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Immunocytochemical Labeling of Rhabdomeric Proteins in *Drosophila* Photoreceptor Cells Is Compromised by a Light-dependent Technical Artifact

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Summary
Drosophila photoreceptor cells are employed as a model system for studying membrane protein transport. Phototransduction proteins like rhodopsin and the light-activated TRPL ion channel are transported within the photoreceptor cell, and they change their subcellular distribution in a light-dependent way. Investigating the transport mechanisms for rhodopsin and ion channels requires accurate histochemical methods for protein localization. By using immunocytochemistry the light-triggered translocation of TRPL has been described as a two-stage process. In stage 1, TRPL accumulates at the rhabdomere base and the adjacent stalk membrane a few minutes after onset of illumination and is internalized in stage 2 by endocytosis after prolonged light exposure. Here, we show that a commonly observed crescent shaped antibody labeling pattern suggesting a fast translocation of rhodopsin, TRP, and TRPL to the rhabdomere base is a light-dependent antibody staining artifact. This artifact is most probably caused by the profound structural changes in the microvillar membranes of rhabdomeres that result from activation of the signaling cascade. By using alternative labeling methods, either eGFP-tags or the self-labeling SNAP-tag, we show that light activation of TRPL transport indeed results in fast changes of the TRPL distribution in the rhabdomere but not in the way described previously. (J Histochem Cytochem 67: 745–757, 2019)

Keywords
immunocytochemistry, protein transport, rhodopsin, SNAP-tag, TRP ion channel, vision

Introduction

Immunocytochemistry is the method of choice for determining the subcellular localization of proteins. The currently used techniques in *Drosophila* vision research typically involve cryosections, ultrathin plastic sections, or whole mounts.[1–4] For detection of bound primary antibodies, fluorescently labeled secondary antibodies are employed which compared to enzymatic staining protocols allow high spatial resolution and simultaneous labeling of multiple proteins by using different fluorophores. It is generally assumed that the observed fluorescent signals reflect adequately the localization of the respective proteins. However, for some components of the *Drosophila* phototransduction cascade striking differences between immunocytochemical studies using fluorescence microscopy and immunogold studies using electron microscopy have been observed.[5–8]

Drosophila photoreceptor cells are a well-established model for studying membrane protein

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transport.[9] The *Drosophila* compound eye is made up of ca. 800 single units, called ommatidia. Each ommatidium is composed of a lens, eight photoreceptor cells (PRCs) R1-8, and accessory cells.[10–12] PRCs are elongated cells that form a rod-like structure, the rhabdomere, containing ca. 40,000 densely packed microvilli. The rhabdomere is positioned along the longitudinal axis of the apical surface of the PRC. Microvilli of *Drosophila* PRCs are fingerlike membrane protrusions of approximately 12 μm in length and 60 nm in diameter which are connected to the cell body through an even narrower base.[10,13] On its edges the rhabdomeric membrane connects to the stalk membrane which is physically separated from the basolateral plasma membrane by adherens junctions at the zonula adherens. While rhabdomeres of R1-6 cells are arranged in a trapezoidal pattern, R7 and R8 cells form the apical and distal portion, respectively, of a common rhabdomere in the center of the ommatidium. In cross sections through the compound eye, seven almost round rhabdomeres of R1-6 cells and either R7 or R8 are observed (Fig. 1A and B). Light that hits the compound eye is directed by the lenses toward the rhabdomeres where it activates rhodopsin. Activated rhodopsin initiates the phototransduction cascade by activating the heterotrimeric visual G protein Gq. The G protein α subunit dissociates from the $\beta\gamma$ subunit, binds to and activates phospholipase Cβ (PLC). PLC in turn cleaves phosphatidylinositol-4,5-bisphosphate (PIP_2) which ultimately triggers opening of the ion channels TRP and TRPL resulting in cation influx and PRC depolarization.[13] Together with photoreceptor-enriched protein kinase C, TRP and PLC are bound in a signaling complex assembled by the PDZ domain containing scaffold protein INAD.[7,14] Presumably due to its interaction with the INAD signaling complex, TRP remains anchored within the rhabdomeric membrane and does not undergo translocation to the cell body upon illumination.

Upon antibody staining of tissue cross sections one would expect that the labeling pattern for proteins embedded in the microvillar photoreceptor membrane perfectly overlaps with the round structure of the rhabdomeres. Indeed, electron microscopy studies using immunogold labeling with antibodies directed against the major rhodopsin Rh1 or the ion channels TRP or TRPL suggest that these proteins are distributed throughout the microvillar membrane and fill the entire rhabdomere.[5–8] In sharp contrast, in immunohistochemical studies fluorescence labeling of the same proteins often reveals a crescent shaped signal at the inner rim of the rhabdomeres, suggesting that these proteins are not evenly distributed throughout the rhabdomere but are concentrated at its rim.[1,4,15–22] In a past report, the apparent localization of TRP at the inner rim of the rhabdomere has been taken as evidence for close proximity of TRP channels to submicrovillar cisternae, thus supporting the meanwhile abandoned hypothesis of a capacitative gating mechanism for TRP via light-triggered Ca^2+ release from these cisternae.[21] Since the crescent labeling pattern appears inconsistently across studies, this phenomenon has since been hypothesized to be an artifact of antibody staining and not representing the actual distribution of the respective proteins within the rhabdomere.[15,16,23] The proposed explanation for this staining pattern states that antibodies might have difficulty to access the tightly packed spaces inside the microvilli in which their specific epitope is located.[15,23] This interpretation is supported by results showing that crescent shaped staining is more prevalent in whole mounts than in cryosections of PRCs.[16,23] The putative staining artifact is particularly problematic when studying the translocation of proteins from the rhabdomere to the cell body, for example, light-induced internalization of Rh1 or TRPL.[1,17,22] Interestingly, labeling of Rh1 is more likely to show the crescent shaped pattern when flies are light-exposed as compared to dark-adapted flies.[3] This has been interpreted as evidence that rhodopsin moves to the base of the rhabdomere upon light exposure from where a fraction of activated rhodopsin becomes internalized by endocytosis.[20] TRPL has been shown to translocate from the rhabdomere to the cell body upon illumination.[15] Previously, the internalization of TRPL has been described as a two-stage process, in which TRPL moves to the stalk membrane as well as to the base of the microvilli within a few minutes after illumination (stage 1) and then becomes internalized into the cell body over the course of several hours (stage 2).[1,17]

Because of the possibility of a labeling artifact inherent to antibody detection of rhabdomeric proteins we compared antibody labeling with alternative methods. We asked whether the reported fast light-triggered redistribution of phototransduction proteins in the rhabdomere is due to an artifact. In the present study, we simultaneously use antibody staining directed against rhabdomeric proteins and labeling by fluorescent proteins or self-labeling tags to investigate subcellular localization. We show that the frequently observed crescent shaped antibody staining indeed is an artifact. This artifact is enhanced by light-activation of the phototransduction cascade, presumably as a result of microvillar contraction. However, we also detect a fast, light-triggered redistribution of TRPL in the rhabdomere using alternative detection methods. In the light of these results, we advocate the use of alternative visualization methods for the investigation of distribution and translocation behavior of rhabdomeric proteins—most importantly during the very first

