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Light-regulated interaction of Dmoesin with TRP and TRPL channels is required for maintenance of photoreceptors

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Abstract

Recent studies in *Drosophila melanogaster* retina indicate that absorption of light causes the translocation of signaling molecules and actin from the photoreceptor's signaling membrane to the cytosol, but the underlying mechanisms are not fully understood. As ezrin-radixin-moesin (ERM) proteins are known to regulate actin–membrane interactions in a signal-dependent manner, we analyzed the role of Dmoesin, the unique *D. melanogaster* ERM, in response to light. We report that the illumination of dark-raised flies triggers the dissociation of Dmoesin from the light-sensitive transient receptor potential (TRP) and TRP-like channels, followed by the migration of Dmoesin from the membrane to the cytoplasm. Furthermore, we show that light-activated migration of Dmoesin results from the dephosphorylation of a conserved threonine in Dmoesin. The expression of a Dmoesin mutant form that impairs this phosphorylation inhibits Dmoesin movement and leads to light-induced retinal degeneration. Thus, our data strongly suggest that the light- and phosphorylation-dependent dynamic association of Dmoesin to membrane channels is involved in maintenance of the photoreceptor cells.

Introduction

The *Drosophila melanogaster* eye is composed of ~800 repeat units, referred to as ommatidia. Each ommatidium is composed of six elongated peripheral photoreceptor cells (R1–6), which extend across the length of the ommatidium, and two shorter central photoreceptors (R7 and

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Online supplemental material

Fig. S1 shows that Dmoesin of eyeless *D. melanogaster* heads does not display light-dependent movement from the membrane to the cytosol. Fig. S2 depicts the kinetics of light-dependent movement of Dmoesin from the rhabdomere to the cell body and time-course analysis of Dmoesin redistribution upon illumination, as observed in Western blot analysis. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200503014/DC1>.

The online version of this article contains supplemental material.

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-8; Ready et al., 1976). Photoreceptors are highly polarized cells composed of two well defined compartments: a cell body and a signaling compartment called the rhabdomere. The rhabdomere contains tightly packed, actin-rich microvilli that harbor the signaling proteins required to generate the photoreceptor potential upon illumination. The *D. melanogaster* transient receptor potential (TRP) protein is a light-sensitive cation channel that provides a major component of the light-induced current (Hardie and Minke, 1992). TRP is also required for anchoring a supramolecular signaling complex that includes the inactivation-no-afterpotential D (INAD) PSD95/DlgA/ZO-1 homology (PDZ) scaffold protein, PLC β , and the eye-specific PKC (eyePKC) to the plasma membrane (Huber et al., 1996; Shieh et al., 1997; Tsunoda et al., 1997; Montell, 1998). TRP-like (TRPL) is a second light-activated channel (Phillips et al., 1992) that, together with TRP, participates in the production of the light-induced current (Hardie and Minke, 1995). Genetic elimination of both TRP and TRPL channels completely eliminates the photoreceptor potential (Niemeyer et al., 1996; Scott et al., 1997).

The photoreceptor potential is only one of several responses to light. Another light-induced response is the translocation of signaling proteins (Gq α and TRPL) and actin between the rhabdomeric membrane and the cell body (Bähner et al., 2002; Kosloff et al., 2003). The molecular mechanisms underlying translocation of these proteins from the microvilli to the cell body remain largely unknown (Minke and Agam, 2003).

