNA samples (P-MEXP-2143) and labeling of the cRNAs (P-MEXP-2178) have been deposited in The European Bioinformatics Institute ArrayExpress database (<http://www.ebi.ac.uk/arrayexpress>). Hybridization, scanning and analysis of Affymetrix *Drosophila* genome arrays were performed according to the manufacturer's instructions. The annotation for the probe set is available at <http://www.affymetrix.com/products/arrays/specific/fly.affx>. Biotinylated oligonucleotide (B2) and *bioB*, *bioC*, *bioD* and *cre* control cRNAs, which were supplied by Affymetrix, were spiked into each sample. The hybridization cocktail consisted of 20 µg of fragmented biotinylated cRNA (final concentration: 0.07 µg/µl), control oligonucleotide B2 (50 pM), control cRNAs *bioB*, *C*, *D* and *cre* (1.5, 5, 25 and 100 pM, respectively), herring sperm DNA (0.1 mg/ml), acetylated BSA (0.5 mg/ml) and hybridization buffer (100 mM MES, 1 M sodium, 20 mM EDTA, 0.01 % Tween 20 in a total volume of 300 µl). The cocktail was heated to 99°C for 5 min before hybridization. The Affymetrix Fluidics Station and Scanner were both controlled by the Affymetrix Microarray Suite version 4 software (MAS4.0) using the default protocols and parameters throughout. The arrays were stained with streptavidin phycoerythrin (SAPE) solution and scanned once. A second stain was then performed with a biotinylated antibody and visualized with SAPE. The image resulting from this second scan was further analyzed with MAS4.0. The samples were then hybridized to the array for 16 h at 45°C in a rotating oven. Three independent RNA samples were analyzed for each sample group.

Analysis of microarray data

A slight variation of the MAS4.0 algorithm was used to calculate expression values for each probe set as described below. The background subtraction was as described for MAS4.0. The expression values reported are essentially the same as the MAS4.0 'average difference' expression metric, except that any probe pairs in a probe set excluded as outliers in a sample (using the MAS4.0 criteria of 3 s.d. from the mean of the probe set) were excluded uniformly from all of the arrays when calculating the fold changes. This uniform masking of outliers is the only way in which the analysis algorithm varies from the standard MAS4.0 software. Each array was scaled (normalized) as in the MAS4.0 algorithm so that the trimmed mean (mean of the middle 96%) expression value for each array was 150. The expression levels for each of three pairs of samples in each group were used to compute the ratio and expression level changes for each probe set. The standard errors for the absolute values and ratios are presented in Tables 1 and 2 and Supplementary Tables 1 and 2. The complete set of microarray data has been deposited in the EBI ArrayExpress database.

Verification of microarray results

Selected eye-enriched genes with different enrichment ratios were used to probe Northern blots. The band intensities were quantitated using a Fuji PhosphorImager (MacBACV2.5 software). *In situ* hybridizations on sections of adult fly heads were performed as described (Schaeren-Wiemers and Gerfin-Moser, 1993) using digoxigenin-labeled RNA probes.

Generation of a sun mutant

The w^+ P-element insertion, EP(2)2425, which was used to generate the deletion in *sun* by imprecise excision of the P-element, inserted 128 base pairs 5' from the transcription start site of CG12143 (*sun*) predicted by the BDGP. EP(2)2425 was mobilized by genetically introducing transposase using the $\Delta 2-3$ line (Robertson *et al*, 1988). In all, 93 individual w^- excision chromosomes were placed over a deficiency that spanned *sun* (Df(2R)Drl[rv25]). Genomic DNAs were combined into 18 pools and screened for deletions that removed *sun* by PCR. Individual DNAs in the positive pools were rescreened by PCR and the deletions were confirmed by DNA sequencing.

Transgenic flies

A 5.0 kb *sun* genomic DNA fragment (nucleotides 37586–42771 in AE003842) was generated by PCR and subcloned into the pCaSpeR4 vector (Thummel and Pirrotta, 1992). This clone, which included 1.5 and 1.0 kb 5' and 3' of the *sun* gene, was sequenced to confirm that there were no mutations in the *sun* coding region. The *sun* cDNA, which was fused to an N-terminal Myc tag, was linked at the 5' end to the *ninaE* (*Rh1*) promoter and the *ninaE* 3' UTR and subcloned into pCaSpeR4. The constructs were injected into w^{1118} embryos and the resulting transgenes were genetically introduced into $w^{1118};sun$ flies.