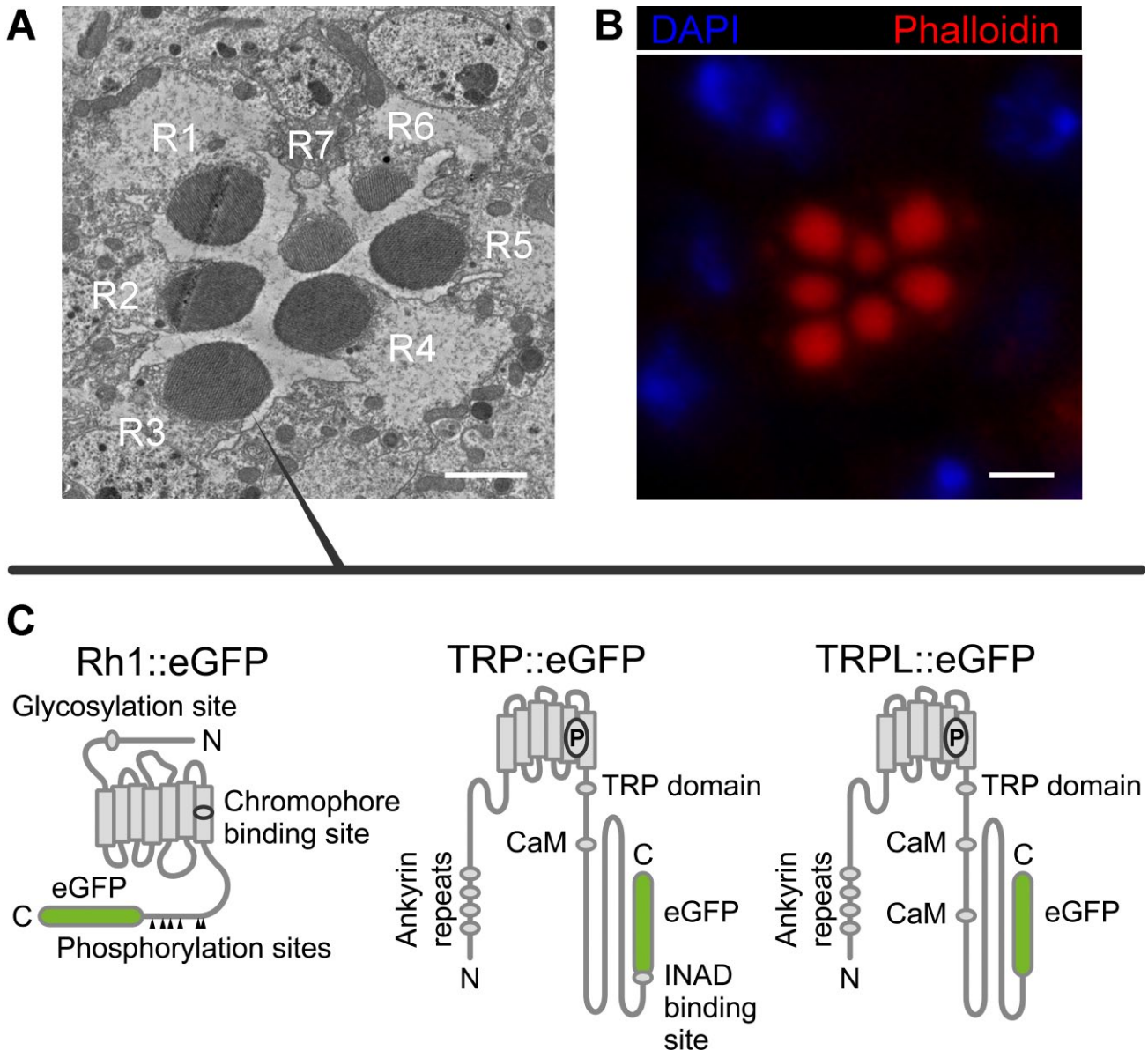


Figure 1. Structure and arrangement of rhabdomeres from *Drosophila* photoreceptor cells. (A) Transmission electron micrograph of a cross section through photoreceptor cells R1-7 from wild type flies as indicated. (B) Light microscopic fluorescence image of an ommatidial cross section from wild type flies stained with Alexa Fluor 546 conjugated phalloidin (red) and DAPI (4',6-diamidino-2-phenylindole, blue) to visualize the rhabdomeric actin cytoskeleton and nuclei of corresponding photoreceptor cells, respectively. (C) Schematics of fluorescence-tagged phototransduction proteins, rhodopsin Rh1 and ion channels TRP (transient receptor potential) or TRPL (TRP-like) that are expressed in photoreceptor cells R1-6 and were used in this study. INAD, inactivation no afterpotential D. Scale bar represents 2 μm.

minutes after application of a light stimulus—to avoid misinterpretations due to antibody staining artifacts.

Materials and Methods

Fly Stocks

Flies were reared on standard cornmeal food and kept at 25C. For dark adaptation, 13-day-old flies were kept in complete dark for 24 h. Internalization was induced by illumination with orange light of wavelength >560 nm (76 μW/cm^2 measured at 590 nm) for the indicated time. Orange light was used because TRPL is most effectively translocated in this light quality. In white or blue light TRPL internalization competes with Rh1 internalization and is less effective.[1] Fly stocks used: Oregon R *w** (referred to as wild type), *y* w*; trpl*^{302}, *y* w*;; trp*^{P343}, *w* norpA*^{P24} (#9048; Bloomington Drosophila Stock

Center, Bloomington, IN), *y* w*;; P[rh1>TRPL::eGFP y+], y* w*; P[rh1>TRP::eGFP y+], P[rh1>Rh1::eGFP w+], y* w*; P[rh1>TRPL::SNAP y+]*.[8,24–27] All combinations and recombinations of these mutant or transgenic alleles were generated by standard genetic methods and confirmed by immunoblotting, electroretinography, or specific PCR genotyping.

Generation of Flies Expressing TRPL::SNAP

The self-labeling variant of TRPL (TRPL::SNAP) was based on a previously reported TRPL::eGFP fusion construct in which the C-terminal GFP-tag was exchanged for a SNAP-tag (New England Biolabs, Frankfurt a.M., Germany).[24] To this end, the coding sequence for SNAP was PCR amplified from the corresponding vector pSNAPf to generate a 5'-*Nco*I and a 3'-NotI restriction site. The SNAP-tag sequence was ligated as a 0.6 kb *Nco*I/*Not*I fragment into a likewise digested *TRPL::eGFP* carrying pENTR vector (Thermo Fisher Scientific, Karlsruhe, Germany), thus replacing the GFP-tag. Using the Gateway System (Thermo Fisher Scientific, Karlsruhe, Germany), *TRPL::SNAP* was shuttled into a modified pYC4 vector for P element mediated transformation of *Drosophila* embryos.[28] *TRPL::SNAP* transgene expression is controlled by a *ninaE* promotor fragment (-833 to +67) and 0.6 kb of the 3' untranslated region of *ninaE*.[25,29]