In several tissues, microvillar organization depends on protein members of the ezrin-radixin-moesin (ERM) family, which form a bridge between the actin cytoskeleton and the plasma membrane (for review see Bretscher et al., 2002). ERM proteins bind to integral membrane proteins either directly or through PDZ scaffold proteins, such as ezrin binding phosphoprotein 50 (EBP50)/Na⁺/H⁺ exchanger regulatory factor and NHE3 kinase A regulatory protein. This binding is a dynamic process, which takes place upon the binding of phosphatidylinositol 4,5-bisphosphate (PIP₂) and phosphorylation of the ERM protein (Hirao et al., 1996; Bretscher et al., 2002). Dmoesin, the unique member of the ERM family in *D. melanogaster* (Polesello and Payre, 2004), is required for the specific organization of different actin-rich structures during development. Furthermore, Dmoesin plays an essential structural role in *D. melanogaster* photoreceptor morphogenesis (Karagiannis and Ready, 2004). Dmoesin mutations disrupt the polarized localization of posterior determinants in oocytes (Jankovics et al., 2002; Polesello et al., 2002). Mutations that disrupt the dynamics of Dmoesin phosphorylation produce severe defects in actin reorganization and cell shape (Polesello et al., 2002; Speck et al., 2003; Karagiannis and Ready, 2004). Although Dmoesin has been shown to accumulate in rhabdomeres (Karagiannis and Ready, 2004), its physiological function in mature photoreceptors and its relationship to light reception is not known.

In this study we used wild-type (WT) and mutant *D. melanogaster* strains to show that Dmoesin only interacts with the TRP and TRPL channels in dark-raised flies. Furthermore, we show that illumination induces dephosphorylation of the conserved COOH-terminal threonine 559 (T559) of Dmoesin, which subsequently dissociates from the channel proteins and moves from the rhabdomeric membrane to the cytosol. Consistent with this conclusion, our results show that mutations that impair phosphorylation of Dmoesin (Polesello et al., 2002; Speck et al., 2003) abolish the movement of Dmoesin upon illumination and result in light-activated degeneration of the photoreceptor cells.

Results

Light induces subcellular redistribution of Dmoesin

We recently showed that actin moves from the rhabdomere to the photoreceptor cell body after the illumination of dark-raised flies (Kosloff et al., 2003). To investigate a possible mechanism that underlies light-activated actin movement in photoreceptors, we examined the role of

Dmoesin, a known regulator of the dynamic reorganization of actin-rich cell structures (Polesello and Payre, 2004). Activation of Dmoesin involves a redistribution of the protein from the cytoplasm (dormant form) to the plasma membrane (active form; Polesello et al., 2002). Thus, modification of Dmoesin intracellular localization might be related to the light-dependent reorganization of actin filaments.

To test this possibility, we examined the distribution of Dmoesin between the membrane and cytosolic fractions in head extracts of dark-raised and illuminated *D. melanogaster*. Although Dmoesin was detected in both the soluble and membrane fractions, the majority of Dmoesin was associated with the membrane fraction in dark-raised flies (Fig. 1 A). In contrast, Dmoesin was predominantly in the soluble fraction in illuminated WT flies (Fig. 1 A). Quantification of the Dmoesin membrane/cytosolic ratio further supports the conclusion that illumination induces a substantial movement of Dmoesin from the membrane to the soluble fraction (Fig. 1 B). Upon illumination, nearly 50% of the membrane-associated Dmoesin of the head moved to the cytosol. By using mutants lacking eyes (Fig. S1, available online at <http://www.jcb.org/cgi/content/full/jcb.200503014/DC1>) we found that the Dmoesin protein present in heads, outside the photoreceptors, remained associated with membranes upon illumination and therefore was not affected by light.

To further test whether the observed intracellular movement of Dmoesin depends on the activation of the visual signaling cascade, we analyzed Dmoesin distribution in mutants that inactivated phototransduction. The *norpA* and *trp* genes encode the PLC β isoform and the major light-activated channel TRP, respectively, and both are required for *D. melanogaster* phototransduction. In contrast to WT flies, no significant changes in the intracellular distribution of Dmoesin were observed upon illumination in head extracts from *trp*^{P343} or *norpA*^{P24} mutants (Fig. 1, A and B). In both mutants, similar amounts of Dmoesin were detected in the membrane and soluble fractions, regardless of the illumination regime. Therefore, the light-induced intracellular movement of Dmoesin is dependent on a functional phototransduction cascade in photoreceptors.

