# Two stages of light-dependent TRPL-channel translocation in *Drosophila* photoreceptors

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There was an error published in *J. Cell Sci.* **119**, 2935-2944.

In the Materials and Methods section, the incorrect light intensities were given. The correct intensities are shown below.

Flies were placed under a white light source (Lambda LS 175W Xenon-arc lamp with 400-700 nm bandpass filter, Sutter Instruments, Novato, CA, or equivalent), at ~2297 lux for stage one TRPL translocation (unless otherwise indicated in figure legend) or ~244 lux for stage two.

The authors apologize for this error.

# Two stages of light-dependent TRPL-channel translocation in *Drosophila* photoreceptors

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## Summary

Transient receptor potential (TRP) channels across species are expressed in sensory receptor cells, and often localized to specialized subcellular sites. In *Drosophila* photoreceptors, TRP-like (TRPL) channels are localized to the signaling compartment, the rhabdomere, in the dark, and undergo light-induced translocation into the cell body as a mechanism for long-term light-adaptation. We show that translocation of TRPL channels occurs in two distinct stages, first to the neighboring stalk membrane then to the basolateral membrane. In the first stage, light-induced translocation occurs within 5 minutes, whereas the second stage takes over 6 hours. The exclusive apical localization of TRPL channels in the first stage of translocation suggests that channels are released from the rhabdomere and diffuse laterally through the membrane into the adjoining stalk membrane. In the second stage, TRPL channels are localized in the basolateral membrane, implicating a different transport mechanism. Genetic analyses suggest that activation of the other light-activated TRP channel and eye-protein-kinase C (eye-PKC) are both required for the second stage of TRPL translocation in R1 to R6 photoreceptor cells, whereas only phospholipase C (PLC) is required for the first stage. Finally, we show that arrestin-2 is required for the rhabdomeric localization and stability of TRPL channels.

Key words: TRP Channels, TRPL Channels, Arrestin, Translocation, *Drosophila*, Phototransduction

## Introduction

In many cells, signaling proteins are segregated into specialized domains to carry out localized and efficient signal transduction. The proteins and molecular mechanisms underlying the subcellular localization of a signaling protein are therefore crucial for function. In this study, we examine the components and mechanisms involved in the subcellular localization and light-dependent translocation of transient receptor potential (TRP)-like channels (TRPL channels) in *Drosophila* photoreceptors. A *Drosophila* photoreceptor, which is a specialized type of epithelial cell, has a plasma membrane that is divided into apical and basolateral domains. Apical and basolateral membranes are separated by adherens junctions (AJs) that join neighboring photoreceptor cells within an ommatidium (Fig. 1A). 800 of these ommatidia or unit eyes make up the compound *Drosophila* eye. The apical membrane consists of the specialized light-sensing rhabdomere and the supporting stalk (Fig. 1A). Phototransduction is localized to the rhabdomere, which consists of ~60,000 tightly packed microvilli and houses most of the signaling components. Here, the G-protein-coupled receptor (GPCR) rhodopsin is photo-converted to the activated meta-rhodopsin state. Meta-rhodopsin activates the $G_q\alpha$-protein, which in turn activates phospholipase-C$\beta$ (PLC), leading to the opening of two light-activated ion channels, the TRP and TRPL channels (Hardie and Raghu, 2001; Ranganathan et al., 1995; Tsunoda and Zuker, 1999). Visual arrestin-2 (Arr-2) and an eye-specific protein kinase-C (eye-PKC) have been shown to be required for normal deactivation of the light-response (Dolph et al.,

1993; Hardie et al., 1993; Ranganathan et al., 1991; Smith et al., 1991).

An interesting twist to compartmentalized signaling in *Drosophila* photoreceptors has emerged in recent years: several signaling components, including the $G_q\alpha$ subunit (Cronin et al., 2004; Kosloff et al., 2003), TRPL channels (Bahner et al., 2002) and visual Arr-2 (Kiselev et al., 2000; Lee et al., 2003; Orem and Dolph, 2002), have been reported to display light-dependent translocation between the rhabdomere and cell body. Vertebrate and crayfish photoreceptors have also been reported to display a similar light-dependent translocation of the G-protein, transducin and visual Arr-2 (Brann and Cohen, 1987; Broekhuyse et al., 1987; Broekhuyse et al., 1985; Mangini and Pepperberg, 1988; McGinnis et al., 1992; Mendez et al., 2003; Philp et al., 1987; Sokolov et al., 2002; Terakita et al., 1998; Whelan and McGinnis, 1988). Dynamic localization of components in and out of the signaling compartment is probably a mechanism for regulating the quantity of components available for signaling, thereby contributing to long-term light adaptation (Bahner et al., 2002; Sokolov et al., 2002).

