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Prolonged G_q activity triggers fly rhodopsin endocytosis and degradation, and reduces photoreceptor sensitivity

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Rapid deactivation of the *Drosophila* light receptor rhodopsin, through a visual arrestin Arr2 and a pathway that involves a transcription factor dCAMTA, is required for timely termination of light responses in the photoreceptor neuron. Here we report that this process is also critical for maintenance of the photoreceptor sensitivity. In both *dCAMTA*- and *arr2*-mutant flies, the endocytosis of the major rhodopsin Rh1 was dramatically increased, which was mediated by a G_q protein that signals downstream of rhodopsin in the visual transduction pathway. Consequently, the Rh1 level was downregulated and the photoreceptor became less sensitive to light. Remarkably, the G_q-stimulated Rh1 endocytosis does not require phospholipase C, a known effector of G_q, but depends on a tetraspanin protein. Our work has identified an arrestin-independent endocytic pathway of G protein-coupled receptor in the fly. This pathway may also function in mammals and mediate an early feedback regulation of receptor signaling.

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Introduction

A variety of neurotransmitters, hormones, cytokines and sensory stimuli trigger intracellular signaling cascades through trimeric G protein-coupled receptors (GPCRs) on the cell membrane (Pierce *et al*, 2002; Kristiansen, 2004). To fine-tune the signaling event, cells express various regulatory molecules to control the activities of GPCR (Gainetdinov *et al*, 2004). Arrestins are a group of primary regulators of GPCR (Shenoy and Lefkowitz, 2003) that, upon binding to the activated receptor, uncouple the receptor from the G protein, a process termed deactivation or desensitization of GPCR. When a GPCR is continuously stimulated, the bound arrestin molecule will recruit clathrin proteins to internalize the receptor through dynamin-dependent endocytosis (Ferguson, 2001; Claing *et al*, 2002). Many internalized GPCRs undergo lysosomal degradation, leading to downregulation of the receptor level (Tsao *et al*, 2001; von Zastrow, 2001). Both internalization and downregulation of a GPCR reduce its density on the plasma membrane, and thus cause long-term desensitization of the cell to the extracellular stimulus. In addition to regulating the signaling, receptor downregulation may protect the cell from overexcitation, and has been implicated in neuronal tolerances to chronically applied drugs like morphine (Zuo, 2005; Marie *et al*, 2006).

Although arrestins are the primary regulators in the deactivation of GPCR, they are not the only molecules that mediate GPCR endocytosis and downregulation. Many GPCRs, such as the endothelin type B, muscarinic, vasoactive intestinal peptide type 1, bradykinin type 2 and cholecystokinin receptors, are internalized independent of arrestin (Claing *et al*, 2002; Prossnitz, 2004). The arrestin-independent pathway of GPCR endocytosis has yet to be characterized *in vivo*.

The *Drosophila* phototransduction cascade is a model pathway (Montell, 1999; Hardie and Raghu, 2001) for genetic dissection of GPCR signaling and regulation. This visual signaling cascade is localized in the rhabdomere (Hardie and Raghu, 2001), a highly packed microvillar structure that is analogous to the outer segment of mammalian photoreceptors. The light receptor rhodopsin activates a G_q protein to stimulate a *norpA* gene-encoded phospholipase C (PLC) (Bloomquist *et al*, 1988; Lee *et al*, 1994), which then opens TRP or TRPL Ca^{2+}/cation channels (Montell, 1999) to depolarize the photoreceptor neuron. To ensure rapid termination of the visual response at the end of light stimulation, rhodopsin needs to be deactivated immediately after stimulating a single G_q molecule (Scott and Zuker, 1997, 1998). An arrestin protein Arr2 plays a pivotal role in the deactivation of rhodopsin (Dolph *et al*, 1993). Nonetheless, this visual arrestin does not appear to mediate rhodopsin endocytosis (Satoh and Ready, 2005) or downregulation in wild-type flies.

We have recently identified a new mechanism underlying the deactivation of rhodopsin. An F-box and leucine-rich repeat protein dFbxl4, which depends on a fly calmodulin-binding transcription activator dCAMTA for expression, facilitates rhodopsin deactivation in an unknown manner (Han *et al*, 2006). In the *dCAMTA* mutant flies *tes[1]* and *tes[2]* (for termination slow 1 and 2), because of the lack of dFbxl4-dependent rhodopsin deactivation, the G_q stimulation was prolonged, which leads to a slow termination of light response. Given that many F-box proteins act as substrate-recruiting subunits of SCF-type E3 ubiquitin ligases (Jin *et al*, 2004), and that several GPCRs undergo activity-dependent ubiquitination (Wojcikiewicz, 2004), it is possible that a reversible, dFbxl4-mediated ubiquitination of rhodopsin

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may abolish the rhodopsin–G_q interaction and thus deactivate the light receptor.

Another interesting phenotype caused by the loss of dCAMTA/dFbxl4 function is that the major rhodopsin protein Rh1, which is expressed in all six peripheral photoreceptors (O'Tousa *et al*, 1985), undergoes light-dependent downregulation (Han *et al*, 2006). Here we demonstrate that the Rh1 downregulation is due to increased endocytosis of Rh1, which is mediated by activity of the G_q protein. Due to the internalization of Rh1, the light sensitivity of the mutant photoreceptor decreased significantly. Thus, rapid deactivation of rhodopsin to prevent overstimulation of G_q is required for the maintenance of Rh1 level and photoreceptor sensitivity, as well as for the termination of light response. Our work has identified an arrestin-independent pathway of GPCR endocytosis in the fly eye, and suggests that G protein, the downstream signaling molecule of GPCR, may have a feedback control on the receptor signaling by triggering internalization and downregulation of the receptor.