To directly visualize the movement of Dmoesin after illumination in the retina, we analyzed the immunocytochemical localization of Dmoesin using anti-Dmoesin antibodies (α Dmoesin; Fig. 2). In dark-raised flies Dmoesin was mainly localized to the base of the rhabdomeres and cortical actin region (Karagiannis and Ready, 2004). After illumination, Dmoesin level of peripheral R1–6 rhabdomeres was much reduced, with a concomitant increase in nonrhabdomeric regions in WT flies (Fig. 2, A–D) that is consistent with the Western blot analysis of Fig. 1. The failure of Dmoesin to move upon illumination in the central R7 cell can be attributed to the inefficient absorption of visible light by the UV rhodopsin of R7 cells (Chou et al., 1996), which constitutes a negative control (Fig. 2, A–D). In agreement with the biochemical data, the light-triggered redistribution of Dmoesin observed by immunocytochemistry required the activation of the phototransduction cascade because it was abolished in the mutant *norpA*^{P24} (Fig. 2, E–H).

Altogether, these results support the conclusion that retinal Dmoesin is predominantly associated with rhabdomeric membranes in dark-raised flies and that activation of the phototransduction cascade induces Dmoesin movement to the cytosol in the photoreceptor cells.

Dmoesin binds TRP and TRPL channels in dark-raised flies, whereas illumination dissociates Dmoesin from the channel proteins

Results from our cell fractionations and immunocytochemistry suggest that illumination regulates Dmoesin dissociation from the membrane proteins of the signaling compartment of photoreceptors. Several studies have reported that ERM/EBP50 proteins interact with members

of the TRP family of proteins, such as TRPC4 and TRPC5 (Tang et al., 2000; Mery et al., 2002; Obukhov and Nowycky, 2004). Therefore, we examined whether Dmoesin interacts with TRP and TRPL, the two major channel proteins of the microvilli in *D. melanogaster* photoreceptors.

To test this hypothesis, we analyzed WT and mutant head extracts by immunoprecipitation with monospecific anti-TRP antibodies (α TRP). Protein complexes precipitated with α TRP were fractionated by SDS-PAGE and probed in Western blots with α Dmoesin. In dark-raised WT flies, a strong Dmoesin signal was observed, indicating that Dmoesin and TRP formed a protein complex in vivo (Fig. 3). In control experiments, no Dmoesin staining was detected in head extracts of dark-raised *trp*^{P343} null mutant or in WT extracts immunoprecipitated with nonimmune serum (NIS; Fig. 3), thus demonstrating the specificity of the Dmoesin–TRP interaction. We then examined the effect of illumination on the Dmoesin–TRP interaction. Interestingly, illumination caused a strong reduction of Dmoesin staining in complexes immunoprecipitated with α TRP, although similar amounts of TRP were immunoprecipitated from dark-raised and illuminated flies (Fig. 3, bottom). Therefore, TRP and Dmoesin interact in vivo only in dark-raised flies, and illumination dissociates the Dmoesin–TRP interaction.

Because TRP is known to bind INAD, which is a multi PDZ-domain scaffold protein (Shieh and Zhu, 1996; Chevesich et al., 1997; Tsunoda et al., 1997), we examined whether INAD is required for Dmoesin–TRP interaction. Head extracts of dark-raised young (<2-d-old) *inaD*¹ null mutants were immunoprecipitated with α TRP. Western blot analysis showed that Dmoesin immunoprecipitation was unaffected in the *inaD*¹ mutant flies (Fig. 3), indicating that Dmoesin interacts with TRP independently from INAD. The observed reduction in TRP level in the *inaD*¹ mutant (Fig. 3) is consistent with the known slow degradation of TRP in *inaD*¹ mutants (Tsunoda et al., 1997). As an additional control, α TRP-precipitated complexes were probed with antibodies against another major membrane protein of the microvilli, Chaoptin (Van Vactor et al., 1988). Western blot analysis did not reveal any Chaoptin signal (unpublished data), providing additional evidence of the specificity of the Dmoesin–TRP interaction.