Here, we show that TRPL channels translocate out of the rhabdomere in two distinct stages. In the first stage, light-induced translocation occurs within 5 minutes, whereas the second stage requires over 6 hours. Each stage exhibits a distinct apical versus basolateral localization, implicating different transport mechanisms. Genetic analyses suggest that activation of the other light-activated channel TRP and an eye-specific PKC are both required for the second stage of TRPL
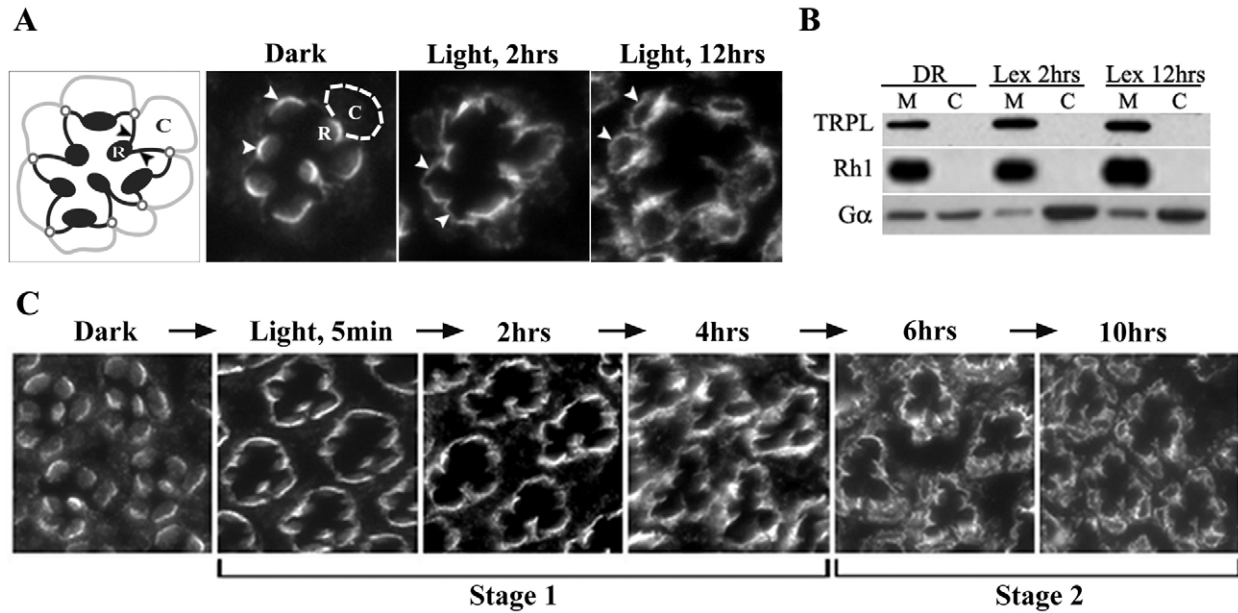
**Fig. 1.** TRPL channels display two stages of light-induced translocation. (A) Diagram of a single ommatidium and cross-sections of single wild-type ommatidia. (Left panel) Single ommatidium illustrating apical and basolateral membranes of the photoreceptor cell. Black features, apical membrane, including the rhabdomere (R) and the supporting stalk membrane (arrowheads); gray, basolateral membrane; ○, adherens junctions separating apical and basolateral membranes. (Right panels) Cross-sections of single wild-type ommatidia, immunostained for TRPL, shown to illustrate typical localization of TRPL channels in the dark, after the first or the second stage of light-induced TRPL translocation. In each ommatidium, seven photoreceptor cells are visible. The rhabdomere (R) and cell body (C) of a single photoreceptor cell are indicated in one ommatidium (Dark); the approximate boundary of the cell body is outlined. TRPL channels are exclusively localized to the rhabdomeres of dark-adapted photoreceptors; arrowheads indicate the concentration of channels at the base of rhabdomeres. After 2 hours of light-exposure (stage 1), TRPL moves into the supporting stalk membrane adjacent to each rhabdomere (arrowheads), forming an apical ring-like localization pattern for each ommatidium. With 12 hours of light-exposure (stage 2), TRPL translocates to the basolateral membrane (arrowheads); light intensity was ~244 lux. Representative ommatidia are shown here and in Figs 3, 5 and 7 from multiple wild-type retinal sections taken from 37 eyes of 35 flies (Dark), 45 eyes of 37 flies (Light, 2hrs), 28 eyes of 26 flies (Light, 12hrs). (B) Representative immunoblot analysis of retinal membrane (M) and cytosolic (C) fractions isolated from wild-type flies that were dark-adapted (DR) or light-exposed for 2 or 12 hours (Lex 2hrs or Lex 12hrs, respectively). In contrast to $G_q\alpha$, which displays a light-dependent shift from membrane to cytosol as previously reported (Cronin et al., 2004), TRPL is found in the membrane fraction during each light condition examined. Rhodopsin (Rh1) was used as a loading control for the membrane fractions. An immunoblot representative for seven different experiments is shown. (C) Time course of the two stages of TRPL-channel translocation from the rhabdomere to the basolateral membrane. Shown are wild-type retinal cross-sections immunostained for TRPL after light-exposures of increasing duration. Dark-raised wild-type flies show rhabdomeric TRPL localization. Within 5 minutes of light-exposure, TRPL rapidly translocates to the stalk membrane, producing the same apical ring-like pattern as seen after 2 and 4 hours of light-exposure; we designate this translocation to the stalk membrane as stage 1. After 6 hours of light-exposure, TRPL is first found localized to the basolateral membrane in some, but not all photoreceptor cells. With 10 hours of light exposure, TRPL is consistently found localized to the basolateral membrane of all photoreceptors. TRPL localization to the basolateral membrane is designated as stage 2 of TRPL translocation. Shown are representative ommatidia from multiple retinal sections, taken from five eyes of five flies (Dark), six eyes of five flies (Light, 5min), seven eyes of six flies (Light, 2hrs), eight eyes of five flies (Light, 4hrs), six eyes of four flies (Light, 6hrs), five eyes of four flies (Light, 10hrs).

translocation, whereas only PLC is required for the first stage. Finally, we show that Arr-2 is required for the localization and stability of TRPL channels.

## Results

### Two stages of light-dependent TRPL-channel translocation

Previously, TRPL channels have been shown to undergo translocation from the rhabdomere to the basolateral membrane of *Drosophila* photoreceptors (Bahner et al., 2002). The underlying mechanism of this translocation, however, remains unknown. Since TRPL channels are integral membrane proteins, we predict that TRPL channels must mobilize in a membrane-restricted manner. Fig. 1B shows that TRPL channels are indeed associated with membrane fractions in the dark and after light-exposure. One possibility is that

TRPL channels are incorporated into vesicles at the base of the rhabdomere, similar to rhodopsin-Arr-2 complexes that accumulate in *norpA* and *rdgC* mutants (Alloway et al., 2000; Kiselev et al., 2000; Orem and Dolph, 2002). Alternatively, TRPL channels might diffuse laterally through the membrane, out of the rhabdomere and into the supporting stalk membrane. To gain insight into how TRPL channels are transported out of the rhabdomere, we set out to examine the light-dependent progression of TRPL translocation in more detail.

We performed immunolocalization studies for TRPL channels in retinal cross-sections from dark-adapted and light-exposed flies. In the dark, TRPL channels consistently localized to the rhabdomeres of photoreceptors, with a higher concentration found at the base of the rhabdomeres (Fig. 1). For all genotypes, flies raised in the dark were indistinguishable from dark-adapted (>10 hours) flies. With

only 5 minutes of light-exposure, TRPL channels translocated out of the rhabdomere and into the adjoining stalk membrane (Fig. 1C). TRPL protein was present both at the base of the rhabdomere and in the stalk membrane, giving an apical, ring-like localization pattern for each ommatidium (Fig. 1). The speed of the light-induced redistribution of TRPL protein suggests that TRPL channels do indeed undergo light-induced translocation, rather than light-induced synthesis and insertion in the stalk membrane. Interestingly, translocated TRPL channels appear to be restricted to the apical membrane since no TRPL immunostaining was present in the basolateral membrane of the photoreceptor cell at this time. We increased the duration of light-exposure and found that at more than 4 hours of light-exposure, translocated TRPL channels were only localized to the stalk membrane, displaying the same apical localization pattern seen after 5 minutes of light-exposure (Fig. 1C). We refer to this localization pattern as the first stage of TRPL-channel translocation (Fig. 1A).