Results

Light-dependent Rh1 endocytosis and downregulation in tes-mutant flies

In dark-reared flies, all Rh1 proteins were localized in the rhabdomeres according to immunostaining results (Figure 1A). Upon exposure to ambient light (~450 lux, from regular fluorescent tubes) for 3 h, a few potential endocytic Rh1 particles (ERPs) were observed in the cell bodies of wild-type photoreceptors. Interestingly, the same light treatment produced many more (~4-fold) ERPs in photoreceptors of a *tes*-mutant fly (*tes*^{2}) (Figure 1A). In electron microscopy images, each ERP appeared to be a collection of small vesicles (Figure 1B), a structure that has been reported to originate from endocytosis of Rh1 (Xu *et al*, 2004; Satoh and Ready, 2005). To provide further evidence that ERPs are derived from the endocytosis, we examined the distribution of Rab5, a small GTPase that mediates early endocytic pathways (Bucci *et al*, 1992), in a *tes, ey-Gal4;p[UAS::GFP-Rab5]* fly. The staining result showed that all ERPs contained Rab5 (Figure 1C), suggesting that ERPs are made of small endocytic vesicles.

Probably due to the excessive endocytosis, the level of Rh1 protein in *tes*-mutant flies was significantly decreased (>2-fold) upon overnight exposure to the ambient light (Figure 1D). In contrast, the levels of all other visual signaling proteins, including TRP, NORPA, an eye protein kinase C INAC and a scaffold protein INAD (Montell, 1999), were not changed by the light stimulation.

If Rh1 downregulation is really due to increased endocytosis and subsequent degradation of Rh1 in *tes*-mutant flies, it should be prevented when the endocytosis is blocked. Endocytosis of most GPCRs depends on the function of dynamin, a large GTPase that cleaves endocytic vesicles from the plasma membrane (Sever, 2002). We introduced a temperature-sensitive mutation of the dynamin gene *shibire* (*shi*) (Poodry and Edgar, 1979; Chen *et al*, 1991; van der Bliek and Meyerowitz, 1991) into the *tes* fly and found that this mutation virtually abolished the endocytosis of Rh1 at a restrictive temperature of 30.5°C (Figure 2A), indicating that Rh1 endocytosis is also dynamin-dependent.

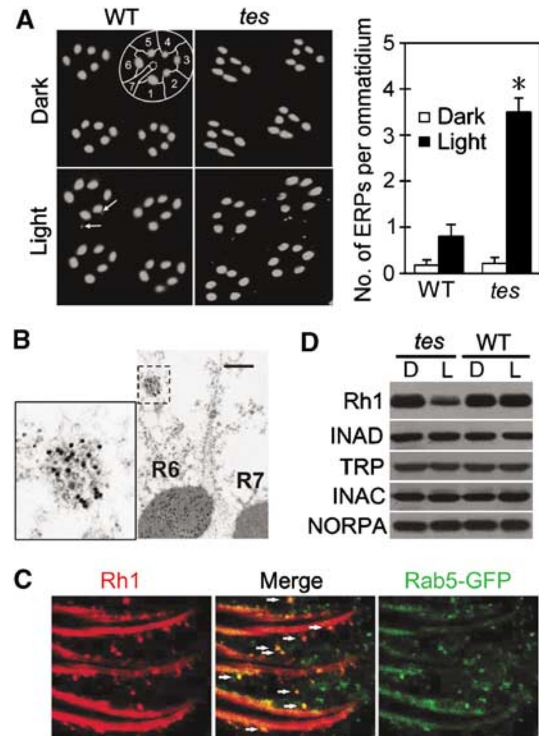

Figure 1 Light-dependent Rh1 endocytosis and downregulation in *tes*-mutant flies. (**A**) Three-hour light stimulation induced more ERPs in *tes* flies than in wild type. The left panel shows cross-sections of eye that have been stained with a monoclonal Rh1 antibody. The cell bodies of photoreceptors in one wild-type ommatidium are outlined. Note that only the six peripheral rhabdomeres, not the central R7 one (dashed circle), contain Rh1. The arrows point to two ERPs in the light-stimulated wild-type section. The number of ERPs per ommatidium was calculated for each genotype and treatment. After averaging three sets of data, the means and s.e.ms (as error bars) are presented in the right panel. Except those in Figures 5 and 6A, all stained flies were exposed to light for 3 h. (**B**) Immunogold electron microscopy reveals that each ERP represents a collection of small endocytic vesicles. An ERP (boxed) in the R6 photoreceptor is enlarged in the left panel. Scale bar, 0.5 μm. (**C**) In a *tes,ey-Gal4;p[UAS::GFP-Rab5]*fly, the ERPs (arrow heads) were colabeled with the Rab5 protein. Whole fly heads were stained with both Rh1 and GFP antibodies. The images show the side view of photoreceptors. (**D**) Western blots showing a reduced level of Rh1 in light exposed *tes*-mutant flies. All other visual signaling proteins had normal levels, indicating that no rhabdomeral degeneration occurred in the examined flies. D: dark-reared, never exposed to light, L: dark-reared but exposed to room light overnight before examination, WT: wild type. In all western blot analyses, each lane was loaded with one fly head. *Indicates that the sample is significantly different from others in the same group.