Immunostaining and Chemical Labeling

For immunohistochemical analyses and chemical labeling, *Drosophila* fly heads were separated from the body, dissected into two halves and incubated in 2% paraformaldehyde (PFA) in PBS (175 mM NaCl, 8 mM Na_2HPO_4, and 1.8 mM NaH_2PO_4, pH 7.2) for 30–60 min at room temperature. Semi-heads were washed twice with phosphate buffer (0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4, pH 7.2) for 10 min and sucrose infiltrated through two washing steps in first 10% sucrose, then 25% sucrose in phosphate buffer, each for 30 min at room temperature. Eyes were finally infiltrated with 50% sucrose in phosphate buffer overnight at 4C and embedded in Shandon™ Cryomatrix™ (Thermo Fisher Scientific, Karlsruhe, Germany). Cryosections of 10 µm thickness of *Drosophila* eyes were obtained at -25C using a CM3050S cryostat (Leica, Wetzlar, Germany). Slices were fixed in 2% PFA in PBS for 5 min at room temperature and then were washed three times in PBS, for 5 min each. For chemical labeling, SNAP-Cell® 505-Star substrate (New England Biolabs, Frankfurt a.M., Germany) was added at a concentration of 1 µM in PBS and incubated for 15 min. After two following washing steps in PBS, for 10 min each, slices were blocked in PBS-T (1% BSA, 0.3% Triton X-100 in PBS) for 2 h at room temperature. After blocking, sections were incubated with primary antibody (1:50 in PBS-T) overnight at 4C. The following primary antibodies were used: rabbit α-TRPL,[15] mouse α-TRP, and mouse α-Rh1 (MAb83F6 and 4C5, Developmental Studies Hybridoma Bank, Iowa City, IA). Sections were subsequently washed three times with PBS and incubated with secondary antibody, either α-mouse Alexa Fluor 660 (Thermo Fisher Scientific) or α-rabbit Cy5 (Jackson ImmunoResearch, UK) 1:100 in PBS-T for 2 h at room temperature. Also Alexa Fluor 546 conjugated phalloidin (Thermo Fisher Scientific; 1:600) was added to secondary antibody solution to stain F-actin in rhabdomeres. After 3 final washing steps in PBS, 5 min each, washing solution was removed and slices were mounted in Mowiol 4-88 (Carl Roth, Karlsruhe, Germany). Sections were analyzed on an AxioImager. Z1m microscope (objective: EC Plan-Neofluar 40×/1.3 Oil) using the ApoTome module (Carl Zeiss, Jena, Germany). Images were captured with the Axiocam 530 mono (Carl Zeiss) camera using the ZEN 2 (blue edition) software (Carl Zeiss).

Immunoblotting and Immunoprecipitation

Fly heads were separated from bodies by freezing in liquid nitrogen and vigorous vortexing. For immunoblotting, 10 heads per genotype were collected and homogenized in 40 µl of SDS extraction buffer, 75 mM Tris-HCl, pH 6.8, 1 mM ethylenediaminetetraacetic acid (EDTA), 4% (w/v) SDS, mixed with 0.2 volumes of 5× SDS sample buffer, 500 mM Tris, pH 6.8, 5% (w/v) SDS, 30% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.05% (w/v) bromophenol blue, and subjected to SDS-PAGE. Immunological detection was achieved by incubation with antibodies α-TRPL,[15] α-Tubulin, and α-Rh1 (E7 and 4C5, Developmental Studies Hybridoma Bank, Iowa City, IA). For immunoprecipitation, 300 heads per genotype were collected and homogenized in 600 µl extraction buffer supplemented with protease and phosphatase inhibitors, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 50 µM (4-amidinophenyl)-methanesulfonyl fluoride hydrochloride monohydrate, 10 µg/ml aprotinin, 50 µg/ml leupeptin, 10 µg/ml pepstatin A, 10 mM benzamidine, 10 mM sodium fluoride, 1 mM orthovanadate, 10 mM βglycerophosphate, 500 nM cantharidine, 10 mM sodium pyrophosphate, using a micropestle (Carl Roth, Karlsruhe, Germany). Head homogenates were extracted on ice for 1 hr. The extracts were centrifuged for 10 min at 16,000 × g and 4C, and the supernatant was used for immunoprecipitation (Input). Immunoprecipitation of TRPL::SNAP fusion protein from *Drosophila* head extracts

was performed with SNAP-tag®-Trap (Chromotek, Planegg-Martinsried, Germany), small recombinant alpaca antibody α-SNAP-tag V_HH coupled to agarose beads. Head extracts were incubated with 20 µl of SNAP-tag agarose beads, preequilibrated in extraction buffer for 1 hr at 4C on a rotating wheel. The beads were collected by centrifugation at 2500 × *g* for 2 min at 4C and washed three times, with 500 µl of ice-cold washing buffer supplemented with protease and phosphatase inhibitors. Precipitated proteins were eluted from SNAP-tag agarose beads with 30 µl of 2× SDS sample buffer, 200 mM Tris, pH 6.8, 2% (w/v) SDS, 12% (w/v) glycerol, 2% (v/v) 2-mercaptoethanol, 0.02% (w/v) bromophenol blue, at 95C for 5 min and subjected to SDS-PAGE.

Electroretinography

Electroretinogram measurements were performed as described previously.[30] In brief, 12-day old flies were immobilized in improvised yokes made from pipette tips, before they were mounted in the center of a Faraday cage. Chlorinated silver wires were inserted into glass micropipettes filled with Davenport solution (100 mM $NaCl_2$, 2 mM KCl, 1 mM $CaCl_2$, 1.8 mM $NaHCO_3$, pH 7.2) and utilized as electrodes. The recording electrode was inserted just beneath the corneas and the reference electrode into the top of the head. Light stimuli were generated by PLED02M (npi electronic GmbH, Germany) driven blue (M3L1-HB-30, 470 nm) and orange (M3L1-HY-30, 590 nm) light emitting diodes (LEDs; Roithner LaserTechnik, Wien, Austria). Light intensities were measured to be at 2.4 mW/cm^2 for the blue LED and at 7.6 mW/cm^2 for the orange LED. Electroretinogram recordings were performed at room temperature after 3 min of dark-adaptation. An EXT 10-2F amplifier (npi electronic, Tamm, Germany) was used with a 700 Hz low pass filter.

Results

Crescent Shaped Staining of Rhabdomeric Proteins Is Light-dependent

Since it is possible that the study of light-triggered TRPL translocation from the rhabdomeres to the cell body is biased by a putative crescent staining artifact, we wanted to clarify this issue. To evaluate whether or not crescent shaped labeling of TRPL in cross sections of rhabdomeres is an artifact, we used immunostaining and eGFP fluorescence of tagged proteins simultaneously (Fig. 2A). The structure of the rhabdomeres was revealed by staining of the actin cytoskeleton with phalloidin. In dark-adapted flies, labeling of cross sections with antibody against TRPL stained the entire rhabdomeres (Fig. 2A, upper row). In these sections the same pattern was observed for eGFP. When using flies that had been exposed to orange light for 5 min, the labeling pattern was different. In this case, the antibody staining against TRPL revealed signals only at the basal rim of the rhabdomeres where they connect to the cell body (Fig. 2A, α-TRPL). This has been previously observed and accordingly interpreted as a rapid translocation of TRPL to the base of the rhabdomeres. However, under the same illumination conditions, TRPL::eGFP fluorescence revealed a localization of these proteins in almost the entire rhabdomeres (Fig. 2A, eGFP). Two explanations for this result are possible: either the eGFP tag hinders TRPL translocation or the antibody is unable to label all rhabdomeric proteins and thus produces a staining artifact. As the antibody detects both, the native protein and the tagged protein, a signal in the entire rhabdomere should still be observed if the tagged TRPL does not move to the base of the rhabdomeres.[24] To determine unambiguously whether antibody labeling does not reveal the real localization of TRPL, we also generated flies that express eGFP-tagged TRPL but no endogenous TRPL (Fig. 2A, lower row). In these flies, the same discrepancy between antibody staining and eGFP fluorescence was observed. We conclude that the putative localization of TRPL at the base of the rhabdomeres after 5 min light exposure is an artifact. Besides TRPL, a crescent shaped staining pattern has previously been observed for other rhabdomeric proteins, specifically rhodopsin Rh1 and TRP. In order to investigate the nature of this labeling pattern for these proteins, we likewise used antibody staining and eGFP fluorescence in parallel. As was observed for TRPL, immunostaining and eGFP fluorescence co-localized in the entire rhabdomeres in dark-adapted flies for Rh1 and TRP (Fig. 2B and C). In 5 min light-adapted flies, antibody staining for both proteins was restricted to the rim of the rhabdomeres, while eGFP fluorescence was still distributed throughout the rhabdomere. Thus, the same crescent shaped labeling artifact that was observed for TRPL applies for Rh1 and TRP as well.