We were, however, puzzled as to why we did not see TRPL translocation to the plasma membrane of the entire cell body – including the basolateral membrane – as previously reported (Bahner et al., 2002). Since this earlier study used light conditions of lower intensity and longer duration (12 hours), we examined whether similar light conditions would induce the translocation of TRPL channels to the basolateral membrane. We found that for light intensities spanning more than three orders of magnitude (from 31 lux to $57 \times 10^3$ lux), TRPL channels still translocated into a similar apical ring-like pattern after 5 minutes of light-exposure (data not shown), suggesting that light intensity was not responsible for the difference in TRPL localization. We found, however, that – for both high- and low-light intensities – TRPL channels localized to the entire plasma membrane, including the basolateral membrane, with longer durations of light-exposure. Although there was some variability from fly to fly, the first appearance of TRPL immunostaining in the basolateral membrane of photoreceptors was observed after 6 hours of light-exposure (Fig. 1C). TRPL displayed consistent localization to the basolateral membrane after 10 hours of light-exposure (Fig. 1C). Thus, longer light-exposure induces TRPL channels to somehow bypass the AJs and localize in the basolateral membrane; we now refer to this as the second stage of TRPL-channel translocation (Fig. 1A). Together, our results show two temporally separable stages of light-induced TRPL translocation with distinct subcellular localization patterns.

We also examined the time-course of TRPL channel relocalization to the rhabdomere with dark-incubation following each stage of light-induced translocation. Wild-type flies were first exposed to light for 2 or 10 hours, inducing translocation to the first or second stage, respectively, and then dark-incubated for increasing times before retinas were sectioned and immunostained for TRPL. Surprisingly, the restoration of TRPL channels to the rhabdomere after the first stage of translocation occurred much more slowly than expected: even after 4 hours of dark incubation, some TRPL staining was still evident in the stalk membrane, and not until 6 hours of dark incubation was TRPL observed exclusively in the rhabdomeres (Fig. 2A). The relocalization of TRPL channels to the apical membrane after the second stage of translocation was first seen after 6 hours of dark incubation, and full recovery of TRPL to the rhabdomeres required 10
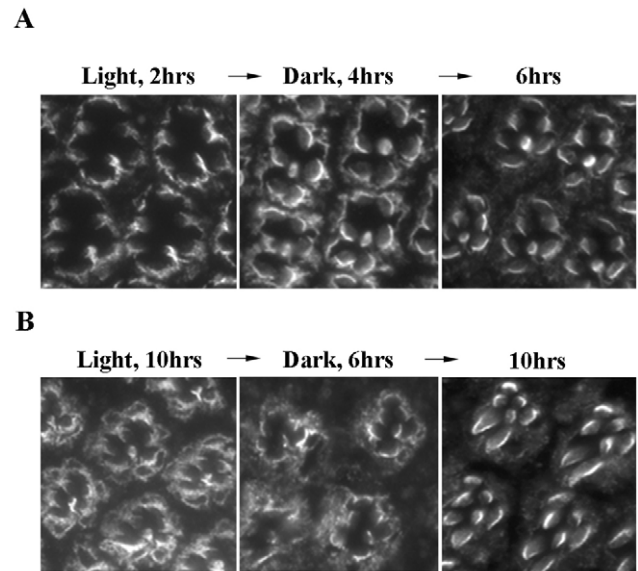


**Fig. 2.** (A,B) Time course of TRPL channel relocalization to the rhabdomere after (A) stage 1 and (B) stage 2 of light-induced translocation. (A) Wild-type flies were light-exposed for 2 hours to induce translocation to the stalk membrane, and then dark-incubated for increasing durations. Full recovery of TRPL localization to the rhabdomere required 6 hours of dark-incubation. Shown are representative ommatidia from multiple retinal sections taken from ten eyes of eight flies (Light, 2hrs), ten eyes of eight flies (Dark, 4hrs), ten eyes of seven flies (Dark, 6hrs). (B) Wild-type flies were light-exposed for 10 hours to induce translocation to the basolateral membrane, and then dark-incubated for increasing durations. After 6 hours of dark-incubation, TRPL gradually appeared in the stalk membrane in some photoreceptor cells. Full recovery of TRPL localization to the rhabdomeres was seen after 10 hours of dark-incubation. For all of the above, wild-type retinal cross-sections were immunostained for TRPL. Shown are representative ommatidia from multiple retinal sections taken from five eyes of three flies (Light, 10hrs), five eyes of four flies (Dark, 6hrs), six eyes of six flies (Dark, 10hrs).

hours of dark incubation (Fig. 2B). Since recovery of TRPL channels to the rhabdomere is quite lengthy from either stage of TRPL localization, it is unclear – based on the reported time of TRPL synthesis (Bahner et al., 2002) – whether TRPL channels do indeed undergo translocation back to the rhabdomere or whether newly synthesized TRPL channels are targeted to the rhabdomeres.

## Signaling the first stage of TRPL translocation is independent of TRP-channel activation

The finding that light-induced translocation of TRPL channels occurs in two distinct stages creates a new framework in which to examine TRPL translocation. We set out to determine which components and events of the phototransduction cascade are required for triggering each stage of translocation. To do this, we examined the localization of TRPL channels in retinal tissue sections from null-mutants of the major rhodopsin (Rh1), the effector PLC and the other light-activated channel TRP ($ninaE^{I17}$, $norpA^{P41}$ and $trp^{P343}$, respectively). All flies were either dark-raised or light-exposed for 2 or 12 hours. $ninaE^{I17}$ mutants display retinal degeneration (Leonard et al.,

1992; O'Tousa et al., 1989) and, although we used young (<24-hour-old) flies to minimize the effects of degeneration, retinal sections still showed significant degeneration, resulting in a higher level of background TRPL immunostaining (see Fig. 3). TRPL immunostaining, however, appeared to be localized primarily in the rhabdomeres of both dark-raised and light-exposed *ninaE*[I17] flies (Fig. 3), suggesting that the light receptor Rh1 is required in signaling both stages of TRPL translocation (Fig. 3).

We next examined the requirement for PLC and TRP channels in the first stage of TRPL-channel translocation. As expected, dark-raised *norpA*[P41] and *trp*[P343] mutants displayed normal rhabdomeric localization of TRPL. After 2 hours of light-exposure, we found that TRPL channels remained entirely rhabdomeric in *norpA*[P41] mutants (Fig. 3), suggesting that activation of the effector PLC is required for the first stage of TRPL translocation. By contrast, we found that TRPL channels translocated normally to the stalk membrane in *trp*[P343] mutants light-exposed for 2 hours, demonstrating that the first stage of TRPL-channel translocation is independent of TRP channels (Fig. 3).