Production of anti-Sun antibodies

A GST fusion protein was generated (pGEX5X-1 vector, Pharmacia), which included the region corresponding to the loop between transmembrane segments 3 and 4 (residues 99–187) and the C-terminus (residues 206–227) of *Sun*. The fusion protein was fractionated by SDS–PAGE, electroeluted as described (Montell and Rubin, 1988) and injected into rabbits (Covance).

Localization of Sun

Immunofluorescence staining of 0.5 μ m sections of P[$w^+;Rh1::myc::sun$];*cn bw* compound eyes was performed as described (Li and Montell, 2000) using rabbit polyclonal anti-Myc antibodies. To perform the immunostaining at the EM level, compound eyes from 6-day-old $w;P[w^+;Rh1::myc::sun]$ flies were fixed, embedded in LR White, thin-sectioned and immunolabeled as described (Tsunoda *et al*, 2001). The double labeling was performed using mouse anti-Rh1 monoclonal antibodies and rabbit anti-Myc primary antibodies and 15 nm colloidal gold-conjugated anti-rabbit IgG and 5 nm colloidal gold-conjugated anti-mouse IgG secondary antibodies. The immunolabeled sections were stained with 1% aqueous uranyl acetate for 30 s and examined using a JEM 1200EX electron microscope. To determine the localization of *Sun* in tissue culture cells, HEK cells were transfected with pcDNA3-*yfp* or pcDNA3-*yfp::sun*. In some experiments the cells were subsequently loaded with LysoTracker Red DND-99 (Molecular Probes). Confocal images were acquired using an UltraView LCI System (Perkin-Elmer).

Analyses of retinal degeneration

The flies used for morphological analyses were reared at 25°C under a 12h light/12h dark cycle. To assay the time course of photoreceptor cell degeneration, the mean number of rhabdomeres per ommatidium was determined using the optical neutralization technique (Franceschini and Kirschfeld, 1971). Each data point was based on examination of ≥ 90 ommatidia. Transmission EM was

performed as described (Li and Montell, 2000) using hemisected fly heads embedded in LR White.

Blue light-induced changes in Rh1 concentration in flies

w^{1118} and $w^{1118};sun$ flies were exposed to 10 mW blue light for 6 h, and the fly heads were homogenized in Tris-buffered saline (20 mM Tris (pH 7.5), 150 mM NaCl) containing 0.5% Triton X-100 and protease inhibitors, and then mixed with an equal volume of 2 $\times$ SDS sample buffer. The extracts equal to 0.2 fly heads were fractionated by SDS–PAGE and the Western blots were probed with anti-Rh1 antibodies. Separate blots containing the same extracts were probed with anti-Arr2, anti-INAD and anti-TRP antibodies.

Co-immunoprecipitations

The *Sun* and Rh1 co-IPs were performed as described (Chevesich *et al*, 1997) with minor modifications. In all, 10 mg fly heads were homogenized in 1 ml buffer A (20 mM Tris–HCl (pH 7.5), 100 mM NaCl, 5 mM MgCl₂, 10% sucrose, 1% glycerol, 1 mM EDTA and 1 $\times$ c[complete protease inhibitors containing 1% CHAPS) on ice. The extracts were centrifuged at 16 000 g for 5 min at 4°C, 200 μ l aliquots of the supernatant were diluted with 800 μ l buffer A without CHAPS, and 1 μ l primary antibody or preimmune serum was added and incubated for 3 h at 4°C. The protein A beads were blocked with buffer A containing 0.2% CHAPS and 1% BSA for 30 min at 4°C and 50 μ l of blocked protein A beads were added to the tubes containing the immune complexes and incubated for 1 h at 4°C. After washing 2 $\times$ with buffer A containing 0.2% CHAPS, the immune complexes were eluted with 2 $\times$ SDS sample buffer and a Western blot was probed with anti-Rh1 monoclonal antibodies.