Crescent Shaped Staining Is Dependent on Hydrolysis of PIP_2

The question arises why this artifact is preferentially observed in light-exposed but not in dark-adapted flies. It has been proposed that the crescent shaped artifact is due to limited access of antibodies to epitopes embedded in the densely packed microvilli of the rhabdomeres.[15,23] Upon activation of the phototransduction cascade by light, there is a profound change in structure of the microvillar membranes, which results

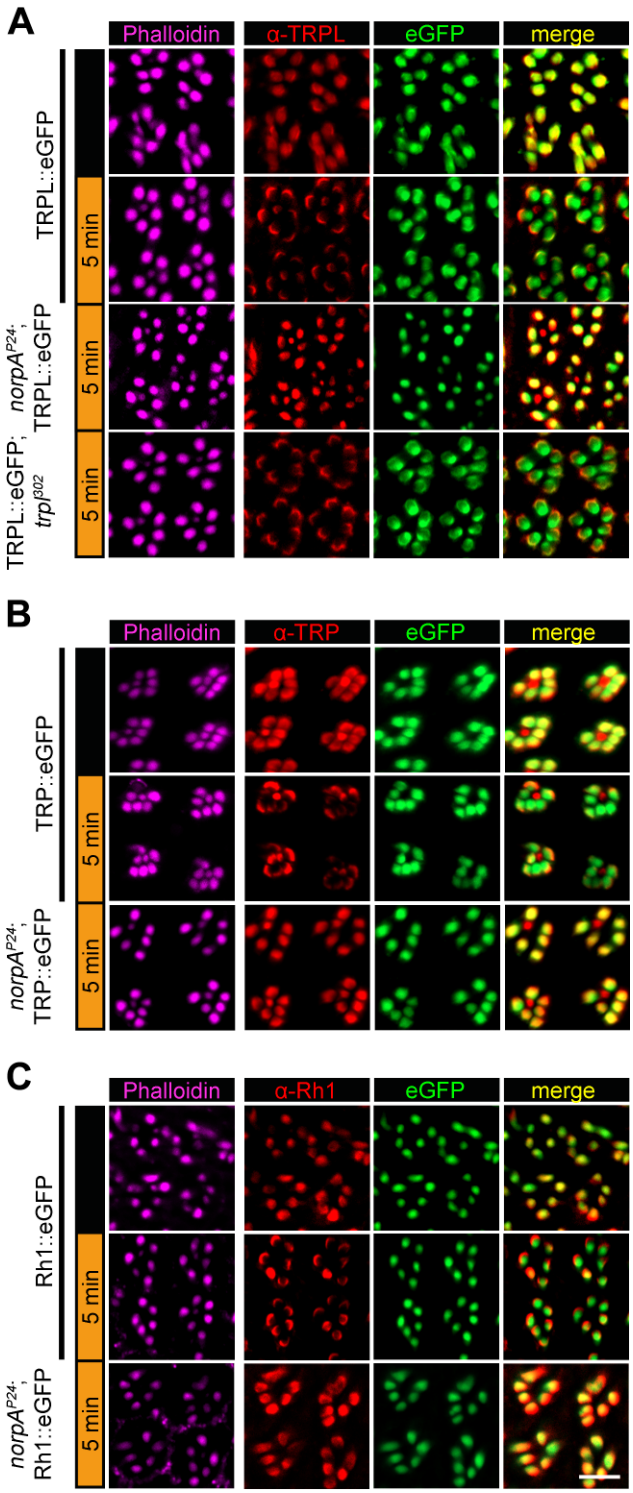


Figure 2. Antibody staining artifact of rhabdomeric proteins is emphasized in illuminated photoreceptor cells. Immunocytochemical analyses of ommatidial cryosections were performed in dark adapted animals and upon 5 min of orange light exposition to initiate phototransduction in flies transgenically expressing either (A) TRPL::eGFP, (B) TRP::eGFP, or (C) Rh1::eGFP heterozygously in

(continued)

Figure 2. (continued)

R1-6 photoreceptor cells. Signal patterns of respective antibody staining and fluorescence tags matched perfectly in case of dark adaptation or inhibition of the phototransduction cascade (*norpA*^{P24}) under illumination, but differed drastically when phototransduction was activated. In the latter case, antibodies detected only proteins at the rhabdomeric base whereas eGFP-tagged variants were evidently still present throughout the entire rhabdomeres. Scale bar represents 5 μm.

in a contraction of the entire ommatidium.[31] This structural change presumably is brought on by the hydrolysis of PIP_2 by PLC which would reduce the diameter of the microvilli by removing the bulky phosphoinositol head group of the membrane lipid and result in even more densely packed microvilli.[31] To test whether the crescent shaped staining pattern depends on the activation of the phototransduction cascade, we utilized the *norpA*^{P24} null mutant which lacks PLC. Without PIP_2 hydrolysis, microvilli do not contract upon light stimulation.[31] As predicted by the above stated hypothesis, the staining pattern of 5-min light-exposed *norpA*^{P24} mutant flies revealed the crescent shaped staining pattern for neither TRPL, TRP, nor Rh1 (Fig. 2). This finding indicates that activation of the phototransduction cascade and concomitant contraction of microvilli enhances the crescent shaped artifact in conventional immunohistochemistry.