### Activation of TRP channels is essential for the second stage of TRPL-channel translocation

We next investigated whether the second stage of TRPL-channel translocation could be induced in *norpA*[P41] and *trp*[P343] mutants. We found that TRPL channels did not translocate to the basolateral membrane of R1-R6 photoreceptor cells in either mutant after a 12-hour light-exposure. TRPL channels remained exclusively rhabdomeric in *norpA*[P41] mutants, wheras TRPL channels remained restricted in the stalk membrane in *trp*[P343] mutants, similar to TRPL localization after a 2-hour light-exposure (Fig. 3). Clear basolateral staining of TRPL was not observed in any R1-R6 photoreceptor cells from *trp*[P343] mutants after 12 hours of light-exposure. Altogether, in contrast to the first stage of TRPL translocation, these findings suggest that the activation of TRP channels is required for signaling apical to basolateral translocation of TRPL channels.

Conversely, Bahner et al. (Bahner et al., 2002) found that TRPL translocation after a 12-hour light-exposure was independent of TRP-channel activation, based on TRPL immunostaining of *norpA* mutants. These findings, however, were contradicted by their own experiments with a TRPL-eGFP transgenic line ('note added in proof') (Bahner et al., 2002), also indicated by Frechter and Minke (Frechter and Minke, 2006), and Minke and Parnas (Minke and Parnas, 2006). Given this discrepancy, we set out to determine whether activation of TRP channels alone is sufficient to trigger translocation of TRPL channels to the basolateral membrane. To do this, we used the *Trp*[P365] mutant, which contains a gain-of-function mutation in the *trp* gene that results in constitutive activation of TRP channels and subsequent $Ca^{2+}$ influx into the photoreceptor cells (Hong et al., 2002; Yoon et al., 2000). Since *Trp*[P365] mutants display massive photoreceptor degeneration, we used young adult [<44-hours after eclosion (AE)] *Trp*[P365]/+ heterozygous flies, which display an almost normal photoreceptor structure (Hong et al., 2002). We examined whether the one copy of *Trp*[P365] in *Trp*[P365]/+ heterozygotes was sufficient to induce TRPL translocation to the basolateral membrane. We first examined young *Trp*[P365]/+ mutant flies
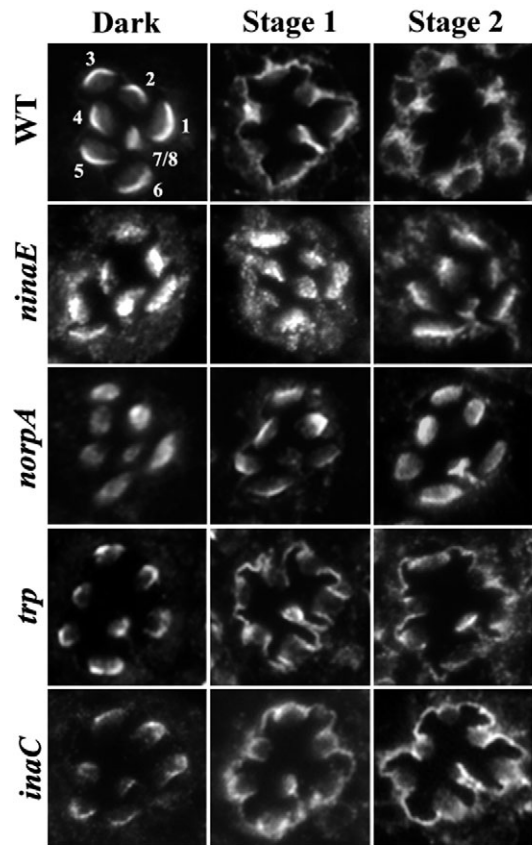


**Fig. 3.** Distinct signaling pathways trigger each stage of TRPL translocation. Shown are representative cross-sections of single wild-type ommatidia and null-mutants of Rh1 (*ninaE*[I17]), PLC (*norpA*[P41]), TRP (*trp*[P343]) and eye-PKC (*inaC*[P109]) immunostained for TRPL. R1 -R7/R8 photoreceptor cells are indicated as1-7/8 in the dark-raised wild-type (WT) ommatidia. (Dark) For all dark-adapted mutants, TRPL channels localized to the rhabdomeres, similar to wild type. Note that, despite using newly eclosed flies, *ninaE* mutants display light-independent retinal degeneration. Because of this degeneration, TRPL immunostaining is not as clear in these mutants as in the others; localization of TRPL in light-exposed flies, however, is no different than in dark-raised flies, suggesting that translocation is blocked in *ninaE* mutants as expected. (Stage 1) After a 2-hour light-exposure, TRPL channels in *trp* and *inaC* mutants translocated to the stalk membrane, similar to wild type, whereas TRPL channels in *ninaE* and *norpA* mutants remained localized in the rhabdomeres. These results signify a requirement for Rh1 and PLC but not TRP or eye-PKC in the first stage of TRPL translocation. (Stage 2) In contrast to wild-type flies, TRPL channels did not translocate to the basolateral membrane in the R1-R6 photoreceptor cells of any of the mutants after a 12-hour light-exposure (see labeling in the WT Stage 2 panel for reference). In *ninaE* and *norpA* mutants, TRPL remained rhabdomeric, whereas in *trp* and *inaC* mutants, TRPL remained restricted to the apical membrane including the stalk membrane. These results show that Rh1, PLC, TRP and eye-PKC are all required for signaling the second stage of TRPL-channel translocation to the basolateral membrane. Shown for each genotype and light condition are representative ommatidia from multiple retinal tissue sections; wild type: see Fig. 1; *ninaE*: 12 eyes of ten flies (Dark), 12 eyes of seven flies (Stage 1), 13 eyes of eight flies (Stage 2); *norpA*: 17 eyes of 14 flies (Dark), 28 eyes of 21 flies (Stage 1), 28 eyes of 21 flies (Stage 2); *trp*: 17 eyes of 13 flies (Dark), six eyes of five flies (Stage 1), 13 eyes of nine flies (Stage 2); *inaC*: seven eyes of four flies (Dark), 14 eyes of nine flies (Stage 1), 12 eyes of seven flies (Stage 2).

(18-22 hours AE) that had been raised in complete darkness. We found that TRPL channels were indeed localized to the basolateral membrane (Fig. 4). To determine whether TRPL channels were initially trafficked and localized to the rhabdomeres of $Trp^{P365}$/+ mutants, we examined dark-raised, newly eclosed (<4 hours AE) $Trp^{P365}$/+ mutants. We found that, indeed, TRPL channels displayed rhabdomeric localization (Fig. 4), demonstrating that TRPL channels in $Trp^{P365}$/+ mutants do not exhibit any defect in trafficking TRPL channels to the rhabdomeres. Taken altogether, we propose that TRP channel activation is both essential and sufficient for signaling apical to basolateral mobilization of TRPL channels.