Next, we examined the effect of *shi* mutation on the downregulation of Rh1. We previously found that it took at least 10 h for ambient light to stimulate Rh1 reduction in the *tes* flies. Since such a long-time treatment of *shi* mutants at 30.5°C could cause damage to the general structure of rhabdomere, we instead stimulated the flies for 3 h with a pure blue light of 700 lux, which is a much stronger stimulation compared with the white ambient light. In *tes*-mutant flies, this treatment reduced the Rh1 level similar to that by overnight exposure to ambient light. In contrast, no significant downregulation of Rh1 was observed in the *shi;tes* double mutants (Figure 2B). These observations indicate that the Rh1 protein is indeed first internalized through

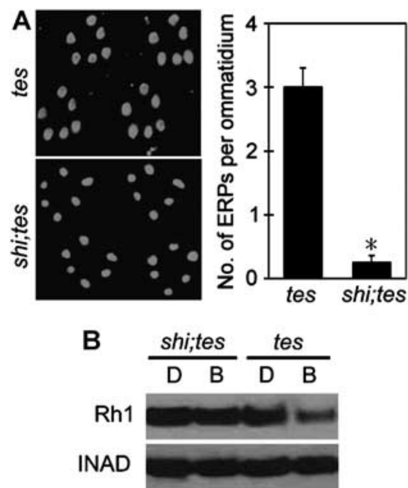

Figure 2 The Rh1 downregulation in *tes*-mutant flies is due to excessive endocytosis of Rh1. (**A**) The Rh1 endocytosis in *tes* flies depends on the *shibire* dynamin. During the 3-h light stimulation, both *tes* and the *shi;tes* double mutants were kept at 30.5°C, a temperature that prevents the mutant *Shibire* dynamin from function. (**B**) The *shi* mutation prevented light-induced Rh1 reduction in *tes* flies. The scaffold protein INAD was probed in parallel. For light stimulation in this particular experiment, the flies were exposed to pure blue light (750 lux) for three hours at 30.5°C. B, blue light. *Indicates that the sample is significantly different from others in the same group.

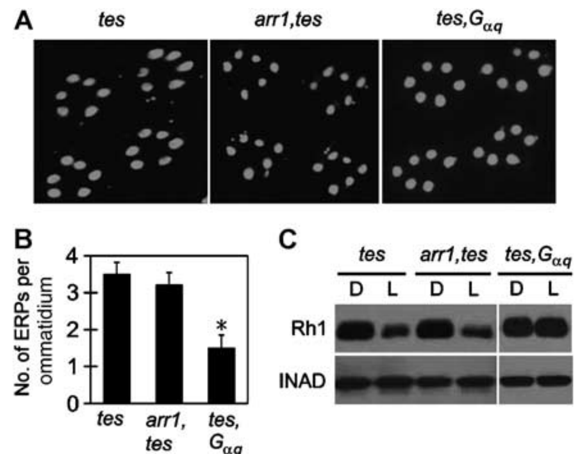

Figure 3 G_q, and not Arr1, is required for Rh1 endocytosis and downregulation in *tes* flies. (**A**) Mutation of G_{α_q}, and not of *arr1*, inhibited endocytosis of Rh1 in *tes* flies. (**B**) Averaged ERP numbers per ommatidium. (**C**) Overnight light stimulation failed to trigger Rh1 downregulation in the *tes*,G_{α_q} double mutant flies. *Indicates that the sample is significantly different from others in the same group.

dynamin-dependent endocytosis and then gets degraded later, likely in lysosomes.

G_q, but not Arr1, mediates endocytosis and downregulation of Rh1 in tes-mutant flies

We further investigated which rhodopsin-interacting molecules are responsible for triggering the endocytosis of Rh1 in *tes*-mutant flies. Although Arr2 is dispensable for rhodopsin endocytosis, another visual arrestin Arr1 has been reported to mediate activity-dependent Rh1 endocytosis during late pupal development (Satoh and Ready, 2005). To examine whether Rh1 is internalized through Arr1 in the adult *tes* fly, we introduced an *arr1* mutation (*arr1*^{1}) into the *tes* chromosome by recombination. Unexpectedly, the mutation of *arr1* did not significantly inhibit the Rh1 endocytosis in the adult *tes*-mutant fly (Figure 3A and B) as it did in pupal photoreceptors (Satoh and Ready, 2005). The downregulation of Rh1 was also not suppressed in the *arr1,tes* flies (Figure 3C). As the Rh1 downregulation in a *tes;arr2* double mutant was at least as severe as that in the *tes* single mutant (see Supplementary data), Rh1 appears to be internalized in the adult mutant flies through an arrestin-independent pathway.

In addition to arrestins, the G_q protein also interacts with Rh1 upon light stimulation. Considering that one consequence of *tes* mutation is the prolonged activation of G_q by Rh1, we hypothesized that the excessive G_q activity may somehow contribute to the increased endocytosis of Rh1. To test this, we recombined a mutant allele ($G^1_{\alpha_q}$) of the G_q α-subunit gene (Scott *et al*, 1995) to the *tes*-mutant chromosome, and examined the Rh1 endocytosis and downregulation in the *tes*,G_{α_q} double mutant flies. The results showed that the G_{α_q} mutation greatly inhibited the endocytosis (Figure 3A and B), and blocked the downregulation of Rh1 (Figure 3C), suggesting that the G_q protein mediates Rh1 endocytosis in adult *tes* flies.