TRPL::SNAP as an Alternative to Immunostainings and eGFP-fusion

In order to reinvestigate the light-induced translocation of TRPL unbiased from the antibody artifact, we created a second tagged TRPL variant next to TRPL::eGFP that also bypasses immunostaining by way of self-labeling but offers flexibility regarding the fluorophores that can be attached to TRPL. A C-terminal fusion of the self-labeling SNAP-tag to TRPL was generated with the goal to increase the sensitivity and contrast of TRPL staining in immunocytochemistry. This was not achieved to our satisfaction by the previously established fusion protein TRPL::eGFP.[24] The SNAP-tag is a 20 kDa large modified version of the human DNA repair protein O-6 alkylguanine-DNA-alkyltransferase (hAGT) that reacts specifically and rapidly with benzylguanine and benzylchloropyrimidine derivates, leading to covalent labeling of the SNAPtag with a synthetic probe (Fig. 3A).[32] In this study, the cell-permeable fluorescent SNAP-tag substrate Cell 505-Star was used as synthetic probe, a photostable green fluorescent substrate that is based on the single isomer 6-carboxyrhodamine 110. We generated transgenic flies expressing TRPL::SNAP in PRCs R1-6 and established a robust

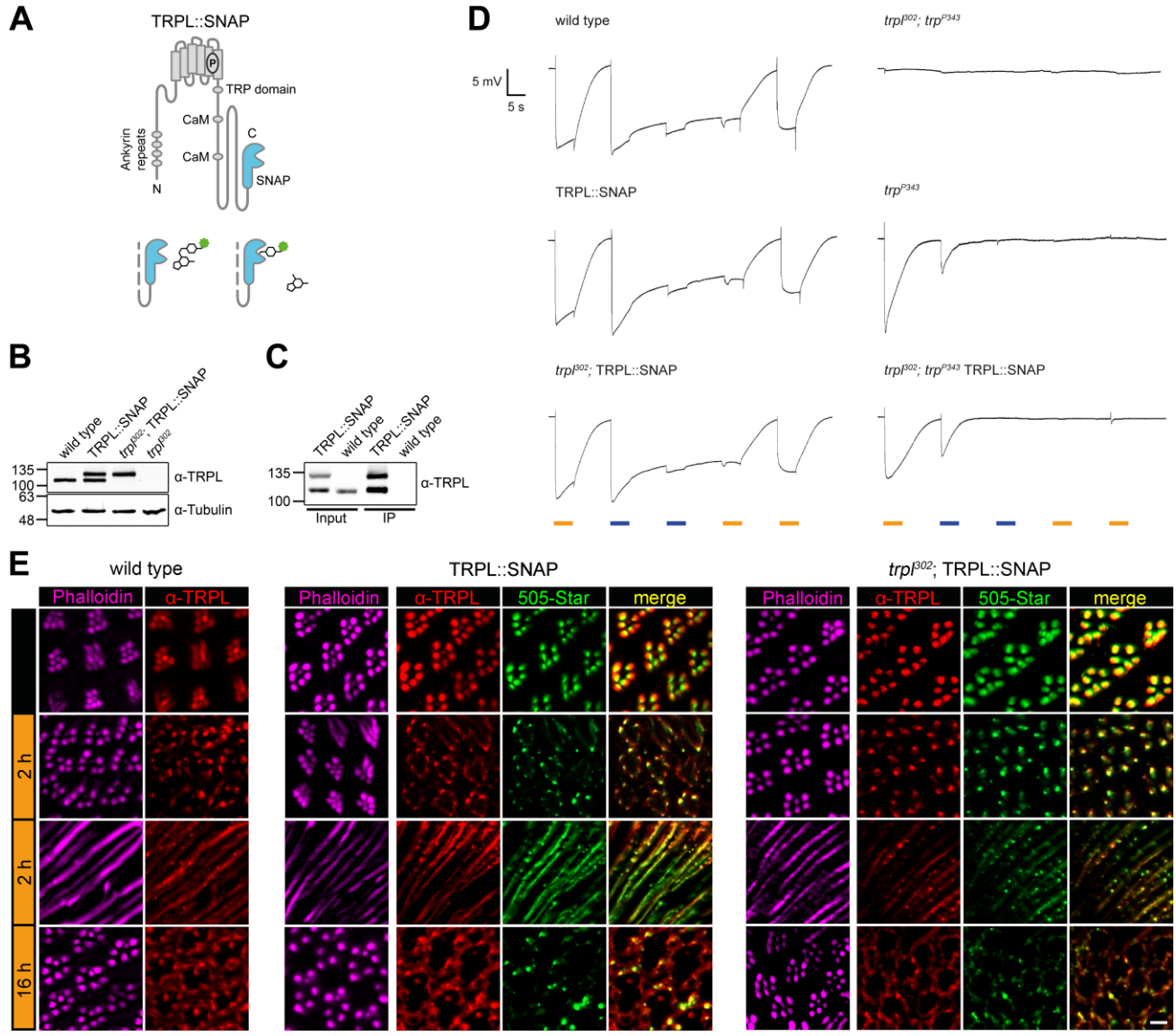


Figure 3. TRPL::SNAP forms functional ion channels that translocate from the rhabdomere to the cell body upon illumination. (A) Schematic of SNAP-tagged TRPL channel for enzymatic self-labeling with fluorophores. This study utilized the synthetic substrate 505-Star which is cleaved by the SNAP-tag's enzymatic activity, covalently attaching the fluorescent probe to itself while releasing a guanine residue. (B) Immunoblot analysis of endogenous TRPL and transgenically expressed TRPL::SNAP in wild type and *trpl*^{302} background. Proteins from 4 *Drosophila* heads probed with polyclonal α-TRPL which detects native TRPL and SNAP-tagged TRPL at ca. 120 and 135 kDa, respectively. Tubulin was used as loading control. (C) Co-Immunoprecipitation assay using antibody directed against SNAP-tag on head extracts of flies transgenically expressing TRPL::SNAP in wild type background or wild type flies. Western Blot was probed with polyclonal α-TRPL, revealing co-precipitation of endogenous TRPL (120 kDa) together with immunoprecipitated TRPL::SNAP (135 kDa). (D) Electroretinograms were recorded from 1-day old flies of the indicated genotypes by using 5-sec-long orange or blue light pulses (indicated by orange and blue bars, respectively) with 10-sec intermissions between light pulses. No obvious deviations from the wild type response were observed upon transgenic expression of the TRPL::SNAP protein in wild type or *trpl*^{302} mutant backgrounds regarding amplitude, on/off-transients, inactivation, deactivation, or prolonged depolarization afterpotential. In *trpl*^{302}; *trp*^{343} double null background, expression of TRPL::SNAP resulted in slightly smaller and less sharp electrophysiological responses compared to the *trp*^{P343} mutant. (E) Immunocytochemical analysis on ommatidial cryosections from 1-3 day old flies was performed at well described time points of TRPL translocation (dark, 2 hr orange light, 16 hr orange light).[1,17,30] Cross sections through ommatidia are shown, except for row three which shows longitudinal sections for better visualization of TRPL vesicles. Cytoskeleton of rhabdomeres was visualized by Alexa Fluor 546 conjugated phalloidin (magenta). At these time points, similar results for TRPL labeling are revealed either with α-TRPL (red) or SNAP-tag labeling using the synthetic substrate 505-Star (green). At 16 hr of light exposure, chemical labeling results in much more distinct signals compared to the staining pattern of α-TRPL. This is not due to a different localization of native TRPL and TRPL::SNAP since a blurred staining pattern of the antibody appears also in the *trpl*^{302} mutant background. Scale bar represents 5 μm.

staining protocol for cryosections of adult eyes. Subsequently, we performed a set of experiments to test the fusion protein's performance with respect to TRPL's native function and behavior.