### Eye-PKC is also required for signaling leading to the second stage of TRPL-channel translocation

To further investigate the signaling pathway that triggers the second stage of TRPL-channel translocation, we sought to identify the signaling events downstream of TRP channel activation. Since activation of TRP channels leads to an influx in $Ca^{2+}$ ions, we tested whether eye-PKC, which is activated by $Ca^{2+}$ and diacyl glycerol (DAG), is also required for signaling the translocation of TRPL channels to the basolateral membrane. Although eye-PKC has been shown to function in deactivation of the light-response and light-adaptation (Hardie et al., 1993; Smith et al., 1991), the exact mechanisms of action as well as additional roles of eye-PKC are unknown. We examined eye-PKC null-mutants ($inaC^{P209}$) that were dark-raised, or light-exposed for 2 or 12 hours. Similar to $trp^{P343}$ mutants, TRPL channels in R1-R6 photoreceptors cells of $inaC^{P209}$ mutants were localized to the rhabdomeres in the dark and translocated to the stalk membrane with 2 hours of light, but were unable to translocate to the basolateral membrane after 12 hours of light exposure (Fig. 3). These results indicate that eye-PKC is required for the second stage of TRPL-channel translocation in R1-R6 photoreceptor cells, specifying perhaps a signaling pathway for basolateral localization that is distinct from the first stage of TRPL translocation.

### Arr-2 is required for the rhabdomeric localization of TRPL channels

In the dark, TRPL channels are anchored exclusively in the rhabdomere by a yet unknown mechanism. One possibility is that light-exposure triggers the disruption or release of this anchor, thus allowing TRPL channels to diffuse out of the rhabdomere and into the stalk membrane during the first stage of TRPL translocation. We hypothesize that there is a light-dependent scaffold protein for TRPL channels in the rhabdomere. Although the scaffolding protein INAD (inactivation-no-afterpotential-D) is required for the rhabdomeric localization of some phototransduction components (reviewed in Hardie and Raghu, 2001; Tsunoda and Zuker, 1999), TRPL channel localization is not affected by the loss of INAD (Tsunoda et al., 1997). Since arrestin proteins in other systems have recently been shown to function as molecular scaffolds (Miller and Lefkowitz, 2001; Perry and Lefkowitz, 2002), we tested whether either of the two photoreceptor-specific arrestin proteins Arr-1 and Arr-2 (Hyde et al., 1990; LeVine et al., 1990; Smith et al., 1990; Yamada et al., 1990), are involved in the localization of TRPL channels in the rhabdomere. We performed immunolocalization studies
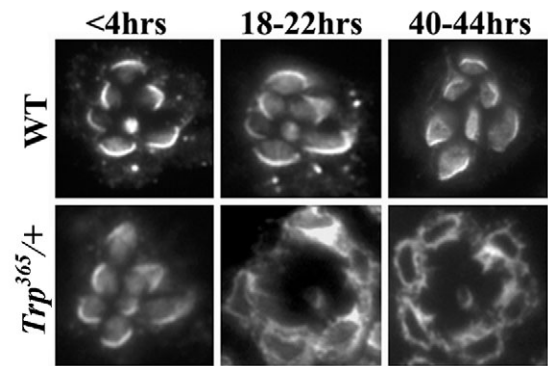


**Fig. 4.** The second stage of TRPL translocation is induced in dark-raised $Trp^{P365}$/+ mutants. Shown are representative cross-sections of single ommatidia from dark-raised wild-type (WT) and $Trp^{P365}$/+ newly eclosed adult flies (<4 hours old), 18-22 hours after eclosion (AE), and 40-44 hours AE, immunostained for TRPL. Wild-type photoreceptors exhibited rhabdomeric localization of TRPL channels in the dark, regardless of age. $Trp^{P365}$/+ flies less than 4 hours old also displayed rhabdomeric TRPL localization, indicating that TRPL channels are initially trafficked to the rhabdomeres similar to wild type. By 18-22 hours AE, TRPL channels in $Trp^{P365}$/+ mutants had translocated to the basolateral membrane of photoreceptors. Shown for each genotype are representative ommatidia from multiple retinal tissue sections; wild-type: two eyes of two flies (<4hrs AE), two eyes of two flies (18-22hrs AE), three eyes of two flies (40-44hrs AE); $Trp^{P365}$/+: five eyes of five flies (<4hrs AE), five eyes of five flies (18-22hrs AE), six eyes of five flies (40-44hrs AE).

for TRPL channels in retinal tissue sections from dark-adapted $arr1$ and $arr2$ mutants. We first examined the $arr1^1$ mutant allele, which expresses ~10% of wild-type levels of Arr-1 protein (Dolph et al., 1993). We found that TRPL channels displayed clear rhabdomeric localization (Fig. 5A), suggesting that Arr-1 is not required for the rhabdomeric localization of TRPL channels. We next examined a null mutant for Arr-2, $arr2^5$. In contrast to $arr1^1$ mutants, TRPL channels in dark-raised $arr2^5$ null-mutants were severely mislocalized (Fig. 5A). These results show that Arr-2 is required for the rhabdomeric localization of TRPL channels in the dark. Interestingly, the subcellular localization of TRPL channels in dark-raised $arr2^5$ mutants was strikingly similar to the localization pattern of TRPL channels after the first stage of light-induced translocation in wild-type photoreceptors (Fig. 1, Fig. 5A).

Since Arr-2 contains an extended C-terminal tail that is absent from Arr-1 (LeVine et al., 1990), we tested whether the C-terminal tail of Arr-2 confers the rhabdomeric localization of TRPL channels. To do this, we examined the $arr2^1$ mutant allele, which encodes a truncated Arr-2 protein with the last 46 amino acids of its C-terminus deleted (Dolph et al., 1993). We found, however, that TRPL channels were localized in the rhabdomeres of dark-adapted $arr2^1$ mutants similar to wild-type (Fig. 5), indicating that the C-terminal tail of Arr-2 is not required for the rhabdomeric localization of TRPL channels. Future structure-function studies should reveal how Arr-2 functions in the rhabdomeric localization of TRPL channels.