Reconstitution of blue light-induced degradation of Rh1 in isolated ommatidia

A transgene expressing Rh1 tagged at the N-terminus with GFP (gift from Dr J O'Tousa) was introduced into a *sun*¹ background. Ommatidia were dissected from adult flies as described (Hardie, 1991) with minor modifications. Ommatidia were dissected in DMEM medium, transferred to the Schneider's *Drosophila* medium and dark adapted for 1 h at room temperature. The ommatidia were either kept in the dark or illuminated with blue light for 1.5 h in Schneider's medium containing 50 nM LysoTracker-Blue DND-22 (Molecular Probes), and then confocal images were acquired.

To assay for light-induced degradation of Rh1, dark-adapted ommatidia were either stimulated with blue light for 4 h or kept in the dark. Western blots containing ommatidial extracts were probed with anti-Rh1 monoclonal antibodies. For experiments using lysosomal and proteasomal inhibitors, the ommatidia were pre-incubated with either 10 μ g/ μ l leupeptin or 10 μ M lactacystin before initiation of the light treatment. To determine the relative concentrations of Rh1 on Westerns, the blots were probed with anti-Rh1 primary antibodies, washed and probed with ¹²⁵I-labeled anti-mouse IgG (ICN). As a control for equal loading, we used rabbit anti-INAD and ¹²⁵I-labeled protein A. The band intensities were quantified using a Fuji PhosphorImager and MacBACV2.5 software.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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References

Adams MD, Celniker SE, Holt RA, Evans CA, Gocayne JD, Amanatides PG, Scherer SE, Li PW, Hoskins RA, Galle RF, George RA, Lewis SE, Richards S, Ashburner M, Henderson SN, Sutton GG, Wortman JR, Yandell MD, Zhang Q, Chen LX,

Brandon RC, Rogers YH, Blazej RG, Champe M, Pfeiffer BD, Wan KH, Doyle C, Baxter EG, Helt G, Nelson CR, Gabor Miklos GL, Abril JF, Agbayani A, An HJ, Andrews-Pfannkoch C, Baldwin D, Ballew RM, Basu A, Baxendale J, Bayraktaroglu L, Beasley EM,