TRPL::SNAP is expressed in fly heads in comparable levels to the native protein, both in the wild type as well as the null mutant background of *trpl*^{302} flies (Fig. 3B). Probing with an antibody raised against TRPL confirms that the C-terminal SNAP-tag does not interfere with the antibody's recognition of its epitope under denaturing conditions. Co-immunoprecipitations with SNAP-tag beads demonstrated interaction between tagged and endogenous TRPL—a prerequisite for the natively occurring homomultimerization of these channels (Fig. 3C).[33] We performed electroretinogram recordings of eyes from TRPL::SNAP expressing flies in various genetic backgrounds using a combination of orange and blue stimuli. According to our data, TRPL::SNAP forms functional TRPL channels and the tag has no obvious effect on amplitude, on-/off-transients, inactivation, deactivation, or prolonged depolarization afterpotential when compared to wild type flies (Fig. 3D). To test the performance of the SNAP-tagged channel in isolation, we generated TRPL::SNAP expressing flies in the double null mutant background of *trpl*^{302}; *trp*^{P343} which lacks both light-activated ion channels in PRCs and has been demonstrated to be unresponsive toward light stimuli.[8,27] Evidently, TRPL::SNAP is able to procure a depolarization of the PRC upon light stimulation to a similar extent as the endogenous TRPL channel. We observed that electroretinogram peaks of these flies were generally less sharp than those of *trp*^{P343} mutants, suggesting a minor influence of the C-terminal tag on TRPL inactivation under reduced Ca^2+ influx. In histochemical analyses of dark-adapted flies and flies exposed to orange light for 2 hr, the same staining pattern for SNAP labeling was observed for transgenic flies expressing TRPL::SNAP in wild type or *trpl*^{302} null mutant background (Fig. 3E). TRPL-staining in these flies corresponded well with TRPL antibody staining in wild type flies, indicating that translocation of TRPL from the rhabdomere to the cell body is not affected by the SNAP-tag. While dark-adapted flies revealed TRPL-staining in the rhabdomeres, 2 hr of illumination resulted in numerous TRPL-containing vesicular structures, as has been documented before.[1,22] Interestingly, regarding the staining pattern after 16 hr of light exposure, we found that the self-labeling technique of TRPL::SNAP resulted in more distinct signals that colocalized with the strongest signals of the immunostaining. Under these light conditions, it has been reported that TRPL is excluded from the rhabdomere and remains in a storage compartment as long as flies are kept in the light .[15,17] To further address the possibility that discrepancies between antibody staining and labeling by TRPL::SNAP are due to differences in subcellular translocation behavior, flies that express SNAP-tagged TRPL but no native TRPL were studied after 16 hr of illumination. The antibody staining pattern in these flies was comparable to the one seen in wild type flies including weak background signals, suggesting that TRPL-staining represents the localization of endogenous TRPL but with better resolution and contrast.

Study of the Initial Phase of TRPL Internalization Using TRPL::eGFP and TRPL::SNAP

From the above described characterization, we concluded that TRPL::SNAP forms a fully functional rhabdomeric ion channel, which is activated through illumination and undergoes light-induced internalization in a manner that very much resembles that of endogenous TRPL. Its detection through self-labeling, however, is superior compared to detection of native TRPL with antibodies regarding both the crescent shaped staining artifact as well as the clarity of intracellular signals.

With this in mind, we conducted experiments to reinvestigate the first minutes of TRPL translocation as a response to light exposure. In this approach, we used flies expressing TRPL::SNAP in a genetic wild type background as well as in a *trpl*^{302} null mutant background and detected TRPL localization by antibody or chemical staining. We illuminated flies for distinct intervals between 1 and 30 min with orange light, prepared retinal cryosections, and performed immunostainings. In dark-adapted flies, both detection methods resulted in consistent signals from the entire rhabdomere, as expected (Figs. 4A to C and 5A). Even though we reduced the initial period of light exposure from 5 min down to just 1 min, we again observed the crescent shaped artifact when performing immunostainings. This stood in stark contrast to the fluorescence signal from the SNAP-tagged TRPL which was still found within the rhabdomeres (Figs. 4B, C and 5A). The disparity between these signal patterns was again observed after 5 min of illumination and subsisted until approximately 10 min of light incubation at which point the signal of TRPL::SNAP shifted from the distal to the basal microvillar regions and finally started to mostly overlap with the antibody pattern. Strikingly, a portion of the fluorescence from the SNAP-tag appeared to remain at the distal end of the rhabdomeric microvilli. According to these results, we conclude that illumination-induced translocation of TRPL to the base of the rhabdomere is slower and more gradual than previously thought. It appears that a first accumulation at