The G_q-dependent endocytosis and downregulation of Rh1 also occur in arr2-mutant flies

If the prolonged activation of G_q is indeed responsible for the endocytosis and downregulation of Rh1 in *tes*-mutant flies, we reasoned that a similar Rh1 downregulation should be observed in other mutants that have impaired deactivation of rhodopsin. Since Arr2 is the primary regulatory molecule required for rhodopsin deactivation (Dolph *et al*, 1993), we examined the internalization and the protein level of Rh1 in an *arr2*-null mutant that had been exposed to ambient light for 3 h or overnight, respectively.

Compared with wild type, the *arr2*-mutant photoreceptors contained many more ERPs (Figure 4A), indicating an increase of Rh1 endocytosis. Similar to *tes* flies, the light-exposed *arr2* mutant had a reduced level of Rh1 protein (Figure 4B). The reduction in Rh1 level was not due to light-dependent retinal degeneration in this mutant background, since the overnight light stimulation did not cause any significant abnormality in rhabdomeral morphology according to electron microscopy. In addition, Rh1 downregulation, as well as the excessive endocytosis, was reversed in the dark (see Supplementary data). Moreover, all other examined rhabdomeral proteins displayed unchanged levels in the light-exposed *arr2* flies (Figure 4B). It is thus highly likely that the excessive endocytosis has caused a specific downregulation of Rh1 in the *arr2*-mutant fly.

Remarkably, the endocytosis and the downregulation of Rh1 were greatly inhibited or suppressed, respectively, by the G_{α_q} mutation in a G_{α_q};*arr2* double mutant fly (Figure 4C–E). In contrast, the *arr1* mutation had no significant effect on Rh1 endocytosis and downregulation in the *arr2* background (Figure 4C–E). Thus, the excessive activity of G_q protein is required for Rh1 endocytosis and downregulation in both *tes*- and *arr2*-mutant flies.

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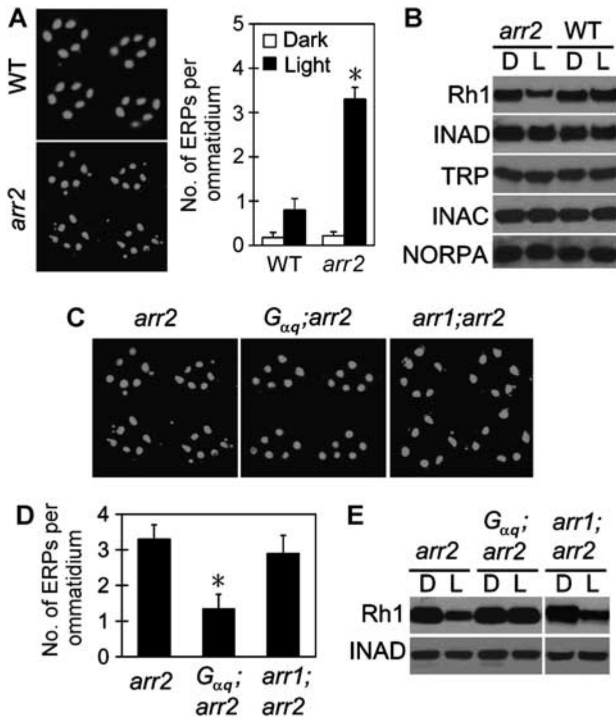

Figure 4 Rh1 undergoes G_q-dependent endocytosis and downregulation in an *arr2*-null mutant fly. (**A**) Ambient light induced more ERPs in the *arr2*-mutant fly compared with wild type. (**B**) Overnight light stimulation caused Rh1 reduction in *arr2* flies. All other rhabdomeral proteins had unchanged levels. (**C**) Mutation of G_{α_q}, and not of *arr1*, inhibited the Rh1 endocytosis in *arr2* flies. (**D**) Averaged ERP numbers per ommatidium. (**E**) Light exposure failed to induce Rh1 downregulation in the G_{α_q};*arr2* double mutant. *Indicates that the sample is significantly different from others in the same group.

G_q-dependent Rh1 endocytosis in the wild-type background

As a low level of Rh1 endocytosis was observed in wild-type flies, we tested whether this basal activity of endocytosis also involves G_q. Exposure of wild-type flies to ambient light for a longer time, 6 h, produced a few more ERPs (1.4 ± 0.2 versus 0.8 ± 0.3 per ommatidium) than the 3-h stimulation. In contrast, the same 6-h exposure only stimulated 0.8 ± 0.2 ERPs per ommatidium in the G_{α_q} mutant fly (Figure 5). These data suggest that G_q-mediated Rh1 endocytosis also occurs in wild-type flies, although the activity is too low to cause any detectable downregulation of Rh1.

The NORPA PLC does not mediate the G_q-dependent Rh1 endocytosis or downregulation

The primary function of G_q protein in fly visual signaling is to stimulate the *norpA*-encoded PLC, whose activity leads to the opening of TRP and TRPL ion channels (Hardie and Raghu, 2001). A possible explanation for the role of G_q in Rh1 endocytosis is that the Ca^{2+} influx through TRP channels might be required for the occurring of endocytosis. Nonetheless, the following observations argue against this hypothesis. A *norpA* mutation, which prevents G_q-dependent activation of TRP and TRPL channels, did not inhibit the endocytosis of Rh1 in either *tes*- or *arr2*-mutant background. Remarkably, in the *norpA;tes* and *norpA;arr2* double mutant flies, a large amount of ERPs even appeared within 1.5 h after light exposure (Figure 6A), earlier than that in the *tes* and *arr2* single-mutant background. As a consequence, the double mutants displayed even lower Rh1 levels upon overnight light exposure (Figure 6B). The *norpA* mutation enhances Rh1 endocytosis and downregulation probably because the NORPA protein is also required to deactivate G_q, and the mutation of *norpA* actually leads to the accumulation of more active G_q proteins (Cook *et al*, 2000). These data indicate that G_q may mediate Rh1 endocytosis through a mechanism that is independent of PLC.