- Beeson KY, Benos PV, Berman BP, Bhandari D, Bolshakov S, Borkova D, Botchan MR, Bouck J, Brokstein P, Brottier P, Burtis KC, Busam DA, Butler H, Cadieu E, Center A, Chandra I, Cherry JM, Cawley S, Dahlke C, Davenport LB, Davies P, de Pablos B, Delcher A, Deng Z, Mays AD, Dew I, Deitz SM, Dodson K, Doup LE, Downes M, Dugan-Rocha S, Dunkov BC, Dunn P, Durbin KJ, Evangelista CC, Ferraz C, Ferriera S, Fleischmann W, Fosler C, Gabriellian AE, Garg NS, Gelbart WM, Glasser K, Glodek A, Gong F, Gorrell JH, Gu Z, Guan P, Harris M, Harris NL, Harvey D, Heiman TJ, Hernandez JR, Houck J, Hostin D, Houston KA, Howland TJ, Wei MH, Ibegwam C, Jalali M, Kalush F, Karpen GH, Ke Z, Kennison JA, Ketchum KA, Kimmel BE, Kodira CD, Kraft C, Kravitz S, Kulp D, Lai Z, Lasko P, Lei Y, Levitsky AA, Li J, Li Z, Liang Y, Lin X, Liu X, Mattei B, McIntosh TC, McLeod MP, McPherson D, Merkulov G, Milshina NV, Mobarry C, Morris J, Moshrefi A, Mount SM, Moy M, Murphy B, Murphy L, Muzny DM, Nelson DL, Nelson DR, Nelson KA, Nixon K, Nusskern DR, Pacleb JM, Palazzolo M, Pittman GS, Pan S, Pollard J, Puri V, Reese MG, Reinert K, Remington K, Saunders RD, Scheeler F, Shen H, Shue BC, Siden-Kiamos I, Simpson M, Skupski MP, Smith T, Spier E, Spradling AC, Stapleton M, Strong R, Sun E, Svirskas R, Tector C, Turner R, Venter E, Wang AH, Wang X, Wang ZY, Wassarman DA, Weinstock GM, Weissenbach J, Williams SM, Woodage T, Worley KC, Wu D, Yang S, Yao QA, Ye J, Yeh RF, Zaveri JS, Zhan M, Zhang G, Zhao Q, Zheng L, Zheng XH, Zhong FN, Zhong W, Zhou X, Zhu S, Zhu X, Smith HO, Gibbs RA, Myers EW, Rubin GM, Venter JC (2000) The genome sequence of *Drosophila melanogaster*. *Science* **287**: 2185–2195
- Alloway PG, Howard L, Dolph PJ (2000) The formation of stable rhodopsin-arrestin complexes induces apoptosis and photoreceptor cell degeneration. *Neuron* **28**: 129–138
- Arbeitman MN, Furlong EE, Imam F, Johnson E, Null BH, Baker BS, Krasnow MA, Scott MP, Davis RW, White KP (2002) Gene expression during the life cycle of *Drosophila melanogaster*. *Science* **297**: 2270–2275
- Bascom RA, Manara S, Collins L, Molday RS, Kalnins VI, McInnes RR (1992) Cloning of the cDNA for a novel photoreceptor membrane protein (rom-1) identifies a disk rim protein family implicated in human retinopathies. *Neuron* **8**: 1171–1184
- Blackshaw S, Fraioli RE, Furukawa T, Cepko CL (2001) Comprehensive analysis of photoreceptor gene expression and the identification of candidate retinal disease genes. *Cell* **107**: 579–589
- Bloomquist BT, Shortridge RD, Schneuwly S, Perdew M, Montell C, Steller H, Rubin G, Pak WL (1988) Isolation of a putative phospholipase C gene of *Drosophila*, *norPA*, and its role in phototransduction. *Cell* **54**: 723–733
- Bonini NM, Leiserson WM, Benzer S (1998) Multiple roles of the eyes absent gene in *Drosophila*. *Dev Biol* **196**: 42–57
- Chevesich J, Kreuz AJ, Montell C (1997) Requirement for the PDZ domain protein, INAD, for localization of the TRP store-operated channel to a signaling complex. *Neuron* **18**: 95–105
- Claing A, Laporte SA, Caron MG, Lefkowitz RJ (2002) Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and β -arrestin proteins. *Prog Neurobiol* **66**: 61–79
- Claridge-Chang A, Wijnen H, Naef F, Boothroyd C, Rajewsky N, Young MW (2001) Circadian regulation of gene expression systems in the *Drosophila* head. *Neuron* **32**: 657–671
- Dolph PJ, Ranganathan R, Colley NJ, Hardy RW, Socolich M, Zuker CS (1993) Arrestin function in inactivation of G protein-coupled receptor rhodopsin *in vivo*. *Science* **260**: 1910–1916
- Ferguson SS (2001) Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol Rev* **53**: 1–24
- Franceschini N, Kirschfeld K (1971) Les phénomènes de pseudopupille dans l'oeil composé de *Drosophila*. *Kybertik* **9**: 159–182
- Fukuda M (1991) Lysosomal membrane glycoproteins. Structure, biosynthesis, and intracellular trafficking. *J Biol Chem* **266**: 21327–21330
- Hardie RC (1991) Whole-cell recordings of the light induced current in dissociated *Drosophila* photoreceptors: evidence for feedback by calcium permeating the light-sensitive channels. *Proc R Soc Lond (B)* **245**: 203–210