To examine whether Arr-1 or Arr-2 play a role in the translocation of TRPL channels, we exposed $arr1^1$, $arr2^5$ and $arr2^1$ mutants to 2 and 12 hours of light. With 2 hours of light-

**A**



**B**
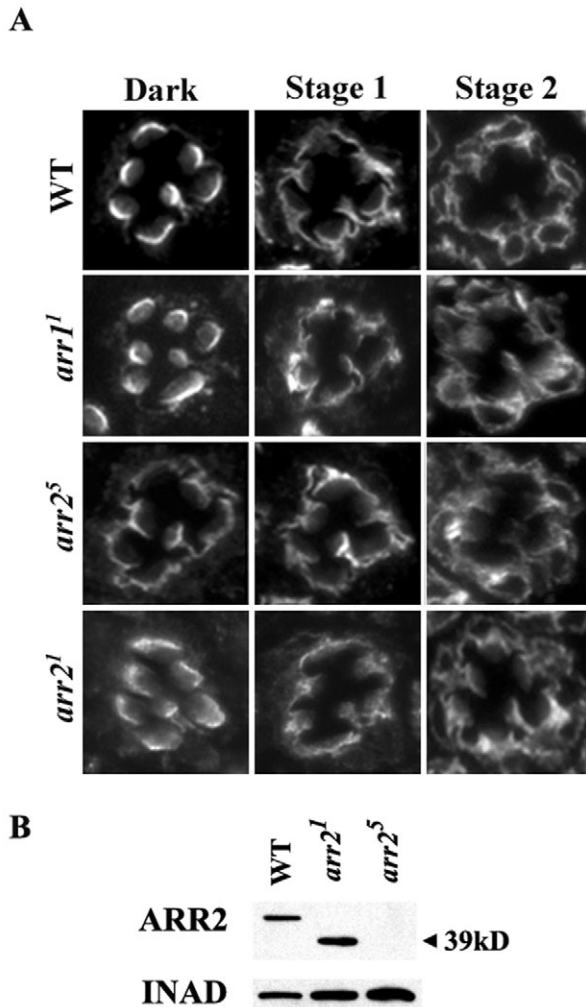


**Fig. 5.** Arr-2 is required for rhabdomeric localization of TRPL channels. (A) Shown are representative cross-sections of single ommatidia from wild-type, *arr1¹*, *arr2⁵*, and *arr2¹* mutants that were either dark-adapted (Dark), or light-exposed for 2 hours (Stage 1) or 12 hours (Stage 2), and then immunostained for TRPL. Whereas wild-type flies, *arr1¹* and *arr2¹* mutants displayed rhabdomeric localization of TRPL in the dark, *arr2⁵* mutants exhibited a mislocalization of TRPL channels in a pattern similar to TRPL channels in wild-type photoreceptors after a 2-hour light-exposure. All arrestin mutants displayed TRPL staining in the stalk membrane after a 2-hour light-exposure and TRPL staining in the basolateral membrane after a 12-hour light-exposure. For stage 1, *arr2⁵* mutants were light-exposed using a $50.7 \times 10^3$ lux white-light source. Shown for each genotype and light condition are representative ommatidia from multiple retinal tissue sections; wild-type: see Fig. 1; *arr1¹*: five eyes of four flies (Dark), six eyes of five flies (Stage 1), four eyes of three flies (Stage 2); *arr2⁵*: 19 eyes of 13 flies (Dark), five eyes of five flies (Stage 1), eight eyes of seven flies (Stage 2); *arr2¹*: 11 eyes of ten flies (Dark), eight eyes of six flies (Stage 1), eight eyes of six flies (Stage 2). (B) Representative immunoblot showing the presence of wild-type Arr-2 protein and the C-terminal truncated Arr-2 (~39 kD), expressed in wild type and *arr2¹* mutants, respectively. The immunoblot was probed with a polyclonal antibody against the N-terminal sequence of Arr-2 (see Materials and Methods). Anti-INAD was used as a loading control.

exposure, all of the arrestin mutants displayed TRPL localization similar to that observed in dark-adapted *arr2⁵* mutants. After 12 hours of light-exposure, we found that TRPL channels were able to translocate to the basolateral membrane in *arr1¹*, *arr2⁵* and *arr2¹* mutants (Fig. 5). These results demonstrate that Arr-2 functions in the maintenance of TRPL channels in the rhabdomeres in the dark, but is not required for their translocation to the basolateral membrane.

### Arr-2 is required for the stability of TRPL channels

The localization of TRPL channels in *arr2⁵* mutants is not only similar to the localization of TRPL channels in the first stage of translocation in wild-type photoreceptors, but it is also reminiscent of the mislocalization of TRP channels in *inaD* null-mutants. In these mutants, TRP channels are primarily located in the stalk membrane flanking each rhabdomere (Tsunoda et al., 1997). These similar localization patterns suggest that the relationship between Arr-2 and TRPL channels is analogous to the relationship between INAD and TRP channels. Since TRP protein levels decay with age in *inaD* mutants (Tsunoda et al., 1997), we examined whether the same is true for TRPL protein levels in *arr2⁵* mutants. Because *arr2⁵* mutants display light-dependent degeneration (Dolph et al., 1993), flies were raised in the dark to prevent degeneration. Indeed, we found that levels of TRPL protein in *arr2⁵* mutants

declined from newly eclosed flies to 10-day-old flies, whereas TRPL levels remained unchanged in wild-type flies (Fig. 6). By contrast, levels of other transduction proteins, including rhodopsin and eye-PKC, remain stable in wild-type flies and *arr2⁵* mutants (Fig. 6). Thus, Arr-2 is required for both the rhabdomeric localization and stability of TRPL channels.

### Does Arr-2 also function in the trafficking of TRPL channels to the rhabdomere?

To gain insight into whether Arr-2 also plays a role in the targeting and/or trafficking of TRPL channels to the rhabdomeres, we performed immunolocalization studies for TRPL in wild-type and *arr2⁵* mutant flies at an earlier developmental stage, when TRPL is first trafficked to the rhabdomeres. At 72 hours after puparium formation (APF; 25°C), the rhabdomeres of photoreceptors were developed and visible but little to no TRPL was present in either wild-type or *arr2⁵* mutant rhabdomeres. Low levels of TRPL signal were observed only in the cell body of photoreceptors (Fig. 7). At 90 hours APF, TRPL was clearly localized to the rhabdomeres of wild-type photoreceptors (Fig. 7). In *arr2⁵* mutants, we observed staining primarily in the rhabdomeres of photoreceptors, with some punctate staining outside of the rhabdomeres; the clear apical localization pattern found in the ommatidia of newly eclosed *arr2⁵* flies was not observed in *arr2⁵* pupae (Fig. 7). Although the role of Arr-2 in the targeting and/or trafficking of TRPL channels to the rhabdomeres is still unclear, some TRPL protein in the late *arr2⁵* pupae can be targeted and trafficked to the rhabdomeres, suggesting that Arr-2 plays its primary role in the maintenance or anchoring of TRPL channels in the rhabdomeres.