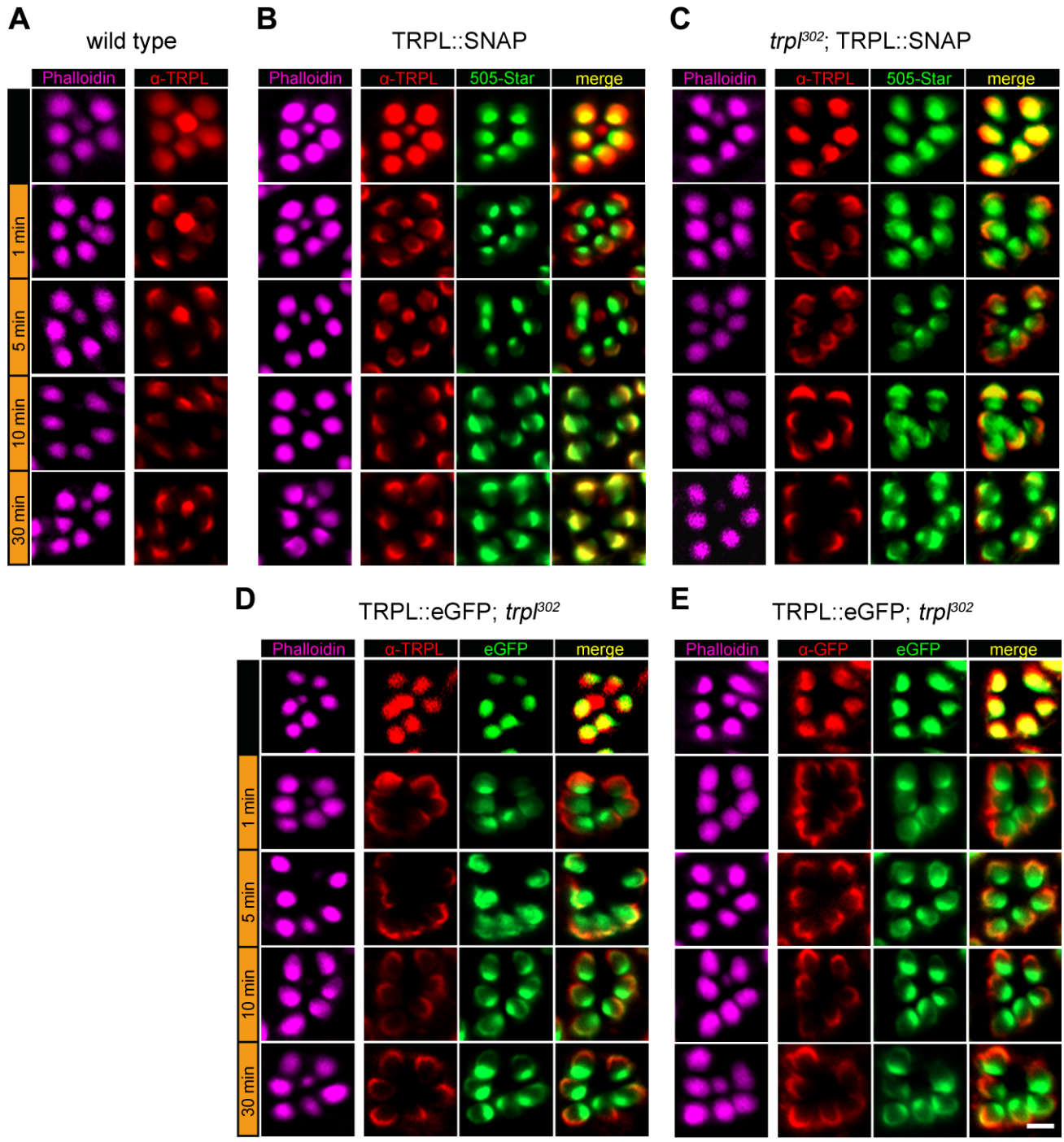
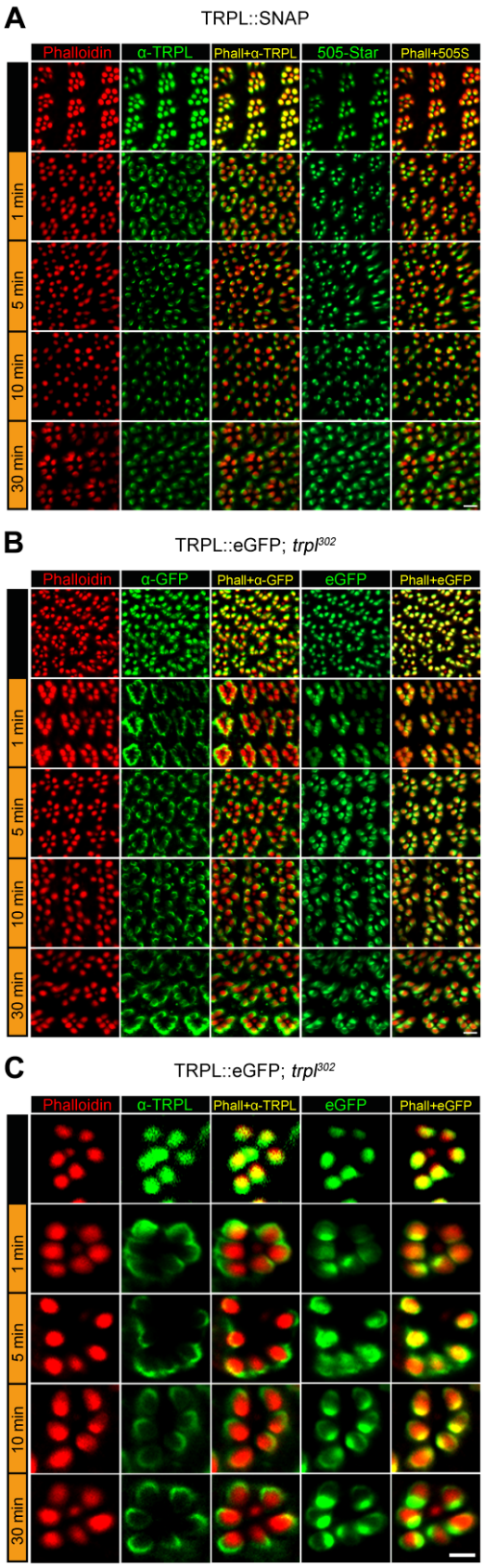


Figure 4. Time course of the initial phase of TRPL translocation as revealed by immunostaining, SNAP-tag, or eGFP fusion. Ommatidial cryosections were prepared from 1-3 day old flies kept in the dark or after 1, 5, 10, or 30 min of illumination with orange light. Sections were stained with Alexa Fluor 546 conjugated phalloidin (magenta) to visualize the cytoskeleton of rhabdomeres. Sections were probed with α-TRPL (red, B-D) or α-GFP (red, E) and visualized by self-labeling of TRPL::SNAP with the fluorescent substrate 505-Star (green, B,C) or by observing eGFP fluorescence directly (green D,E). Staining patterns of wild type flies (A) were compared to TRPL::SNAP expressing flies in a genetic wild type (B) or a $trpl^{302}$ null mutant background (C) as well as TRPL::eGFP expressing flies in a $trpl^{302}$ null mutant background (D,E). After 1 min of orange light incubation, all immunostainings were confined to the inner rim of rhabdomeres. This antibody staining artifact of illuminated ommatidia subsisted throughout every time point imaged. Following illumination the fluorescence signal from TRPL::eGFP as well as that of chemically labeled TRPL::SNAP revealed a bipartite pattern at the base and at the tip of the rhabdomeres that gradually shifted from the rhabdomere tip to the basal region at around 10–30 min. Scale bar represents 2.5 μm.



(continued)

Figure 5. Time course of stage I TRPL translocation as revealed by immunostaining, SNAP-tag, or eGFP fusion. Ommatidial cryosections were prepared from 1-3 day old TRPL::SNAP expressing flies or TRPL::eGFP expressing flies in a *trpl*-mutant background. Flies were kept in the dark or after 1, 5, 10, or 30 min of illumination with orange light before preparation. Sections were stained with Alexa Fluor 546 conjugated phalloidin (red) to visualize the cytoskeleton of rhabdomeres. Sections were probed with α-TRPL and Cy5-coupled secondary antibodies (green/left column, A,C) or α-GFP and Alexa Fluor 660-coupled secondary antibodies (green/left column, B), or were visualized by self-labeling of TRPL::SNAP with the fluorescent substrate 505-Star (green/right column, A), or by observing eGFP fluorescence directly (green/right column B, C). After orange light illumination for 1, 5, 10, or 30 min immunostainings were confined to the inner rim of rhabdomeres. (A) The fluorescence signal of chemically labeled TRPL::SNAP remained longer within the rhabdomere and gradually shifted from the rhabdomere tip to the basal region at around 10-30 min revealing a bipartite pattern. (B,C) A similar behavior was observed for direct eGFP fluorescence. Scale bar represents 5 μm (A,B) and 2.5 μm (C).

the rhabdomeric base is reached between 10 and 30 min after the initial light stimulus.

Due to the peculiar bipartite patterning of the SNAP-tagged TRPL protein, we also performed these experiments with flies expressing TRPL::eGFP in a genetic *trpl*^{302} null mutant background and detected the fusion protein either by direct fluorescence of eGFP or with antibodies directed against TRPL or eGFP. The general outcome, however, was the same: The localization of the TRPL::eGFP signal also differed between dark and light-exposed flies after short light exposures (Figs. 4D, E and 5B, C). Labeling patterns of TRPL::eGFP were not entirely uniform within the rhabdomere after onset of illumination. We rather observed a bipartite pattern at the distal and basal regions of the rhabdomere with significantly weaker signal from the central portion of the rhabdomere. This signal distribution was particularly pronounced between 5 and 30 min of light exposure.