- Hyde DR, Mecklenburg KL, Pollock JA, Vihtelic TS, Benzer S (1990) Twenty *Drosophila* visual system cDNA clones: one is a homolog of human arrestin. *Proc Natl Acad Sci USA* **87**: 1008–1012
- Kalidas S, Smith DP (2002) Novel genomic cDNA hybrids produce effective RNA interference in adult *Drosophila*. *Neuron* **33**: 177–184
- Kiselev A, Socolich M, Vinos J, Hardy RW, Zuker CS, Ranganathan R (2000) A molecular pathway for light-dependent photoreceptor apoptosis in *Drosophila*. *Neuron* **28**: 139–152
- Kopczynski CC, Davis GW, Goodman CS (1996) A neural tetraspanin, encoded by late bloomer, that facilitates synapse formation. *Science* **271**: 1867–1870
- Kosloff M, Elia N, Joel-Almagor T, Timberg R, Zars TD, Hyde DR, Minke B, Selinger Z (2003) Regulation of light-dependent Gq α translocation and morphological changes in fly photoreceptors. *EMBO J* **22**: 459–468
- Lee SJ, Xu H, Kang LW, Amzel LM, Montell C (2003) Light adaptation through phosphoinositide-regulated translocation of *Drosophila* visual arrestin. *Neuron* **39**: 121–132
- Li HS, Montell C (2000) TRP and the PDZ protein, INAD, form the core complex required for retention of the signalplex in *Drosophila* photoreceptor cells. *J Cell Biol* **150**: 1411–1422
- Maecker HT, Todd SC, Levy S (1997) The tetraspanin superfamily: molecular facilitators. *FASEB J* **11**: 428–442
- McDonald MJ, Rosbash M (2001) Microarray analysis and organization of circadian gene expression in *Drosophila*. *Cell* **107**: 567–578
- Minke B, Selinger Z (1992) Intracellular messengers in invertebrate photoreceptors studied in mutant flies. In *Neuromethods*, Boulton A, Baker G, Taylor C (eds), pp 517–563. Clifton, NJ: The Humana Press Inc.
- Montell C (1999) *Drosophila* visual transduction. *Ann Rev Cell Dev Biol* **15**: 231–268
- Montell C, Rubin GM (1988) The *Drosophila ninaC* locus encodes two photoreceptor cell specific proteins with domains homologous to protein kinases and the myosin heavy chain head. *Cell* **52**: 757–772
- Pak WL (1995) *Drosophila* in vision research. *Invest Ophthalmol Vis Sci* **36**: 2340–2357
- Penn JS, Anderson RE (1992) Effects of light history on the rat retina. In *Progress in Retinal Research*, Osborne N, Chader G (eds), Vol. 11, pp 75–98. New York: Pergamon Press
- Robertson HM, Preston CR, Phillis RW, Johnson-Schlitz DM, Benz WK, Engels WR (1988) A stable genomic source of P element transposase in *Drosophila melanogaster*. *Genetics* **118**: 461–470
- Rong YS, Golik KG (2000) Gene targeting by homologous recombination in *Drosophila*. *Science* **288**: 2013–2018
- Schaeren-Wiemers N, Gerfin-Moser A (1993) A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: *in situ* hybridization using digoxigenin-labelled cRNA probes. *Histochemistry* **100**: 431–440
- Suzuki E, Hirokawa K (1991) Immunoelectron microscopic study of the opsin distribution in the photoreceptor cells of *Drosophila melanogaster*. *J Electron Microscop* (Tokyo) **40**: 187–192
- Takeshima H, Komazaki S, Nishi M, Iino M, Kangawa K (2000) Junctophilins: a novel family of junctional membrane complex proteins. *Mol Cell* **6**: 11–22
- Thummel CS, Pirrotta V (1992) New pCaSpeR P element vectors. *Dros Info Serv* **71**: 150
- Todres E, Nardi JB, Robertson HM (2000) The tetraspanin superfamily in insects. *Insect Mol Biol* **9**: 581–590
- Travis GH, Sutcliffe JG, Bok D (1991) The retinal degeneration slow (*rds*) gene product is a photoreceptor disc membrane-associated glycoprotein. *Neuron* **6**: 61–70
- Tsunoda S, Sun Y, Suzuki E, Zuker C (2001) Independent anchoring and assembly mechanisms of INAD signaling complexes in *Drosophila* photoreceptors. *J Neurosci* **21**: 150–158
- Vihtelic TS, Hyde DR, O'Tousa JE (1991) Isolation and characterization of the *Drosophila* retinal degeneration B (*rdgB*) gene. *Genetics* **127**: 761–768
- Xu XZ, Chien F, Butler A, Salkoff L, Montell C (2000) TRP γ , a *Drosophila* TRP-related subunit, forms a regulated cation channel with TRPL. *Neuron* **26**: 647–657