### Discussion

In this study, we show that the light-induced translocation of TRPL channels from the rhabdomere to the cell body occurs in two temporally and genetically separable stages. Each stage
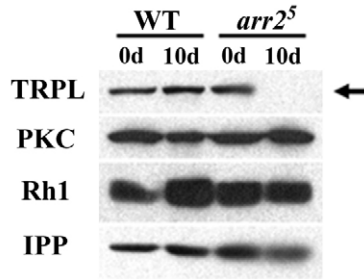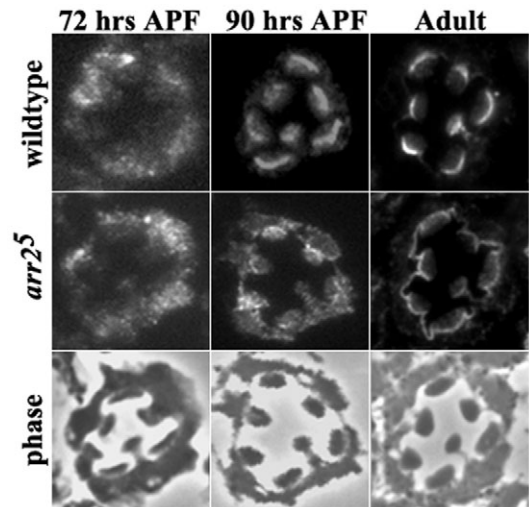
**Fig. 6.** (A) Arr-2 is required for the stability of TRPL channels. Representative immunoblot of TRPL, eye-PKC (PKC), rhodopsin (Rh1), and inositol polyphosphate 1-phosphatase (IPP) in newly eclosed (0d) and 10 day old (10d) dark-raised wild type (WT) and *arr2^5* mutant (*arr2^5*). In *arr2^5* mutants is an age-dependent decline in the steady-state level of TRPL protein. By contrast, levels of eye-PKC and rhodopsin remain constant in wild type and *arr2^5* mutant. Anti-IPP was used on all blots as a loading control. Representative immunoblot from a total of 20 different experiments.



**Fig. 7.** The localization of TRPL channels in *arr2^5* pupae. Representative cross-sections of single ommatidia from dark-adapted wild type and *arr2^5* null-mutants at 72 hours and 90 hours APF, and post-eclosion, immunostained for TRPL. At 72 hours APF, the rhabdomeres of photoreceptors were visible (phase) with low levels of TRPL staining present in the cell body of photoreceptors. At 90 hours APF, TRPL was localized at the base of the rhabdomeres of wild-type photoreceptors, similar to the adult. At 90 hours APF, TRPL staining in the *arr2^5* pupae appeared in both the rhabdomeres and dispersed in the cell body. This is in contrast to the adult *arr2^5* null-mutant, which displayed TRPL staining in the stalk membranes and at the base of the rhabdomeres. Interestingly, puncta of TRPL staining were present in *arr2^5* pupae 90 hours APF that were not seen in wild type. The corresponding phase-contrast images for the *arr2^5* mutants are shown below (phase). Shown for each genotype and light condition are representative ommatidia from multiple retinal tissue sections; wild-type: five eyes of four flies (72hrs APF), six eyes of five flies (90hrs APF); *arr2^5*: four eyes of three flies (72hrs APF), seven eyes of six flies (90hrs APF). For number of eyes and flies used for adult ommatidia, see Figs 1 and 5.

is marked by a distinct subcellular localization. In the first stage, which occurs within only 5 minutes and continues for over 4 hours, TRPL channels migrate to the stalk membrane flanking each rhabdomere and appear restricted to the apical membrane. In the second stage, TRPL channels are localized to the basolateral membrane. Our genetic analysis showed that the first stage of TRPL-channel translocation is dependent on the phototransduction effector protein PLC, but not the downstream TRP channels. Activation of PLC might be crucial for $PIP_2$ hydrolysis, the creation of second messengers and/or the activation of TRPL channels themselves. Future studies will need to determine which of these options signal the release of TRPL channels from the rhabdomere, permitting translocation into the stalk membrane.

For the second stage of light-induced TRPL translocation, we propose that activation of TRP channels is both essential and sufficient for signaling TRPL channel mobilization from apical to basolateral membrane. In addition, we show that eye-PKC is required for this mobilization. Our results specify a light-induced signaling pathway for the second stage of TRPL-channel translocation that is distinct from that of the first stage. One possibility is that influx of $Ca^{2+}$ through the more $Ca^{2+}$-selective TRP channels activates eye-PKC, whose targets are likely to play key roles in the mobilization of TRPL channels to the basolateral membrane. Since the major role of eye-PKC has been thought to be in the deactivation of the phototransduction cascade, studies have investigated the phosphorylation of known phototransduction components by eye-PKC, identifying TRP and INAD as targets (Huber et al., 1996; Liu et al., 2000). Future studies, however, searching for new targets of eye-PKC, might reveal important players and mechanisms involved in the transport of TRPL channels to the basolateral membrane. Fig. 8 illustrates a model of the two stages of light-induced TRPL translocation and the signaling proteins required for each stage.

The subcellular localization of TRPL channels during these two stages of translocation suggests two distinct mechanisms of transport. The mobilization of TRPL channels from the rhabdomere into the adjacent stalk membrane is significant because it indicates that TRPL channels do not translocate directly from the rhabdomere to the basolateral membrane – as might have been expected. Instead, TRPL channels appear to diffuse laterally from the rhabdomeric membrane directly into the neighboring stalk membrane. There, TRPL channels appear to be restricted to the apical plasma membrane by the AJs. In the second stage of translocation, which occurs with longer light-exposures, TRPL channels must negotiate their way past the AJs by some other mechanism and localize in the basolateral membrane of the photoreceptor. One possibility is that TRPL channels are incorporated into vesicles from the apical membrane, transported to the basolateral membrane where they are then reinserted into the membrane.

The two stages of light-induced TRPL-channel translocation may also serve different physiological functions. For example, TRPL channels in the stalk membrane may undergo bi-directional transport as a mechanism for regulating the number of channels available for signaling, whereas TRPL channels in the basolateral membrane may be targeted for degradation. The extended duration of dark-incubation required for full recovery of TRPL channels to the rhabdomeres from either stage was unexpected and thus allows for the possibility that TRPL channels do not undergo translocation back to the
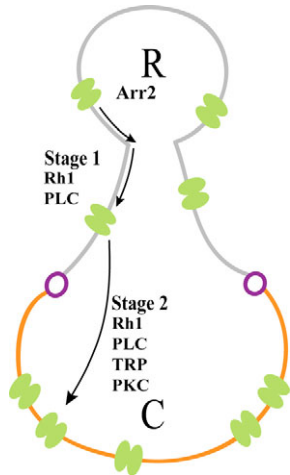
**Fig. 8.** Proposed model for the two stages of light-induced TRPL-channel translocation. A diagram of a single photoreceptor cell with rhabdomere (R) and cell body (C) is shown. The apical (gray) and basolateral (orange) membranes are also indicated. Arr-2 is required for maintaining TRPL channels (green) in the rhabdomeres. During Stage 1, TRPL channels are released from the rhabdomere and translocate by lateral diffusion into the stalk membrane, where the channels are restricted to the apical membrane by the AJs (open circles). Stage 1 requires Rh1 and the effector PLC. In Stage 2, TRPL channels bypass the AJs and translocate to the basolateral membrane. Stage 2 requires Rh1, PLC, TRP and eye-PKC.

rhabdomeres. These results also suggest that TRPL translocation plays physiological roles other than light-adaptation.