Discussion

By using antibody staining in parallel with two alternative labeling methods, we show that immunocytochemical labeling of proteins embedded in the light-absorbing rhabdomeres of *Drosophila* photoreceptors can be compromised by a staining artifact. This crescent-shaped antibody staining artifact applies to TRPL, rhodopsin Rh1, TRP, and possibly other rhabdomeric proteins, for example, the scaffold protein INAD and the PIP_2 re-synthesizing kinase dPIP5K.[34,35] Chaoptin, a membrane protein involved in rhabdomere development and microvillar organization, also produces artifactual staining patterns in immunohistochemical labelings.[36,37] For all three proteins investigated here, the artifact depended on the

light conditions to which the flies were subjected. The staining artifact was much more prevalent in light-treated animals. This finding may be explained by the light-triggered structural changes in the rhabdomere that presumably result in even more densely packed microvilli providing even less access for antibodies to their epitopes. In addition, the artifact appears to depend on the exact conditions of preparation with respect to fixation and antibodies used. For example, we observed the artifact also in dark-adapted flies in some instances and sometimes observed antibody staining throughout the rhabdomere even in light-exposed flies (data not shown).

Light-dependency of this staining artifact is especially problematic when investigating light-triggered internalization of rhabdomeric proteins like the TRPL ion channel. Accordingly, re-evaluation of the light-triggered internalization of TRPL from the rhabdomere revealed that the previous assumption of a relatively fast translocation of TRPL to the base of the rhabdomeres and to the rhabdomeric stalk membrane probably results from a misinterpretation of immunocytochemical results due to this antibody staining artifact.[1,17] As revealed by alternative detection methods that avoid the light-dependent crescent shaped staining artifact, the translocation of this ion channel seems to occur much slower (30 min rather than 5 min) and more gradual than previously assumed. A slower and more gradual TRPL movement within the rhabdomeres could be achieved by gradual removal of TRPL at the rhabdomere base through endocytosis that lowers the local TRPL concentration at the rhabdomere base and supports a diffusion driven redistribution of TRPL molecules, which then in turn can be internalized via endocytic vesicles after 2 hr of illumination.

Phototransduction mutants have been described previously, in which the assumed movement of TRPL to the stalk membrane and rhabdomere base (stage 1 of TRPL translocation) was inhibited.[17] The study by Cronin and colleagues reported that null mutants of phototransduction components that prevented PLC activity, namely *ninaE*^{17} and *norpA*^{P41}, hindered stage 1 of TRPL translocation, since the antibody staining pattern was clearly rhabdomeric. On the other hand, *trp*^{P343} and *inaC*^{P109} mutants which have defects in the phototransduction cascade but do not hinder hydrolysis of PIP_2 were reported to not inhibit stage 1 translocation of TRPL.[17] Accordingly, the antibody staining pattern was a crescent labeling of the rhabdomeric base and stalk membrane after 2 hr of illumination.[17] In the light of the data presented here, these results can be reconciled by assuming that the *ninaE*^{17} and *norpA*^{P41} mutations prevented microvillar contractions and thereby the crescent shaped artifact while *trp*^{P343} and *inaC*^{P109} mutations did not.

Although the results using TRPL::eGFP or TRPL::SNAP argue against a fast, light-triggered

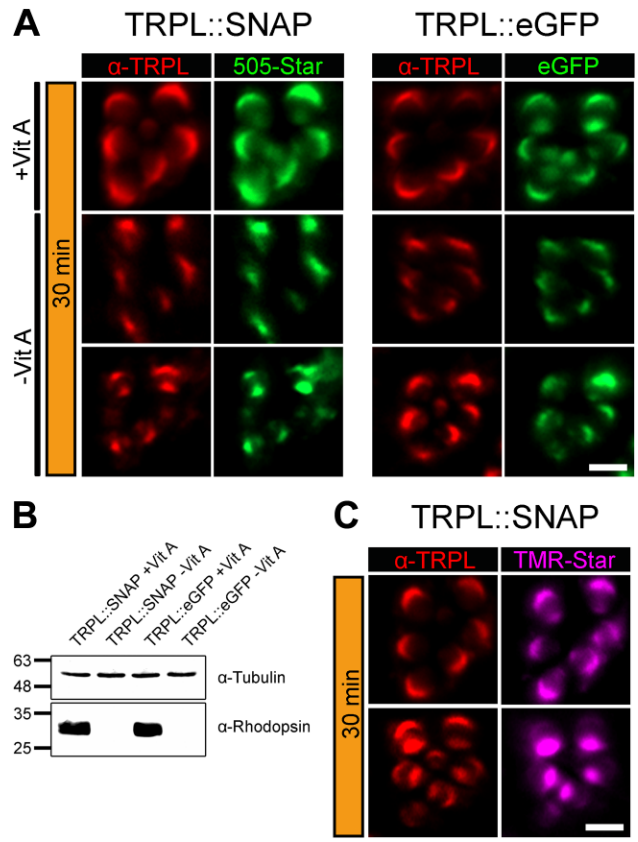


Figure 6. Light-induced staining patterns of TRPL fusion proteins after vitamin A deprivation or labeled with alternative chromophores. (A,C) Ommatidial cryosections were prepared from 1-3 day old TRPL::SNAP or TRPL::eGFP expressing flies after 30 min of illumination with orange light. Sections were stained with α-TRPL or by self-labeling of TRPL::SNAP with the fluorescent substrates 505-Star (green) or TMR-Star (magenta). (B) Immunoblot analysis of endogenous Rh1 expression in *TRPL::SNAP* and *TRPL::eGFP* flies with and without vitamin A deprivation. Proteins from 4 *Drosophila* heads probed with antibody α-Rh1 which detects rhodopsin at ca. 30 kDa. Tubulin was used as loading control. Scale bar in A and C represents 2.5 μm.

translocation of TRPL to the rhabdomere base, labeling with these methods revealed a clear difference in TRPL staining of dark- and short-time light-adapted flies. While there is an even distribution of TRPL throughout the rhabdomere in the dark, following illumination a bipartite labeling pattern at the base and at the tip of the rhabdomere is observed while the rhabdomere center appears dark. Since eGFP is directly attached to TRPL, the observed uneven distribution of eGFP fluorescence in these sections cannot result from problems with access of the staining agent to the TRPL channel. Using vitamin A deprived flies and different SNAP-tag chromophores, we also excluded a quenching effect by metarhodopsin that is generated from rhodopsin upon illumination (Fig. 6). Interestingly, with reduced

Rh1 levels we frequently observed the bipartite signal pattern also with TRPL antibody staining. We assume that this results from a better access for antibodies to the rhabdomeric lumen due to the reduction in Rh1.[38] Our experiments in a *norpA*^{P24} null mutant background in which the bipartite staining pattern of TRPL::eGFP was resolved in favor of a uniform rhabdomeric signal also argue against metarhodopsin as potential quencher (Fig. 2A). Other technical reasons that might generally lead to the artificial appearance of an uneven staining pattern in the rhabdomere can be excluded as we observed an even distribution throughout the rhabdomere of TRPL, TRP, and Rh1 in dark-adapted flies by using the same method. Furthermore, TRP and Rh1 were observed as evenly distributed throughout the rhabdomere also in illuminated flies by using TRP::eGFP or Rh1::eGFP. We therefore conclude that this bipartite rhabdomeric staining patterning actually reflects TRPL distribution within the first 30 min of light exposure. A mechanism explaining how this ion channel becomes redistributed upon illumination into the observed bipartite pattern remains to be determined.

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Competing Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author Contributions

KS and AH conceived the experimental design. KS executed the immunocytochemistry, immunoblottings, and co-immunoprecipitations. TS generated the TRPL::SNAP expressing transgenic flies and performed electroretinograms. AH, TS, and KS interpreted the data and drafted the manuscript. All authors have read and approved the final manuscript.

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