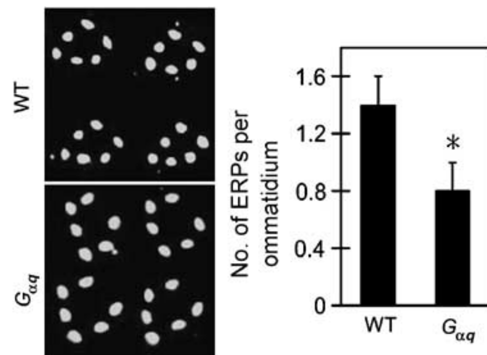

Figure 5 Mutation of G_{α_q} partially inhibited Rh1 endocytosis in the wild-type background. Six-hour light exposure produced less ERPs in the G_{α_q} mutant fly compared with wild type. *Indicates that the sample is significantly different from others in the same group.

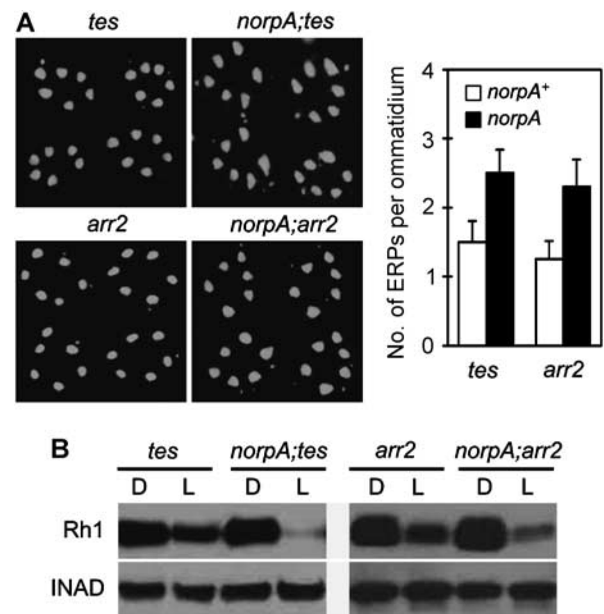

Figure 6 PLC does not mediate the G_{α_q}-dependent endocytosis of Rh1. (**A**) The mutation of *norpA* caused more ERPs in *tes*- and *arr2*-mutant flies after 1.5 h of light exposure. (**B**) After overnight light stimulation, the Rh1 levels in the *norpA;tes* and the *norpA;;arr2* flies were even lower than those in the *tes* and *arr2* single mutants.

A tetraspanin Sunglass is involved in the G_q-dependent Rh1 endocytosis

It is reported that Sunglasses (Sun), a lysosomal tetraspanin protein, is required to transfer Rh1 to the lysosome for degradation upon blue light stimulation (Xu *et al*, 2004). In an attempt to block the Rh1 degradation and accumulate

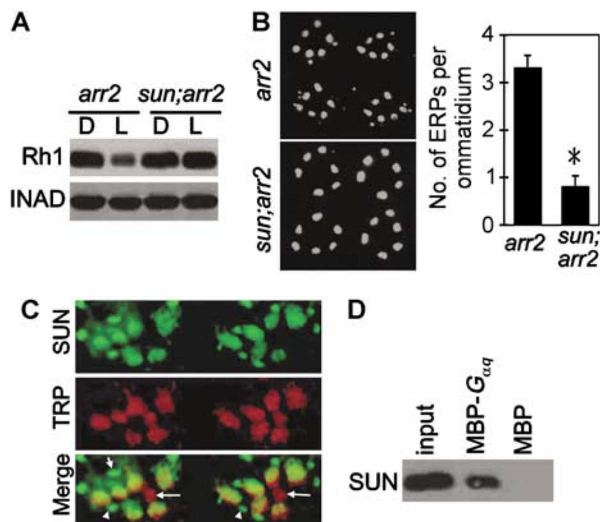

Figure 7 The tetraspanin Sun is involved in the G_{α_q}-dependent Rh1 endocytosis. (**A**) A *Sun*-null mutation suppressed the downregulation of Rh1 in light-exposed *arr2* flies. (**B**) The light stimulation failed to induce Rh1 endocytosis in the *sun;arr2* double mutant flies. (**C**) Sun protein was detected in the peripheral rhabdomeres of *p[rh1::Myc-Sun]* flies. The R7 one (arrow) had negative staining. The TRP protein was labeled to show the locations of all rhabdomeres. Three Sun-enriched areas in the cell bodies are indicated by arrowheads. (**D**) An MBP-G_q fusion protein, not the MBP alone, pulled down Myc-Sun from the head extracts. Lane one was loaded with 1/5 of input. The western blot was probed with an Myc antibody. *Indicates that the sample is significantly different from others in the same group.

more ERPs in *arr2*-mutant photoreceptors, we introduced a *sun*-null mutation into the *arr2* background. As expected, the *sun* mutation abolished the ambient light-induced Rh1 downregulation in the *sun;arr2* flies (Figure 7A). Nonetheless, the number of ERPs did not increase but was dramatically reduced in these double mutant flies compared with that of the *arr2* single mutant (Figure 7B). This observation suggests that Sun is required for the G_q-stimulated endocytosis, as well as for the lysosomal targeting, of Rh1.