To address whether activation of the phototransduction cascade is required for the observed dissociation of the Dmoesin–TRP complex, we examined the coimmunoprecipitation of Dmoesin in a visual transduction mutant. In the PLC β mutant (*norpA*^{P24}), a strong Dmoesin signal was observed in protein complexes precipitated with α TRP, regardless of illumination (Fig. 4). Western blot analysis of the same head extracts probed with α INAD revealed that roughly equal amounts of signaling proteins were coprecipitated in each lane (Fig. 4, bottom). These data show that in *norpA*^{P24} eyes, TRP interacts with Dmoesin equally in both dark- and light-raised flies, thus indicating that the dissociation of the Dmoesin–TRP complex depends on activation of the phototransduction cascade.

We then addressed whether Dmoesin also interacts in vivo with TRPL, the second light-activated channel of the microvilli. Analysis of protein complexes immunoprecipitated with anti-TRPL antibodies revealed that Dmoesin specifically interacts with TRPL in WT dark-raised flies (Fig. 5). Consistent with this conclusion, Dmoesin is undetectable in similar conditions with extracts from *trpl* null mutants, or in WT extracts when using NIS (Fig. 5). As observed with TRP, the Dmoesin–TRPL interaction occurs specifically in dark-raised flies, whereas illumination dissociates the Dmoesin–TRPL complex (Fig. 5).

Altogether, these results show that Dmoesin interacts specifically with both TRP and TRPL. Moreover, activation of the light-induced signaling cascade disrupts the Dmoesin–TRP interaction, leading to movement of a portion of Dmoesin to the soluble fraction.

Light-dependent movement of Dmoesin from the membrane to the cytosol is regulated by T559 phosphorylation

Phosphorylation of a conserved threonine residue located in the actin-binding domain of ERM proteins has been shown to regulate both mammalian ERM (Bretscher et al., 2002) and *D. melanogaster* Dmoesin activity and subcellular localization (Polesello and Payre, 2004). Therefore, we examined if the light-sensitive interaction of Dmoesin with the photoreceptor channel depends on Dmoesin phosphorylation.

To address the influence of Dmoesin phosphorylation, extracts were immunoprecipitated with an antibody directed against an evolutionarily conserved COOH-terminal peptide of ERM (Polesello et al., 2002) that specifically recognizes the phosphorylated T559 of Dmoesin (designated hereafter as α -phospho-ERM). Protein complexes precipitated with α -phospho-ERM were then analyzed on Western blots probed with α Dmoesin. Although Dmoesin was readily detected in extracts of dark-raised flies, Dmoesin staining was strongly reduced in extracts from illuminated WT flies treated under identical conditions (Fig. 6 A, left). In addition, the TRP channel and the INAD scaffold protein that binds to TRP were also detected only in head extracts of dark-raised flies immunoprecipitated with α -phospho-ERM (Fig. 6 A, right). These results are consistent with our previous findings, which demonstrated that Dmoesin only interacts with TRP in dark-raised flies. To support this notion, extracts were immunoprecipitated with α TRP and analyzed on Western blots probed with α -phospho-ERM (Fig. 6 B). Although α -phospho-ERM staining was detected in the protein complexes of dark-raised head extracts precipitated with α TRP, α -phospho-ERM staining was strongly reduced in the protein complexes of illuminated flies (Fig. 6 B, left). In control experiments, no α -phospho-ERM staining was detected in head extracts of dark-raised *trp*^{P343} null mutant or in NIS-precipitated WT membranes (Fig. 6 B, middle and right).

Together, the results suggest that light-activated dephosphorylation of channel-bound Dmoesin triggers its dissociation and movement from the membrane to the cytosol.