In this report, we also demonstrate that TRPL channels are mislocalized and unstable in *arr2* null-mutants. How does Arr-2 function in the localization of TRPL channels? Interestingly, the localization of TRPL channels in *arr2* mutants is similar to the localization of TRPL channels after the first stage of light-induced translocation in wild-type photoreceptors. This observation suggests that the requirement for Arr-2 in the rhabdomeric localization of TRPL channels is mechanistically tied to the first stage of light-induced translocation. That is, when Arr-2 is not present to maintain TRPL channels in the rhabdomere, TRPL channels appear to undergo the same migration as in the first stage of light-dependent translocation. One possibility is that Arr-2 functions as part of a light-dependent anchoring complex for TRPL channels in the rhabdomere. The dissolution of this anchor may then allow free diffusion of TRPL channels in the apical membrane, leading to the apical localization pattern seen after the first stage of TRPL translocation in wild-type flies and dark-incubated *arr2* null-mutants.

The classical function of arrestin proteins is to deactivate GPCRs: visual arrestins deactivate meta-rhodopsin, whereas β-arrestins deactivate β-adrenergic receptors (Arshavsky, 2002; Claing et al., 2002; Dolph, 2002; Pierce and Lefkowitz, 2001). Recent studies have shown that arrestin proteins can also function as molecular scaffolds (Miller and Lefkowitz, 2001; Perry and Lefkowitz, 2002). The relationship between Arr-2 and TRPL is complicated, however, by the fact that Arr-2 and TRPL undergo light-dependent translocation between the rhabdomere and cell body in opposite directions (Kiselev et al.,

2000; Lee et al., 2003). Although a previous study has found that 35% of Arr-2 is present in the rhabdomeres of dark-adapted photoreceptors (Lee et al., 2003) that could, in theory, act as a scaffold for TRPL channels, no such binding between Arr-2 and TRPL has yet been detected. Another possibility is that Arr-2 functions indirectly in the rhabdomeric localization of TRPL channels. β-arrestin, which binds and deactivates the β2 adrenergic GPCRs, has been shown to recruit the receptor tyrosine kinase Src, thereby establishing a link with MAP kinase signaling pathways (Luttrell et al., 1999; Luttrell and Lefkowitz, 2002; Miller and Lefkowitz, 2001; Pierce and Lefkowitz, 2001; Zuker and Ranganathan, 1999). Thus, another possibility is that the loss of Arr-2 affects other signaling proteins in photoreceptors that might interact with Arr-2 and play a role in the localization of TRPL channels.

The major phenotype of *arr2* mutants is impaired deactivation of active metarhodopsin (Dolph et al., 1993). Taking the results of this study, we now know that *arr2* mutants also exhibit a loss of TRPL channels from the rhabdomeres of photoreceptors, which may have additional, more subtle, effects on the *arr2* phenotype. Since *trpl* mutants display defects in light-adaptation (Leung et al., 2000) and the light-dependent translocation of TRPL channels has been shown to correlate with long-term light-adaptation (Bahner et al., 2002), defects in adaptation displayed by *arr2* mutants (Lee et al., 2003) might be partially due to the loss of TRPL channels from the rhabdomeres.

Recent findings in vertebrates and invertebrates have shown that the subcellular localization of some phototransduction components is in fact dynamic. These studies have shed new light on how signaling is regulated by the subcellular translocation of transduction components. The identification of two distinct stages of TRPL translocation has given us a framework in which to examine the signaling pathways triggering each stage of TRPL translocation, and the transport mechanisms involved. Future studies are likely to identify additional signaling and structural proteins involved in these processes, as well as the different physiological roles played by each stage of translocation.

## Materials and Methods

### Fly stocks

### Antibodies

To generate an N-terminal Arr-2 and TRPL polyclonal antibody, we used peptide sequences corresponding to Arr-2 residues 9-25 (KKATPNGKVTFYLGRRD) and TRPL residues 1083-1097 (DNSNFDIHVVDLDEK); an additional N-terminal cysteine was added to each peptide for conjugation to the KLH carrier protein. Immunization of rabbits was performed by ProSci, Inc. (Poway, CA). Sera were ammonium sulfate precipitated and affinity-purified. Specificity was tested using wild-type and null mutant controls for both tissue sections and immunoblots. The Arr-2 C-terminal antibody (Dolph et al., 1993), and antibodies against eye-PKC, rhodopsin, INAD and IPP were used as previously described (Tsunoda et al., 1997).

### Immunolocalization studies

Flies were placed under a white-light source (Lambda LS 175W Xenon-arc lamp with 400-700 nm bandpass filter, Sutter Instruments, Novato, CA, or equivalent), at ~$57 \times 10^3$ lux (unless otherwise indicated), for given times. Light intensity was measured by an EXTECH 403125 digital light-meter. All experiments were conducted at 24°C. After illumination, fly heads were fixed in 3% paraformaldehyde

in PBS, washed four times with PBS, infiltrated with 2.3 M sucrose overnight at 4°C, and frozen on stubs in liquid nitrogen as previously described (Cronin et al., 2004). 1-1.5 µm thick sections were cut from retinas using a Leica Ultracut UCT with EM FCS cryo unit at –82°C (Leica Microscopy and Scientific Instruments Group, Heerbrugg, Switzerland). Dark-adapted flies and pupae were fixed under a dim red light before sectioning. Sections were blocked in 1% BSA, 0.1% sapponin in PBS for 30 minutes and immunostained as previously described (Cronin et al., 2004). Anti-TRPL antibody (1:500) was used overnight at 4°C, followed by Rhodamine-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Jackson ImmunoResearch, West Grove, PA) (1:200, 1 hour, room temperature). Slides were mounted with 90% glycerol and p-phenylenediamine (Sigma Aldrich, St Louis, MO).

## Membrane and cytosol isolation
For each light condition, 30 fly heads were collected on minutien pins, placed in Eppendorf tubes, and stored at –80°C. Membranes were separated from cytosol as previously described (Cronin et al., 2004).

## SDS-PAGE and immunoblot analysis
10% polyacrylamide gels were used for all immunoblot analyses. Samples contained three fly heads sonicated in 20 µl SDS loading buffer (Figs 5, 6) or membrane and/or cytosol isolated from the equivalent of 3.75 fly heads (Fig. 1) as described above. Protein was separated by standard SDS-PAGE, transferred to nitrocellulose membranes that were subsequently blocked in 5% dried milk in PBS and probed with the indicated antibodies.

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