It is difficult to explain why the lysosomal protein Sun has a role in the endocytosis of a plasma membrane protein. As the previous study used plastic sections to investigate the location of Sun (Xu *et al*, 2004) and a 24-h high-temperature step in the section preparation could have greatly reduced the immunoreactivity of the antigen protein, we reexamined the subcellular distribution of Sun by whole-head staining of intact eyes. In the same *p[rh1::Myc-Sun]* transgenic fly used by Xu *et al* (2004), we detected a significant amount of Sun protein in the rhabdomeres of peripheral photoreceptors (Figure 7C). Thus, Sun is located on the plasma membrane of rhabdomere as well as in the lysosome. This observation has provided additional support for a role of Sun in the endocytosis of Rh1.

As both Sun and G_q are involved in the Rh1 endocytosis, it is possible that these two proteins may physically interact with each other. To test this, we coupled a maltose-binding protein (MBP)-tagged G_{α_q} protein covalently to Sepharose 4B beads, and used these beads to pull down proteins from head extracts of the *p[rh1::Myc-Sun]* fly. Using western blot, we successfully detected the Myc-Sun protein in the precipitants (Figure 7D). In control experiments, MBP alone, also coupled to Sepharose 4B, failed to pull down any Myc-Sun protein.

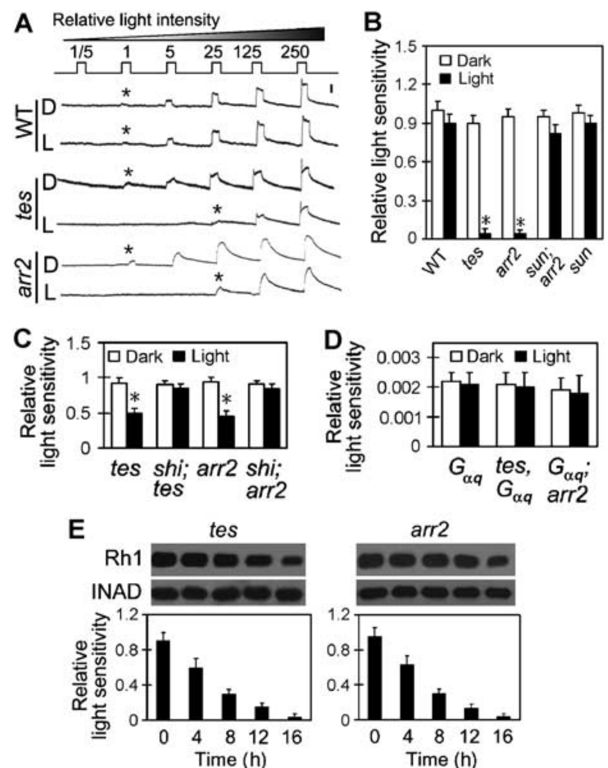

Figure 8 Excessive endocytosis and downregulation of Rh1 leads to reduced light sensitivities of photoreceptor in *tes*- and *arr2*-mutant flies. (**A**) Intracellular recordings revealed decreased light sensitivities of photoreceptor in *tes* and *arr2* flies that had been exposed to ambient light overnight. The fly eye was stimulated with a series of 1-s light pulses of increasing intensities as labeled on the top. The relative intensity 5 is approximately 1 lux. The first appearing response is marked with an asterisk. The scale bar next to the top trace is 10 mV. (**B**) Quantitation of light sensitivities of photoreceptor. The light groups had been exposed to light overnight. Note that mutation of *sun* prevented the reduction of sensitivity. The shown mean relative sensitivities were calculated as described in Materials and methods. The error bars represent s.e.m. (**C**) Mutation of *shi* prevented light-dependent sensitivity reduction in both *tes*- and *arr2*-mutant flies. The light group had been exposed to light for 4 h. All flies were kept at 30.5°C during the 4-h exposure. (**D**) Mutation of *tes* and *arr2* failed to reduce the light sensitivity in the G_{α_q}-mutant background after overnight light exposure. (**E**) Time courses of Rh1 downregulation (top) and photoreceptor sensitivity reduction (lower panels) in *tes* and *arr2* flies during light exposure. *Indicates that the sample is significantly different from others in the same group.

Excessive Rh1 endocytosis reduces the sensitivity of photoreceptor to light in tes- and arr2-mutant flies

The light sensitivity of fly photoreceptor is determined by the density of rhodopsin on the rhabdomeral membrane (Johnson and Pak, 1986) as well as by the number of available G_q molecules (Scott *et al*, 1995). Both the endocytosis and the degradation of Rh1 in *tes* and *arr2* flies will decrease the rhodopsin level on the membrane and thus could lead to reduced sensitivity of photoreceptor to light. By conducting intracellular recordings, we examined the sensitivities of photoreceptor after exposing the flies to light overnight. This overnight stimulation did not significantly change the light sensitivity of photoreceptor in wild-type flies (Figure 8A and B), probably because the amount of internalized Rh1 is too small. In contrast, light sensitivities in both

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tes- and *arr2*-mutant photoreceptors decreased approximately 25-fold after the light exposure (Figure 8A and B).

Several observations suggest that the light-induced sensitivity reduction in *tes* and *arr2* photoreceptors is due to the G_q-mediated Rh1 endocytosis and/or degradation. First, significant reduction of sensitivity was observed as early as 4 h after light exposure, and was abolished by the temperature-sensitive *shi* mutation in both *tes* and *arr2* mutants at 30.5°C (Figure 8C). Second, in the G_{α_q} -mutant fly, which already has a very low sensitivity to light due to the lack of G_q-mediated phototransduction (Scott *et al*, 1995), mutation of either *tes* or *arr2* did not further reduce the photoreceptor sensitivity upon overnight light exposure (Figure 8D). Finally, the light-induced sensitivity reduction was not observed in the *sun;arr2* double mutant fly (Figure 8B).

To investigate whether the degradation of Rh1 is the primary cause of sensitivity reduction, we examined the light sensitivity and the Rh1 protein level at a series of time points during the light exposure. In both *tes* and *arr2* flies, the sensitivity started to decrease at about 4 h (Figure 8E), immediately after large amount of Rh1 had been internalized. Before the Rh1 level was significantly downregulated, the sensitivities had reduced at least fourfold (Figure 8E). Thus, the decrease of sensitivity in the mutant flies is due to the excessive endocytosis as well as the degradation of Rh1.

Discussion

Our data indicate that prolonged activation of the G_q protein stimulates Rh1 endocytosis and downregulation, which lead to decreased light sensitivity of fly photoreceptor. Interestingly, the G_q-mediated Rh1 endocytosis requires a tetraspanin protein Sun, but is independent of PLC and the visual arrestins.

Importance of rhodopsin deactivation for the maintenance of light sensitivity

The identification of G_q protein as a mediator of Rh1 endocytosis and downregulation has revealed a new role of rhodopsin deactivation. Since excessive activity of rhodopsin triggers the G_q-mediated endocytosis of Rh1, the deactivation of rhodopsin helps to concentrate Rh1 in the rhabdomere, and thus is critical for the maintenance of photoreceptor sensitivity. Therefore, molecules that promote rhodopsin deactivation, such as Arr2 and dCAMTA/dFbxl4, are important for both the sensitivity of photoreceptor and the termination of light response.

G_q/Sun-dependent Rh1 endocytosis and downregulation in adult flies

Although normal ambient light does not cause significant change in the overall level of Rh1, a low activity of rhodopsin degradation still occurs in wild-type flies. Stimulation with intense blue light enhances the degradation, leading to detectable downregulation of Rh1 (Xu *et al*, 2004). The degradation pathway, which involves the Sun protein, may help to remove photo-damaged rhodopsin molecules that cannot be deactivated otherwise, and thus protect the photoreceptor from overexcitation. In the *sun*-mutant fly, the photoreceptors undergo light-dependent degeneration (Xu *et al*, 2004) probably due to the existence of unregulated rhodopsin molecules. It is plausible that the G_q/Sun-mediated endocytosis may initiate the degradation of Rh1 in light-exposed wild-type flies.

Sun is a member of the tetraspanin family that have been implicated in cell adhesion, motility, invasion and proliferation (Hemler, 2005). Two human tetraspanins CD9 and CD81 have been shown to form dynamic complexes with a G_q protein and an orphan GPCR GPR56 on the membrane of cultured cells (Little *et al*, 2004). Although it has previously not been reported that a tetraspanin mediates signaling or endocytosis of any GPCR, tetraspanins including CD63, CD82 and CD151 have been found to promote internalization of other associated membrane proteins such as integrins, the EGF receptor and a H^+–K^+ ATPase, by mechanisms depending on either clathrin or protein kinase C (Berditchevski and Odintsova, 2007). It is possible that prolonged interaction between active G_q protein and Sun may trigger one of these mechanisms to internalize Rh1 together with Sun in the fly photoreceptor. In the future, it will be important to further characterize this G_q/Sun-dependent endocytosis.

The visual arrestin Arr2 is critical for the deactivation of rhodopsin, but does not mediate endocytosis of Rh1 in wild-type flies (Satoh and Ready, 2005). This could be due to a rhodopsin phosphatase RDGC (Steele *et al*, 1992) that keeps dephosphorylating Rh1 and the dephosphorylated Rh1 cannot form stable association with Arr2 (Kiselev *et al*, 2000). Although the other arrestin Arr1 is involved in Rh1 endocytosis during late pupal development (Satoh and Ready, 2005), it does not mediate the excessive endocytosis or the degradation of Rh1 in the adult mutant flies. It is likely that the Arr1-dependent endocytic activity is relatively lower in the adult and is not enhanced by the *tes* or *arr2* mutation. In addition, given that many GPCRs internalized through arrestins are targeted for recycle instead of degradation (Yu *et al*, 1993; Pippig *et al*, 1995; Vines *et al*, 2003), the Arr1-mediated endocytosis of Rh1 may not lead to its downregulation.

A new role of G_q protein in GPCR signaling and regulation

A variety of mammalian GPCRs also undergo endocytosis independent of arrestin (Claing *et al*, 2002). Most of these, such as endothelin, muscarinic, bradykinin, cholecystokinin and thromboxane A_2 receptors, are able to activate G_q proteins (Shenker *et al*, 1991; Hubbard and Hepler, 2006; Alexander *et al*, 2007). It has been shown in an *in vitro* system that the activity of G_q protein induces endocytosis of a thromboxane A_2 β receptor (Rochdi and Parent, 2003). Our work here has provided *in vivo* evidence that a G_q protein mediates endocytosis of the fly light receptor Rh1. We propose that the G_q-dependent endocytosis could be a common mechanism that functions in all animal cells for the internalization of G_q-coupled receptors. As G protein is the molecule that signals immediately downstream of GPCR, this G_q-stimulated endocytosis of receptor may mediate an early feedback control of the receptor signaling on plasma membrane.

Remarkably, the G_q proteins internalize both Rh1 and the *in vitro* expressed thromboxane A_2 β-receptor (Rochdi and Parent, 2003) independent of PLC, the known effector of G_q. Thus, the G_q stimulation of GPCR endocytosis represents a new branch of G_q signaling. In addition to controlling the receptor signaling, this new G_q pathway could also be involved in other cellular processes such as apoptotic neuronal

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degeneration (Iakhine *et al*, 2004) and deformation of membrane structures (Kosloff *et al*, 2003). A recent *in vitro* work on the thromboxane A_2 receptor has shown that an ADP-ribosylation factor 6 could be included in this PLC-independent G_q-signaling pathway (Giguere *et al*, 2006). Our study of Rh1 endocytosis suggests that this G_q pathway also involves the tetraspanin protein Sun. It will be interesting to further characterize this endocytic G_q function in the future.

Materials and methods

Fly genetics and light treatment

All examined flies except the *w;p[rh1::Myc-Sun]* transgenic fly had white eyes. Both wild-type flies and those containing the *tes* mutation were in a *cn,bw* background. All *arr2* mutation-containing flies and the *sun*-mutant were in the *white*$^-$ background. The mutant alleles used for each gene in this work are *arr1*^{1}, *arr2*^{5}, $G^1_{\alpha_q}$, *norpA*^{24}, *shi*^{2}, *sun*^{1} and *tes*^{2}. To avoid age-dependent retinal degeneration in some mutant flies, all flies were examined at 1–2 days old.

All flies were reared in the dark at 22°C and transferred to new vials (with food) before light treatment. To induce Rh1 endocytosis, the flies in Figures 5 and 6A were exposed to room light (∼450 lux, from regular fluorescent tubes) for 6 and 1.5 h, respectively, and all the others for 3 h. To induce the downregulation of Rh1, except those in Figure 2B (as specified in the Results), all flies were exposed to room light for 16–18 h (overnight) before collecting the heads for western blot assay.

Immunostaining

For immunofluorescence staining of Rh1, fly heads were fixed with 4% paraformaldehyde in PBS, dehydrated with acetone and embedded in LR White resin as described (Porter and Montell, 1993). One-micrometer sections were cut across the top half of the eye and stained with a monoclonal Rh1 antibody (1:50, DSHB) and FITC-conjugated secondary antibodies. The activity of Rh1 endocytosis was quantitated as the number of ERPs per ommatidium. At least three sets of data were averaged for each genotype and light treatment.

Whole-head staining was performed to locate the GFP-Rab5 protein in the *tes,ey-Gal4;p[UAS::GFP-Rab5]*fly, and the Myc-tagged Sun protein in the *w;p[rh1::Myc-Sun]* fly. After fixation, the heads were double stained with the Rh1 and a GFP antibody (1:100), or with an Myc (1:200, Roche) and a TRP antibody (1:200), respectively, following a protocol described previously (Satoh and Ready, 2005). The stained eyes were examined under a LSM5 confocal microscope.

To examine ERPs with electron microscope (EM), fly heads were fixed and embedded in LR White as described (Li and Montell, 2000), except that ethanol was used for the dehydration. Thin sections of eye were cut and immunostained with the Rh1 antibody and anti-mouse IgGs conjugated with 15-nm gold particles. After staining with 1% aqueous uranyl acetate, the sections were examined using a transmission EM.

G_{α_q}-binding assay

An MBP-G_{α_q} fusion protein was expressed in BL-21 cells, purified with amylase resin (NEB) and coupled to CNBr-activated Sepharose 4B beads (Amersham). Approximately 50 μg protein on 50 μl beads were incubated with head extracts of 50 *p[ninaE::myc-sun];sun*^{1} flies in PBS that contains 1% Triton X-100 and protease inhibitors. After three washes with PBS, proteins were eluted with 100 mM glycine in PBS and subjected to SDS–PAGE and western blotting.

Intracellular recording

Intracellular recording was performed as previously described (Wes *et al*, 1999), with minor modifications. In brief, flies were fixed with stripes of tape and a small opening was made on surface of the eye using fine tweezers. A glass microelectrode with resistance >30 MΩ (filled with 2 M KCl) was gradually inserted into the opening until light-induced membrane depolarization was observed. The reference electrode, filled with Ringer's solution, was put either on the thorax (for the data shown) or inside the eye at the retina layer, without causing difference in the measured sensitivities. Orange light of different intensities (through passing a series of neutral density filters) was used to trigger responses in the photoreceptor cells. Before recording, the flies were allowed to adapt for 3 min in the dark. The signal was amplified and recorded using a Warner IE210 Intracellular Electrometer.

The relative light sensitivity of a photoreceptor is defined as I_{WT}/I, where I_{WT} represents the mean light intensity required to stimulate a detectable response in wild-type photoreceptors that had previously not been exposed to light, and I is the lowest light intensity that is necessary for stimulation of the examined photoreceptor. For each genotype and condition, cells from >8 flies were examined and the relative sensitivities were averaged to obtain a mean. The standard error of means (s.e.m.) were calculated and presented as error bars in the figures.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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