To directly visualize intracellular movements of Dmoesin in photoreceptors upon illumination, we made use of transgenic lines that allow the expression of functional Dmoesin fused to GFP (Polesello et al., 2002). Dmoesin-GFP fusions were expressed under the control of the Rh1-Gal4 driver, which is specific to mature peripheral R1–6 photoreceptors. Upon application of long wavelength excitation light that elicits a strong autofluorescence of the rhabdomeres (without excitation of the GFP), locations and dimensions of the rhabdomeres in each ommatidium are readily observed in the living retina. The typical structure of the ommatidium is visible as seven red circles, representing the R1–6 peripheral rhabdomeres and the smaller R7 rhabdomere, at the center. Live retinæ were dissected under dim red light, and the subcellular localization of Dmoesin-GFP was examined with confocal microscopy. Fig. 7 A shows a representative image of ommatidia from dark-raised flies expressing Dmoesin-WT-GFP, which localizes to the rhabdomeres and cortical actin of R1–6 photoreceptors. Because of variability in the expression levels of Dmoesin-GFP in the various ommatidia, some photoreceptor cells did not express Dmoesin (Fig. 7 A). In the photoreceptor cells that did express Dmoesin-GFP, the intense fluorescence of the GFP masked the weaker red autofluorescence and the merged images appeared green. When the level of Dmoesin-GFP in the rhabdomeres was reduced (Fig. 7 F) a yellow color appeared in the merged images. The lack of green fluorescence from the central R7 rhabdomere (which does not express Dmoesin-GFP) provides an internal negative control. After illumination a marked redistribution of Dmoesin-GFP is observed, with the green fluorescence moving from the rhabdomeres to the cell body region (Fig. 7 B). Although there are some differences in the detailed localization and movement of the native Dmoesin (Fig. 2) and Dmoesin-WT-GFP, the results of Fig. 7 confirm our interpretation and clearly indicate that illumination induces a redistribution of the Dmoesin protein from the rhabdomere to the cytoplasm of the cell body.

To address the influence of T559 phosphorylation on Dmoesin light-induced movement as observed in vivo, we expressed variant Dmoesin proteins with point mutations. The T559A mutation prevents phosphorylation of Dmoesin to keep it in the dormant state, whereas the T559D mutation is a phosphomimetic mutation that is expected to prevent phosphorylation but to keep the molecule in the “open” (presumably active) state (Polesello et al., 2002). When compared with Dmoesin-WT-GFP, distribution of Dmoesin-T559A-GFP in dark-raised flies showed a marked difference, with the major fraction of the GFP fluorescence in the cell body (Fig. 7 C), which is reminiscent of illuminated Dmoesin-WT-GFP flies (Fig. 7 B). In addition, illumination did not induce a significant change in the subcellular distribution of the Dmoesin phosphomutant T559A (Fig. 7 D). Dmoesin-T559D-GFP of dark-raised mutants showed intermediate distribution between rhabdomeres and the cell body. Although GFP fluorescence in the rhabdomere and cortical actin regions was significantly reduced relative to WT retina, it was much higher relative to the Dmoesin-T559A mutant (Fig. 7 E). Quantification of GFP colocalization with the rhabdomere autofluorescence confirmed that the Dmoesin-WT present in rhabdomeres of dark-raised flies was drastically reduced (greater than fivefold) upon illumination (Fig. 7 G). In contrast, most of Dmoesin-T559A fluorescence was not confined to the rhabdomeres in dark-raised retinæ, and there was no significant movement after light exposure. Also, similar levels of Dmoesin-T559D were observed in both dark- and light-raised retinæ. Thus, these data support the conclusion that phosphorylation of T559 controls the rhabdomeric localization of Dmoesin and that dephosphorylation controls its light-activated movement to the cell body.

To support this interpretation using a different approach, we analyzed the impact of T559 mutations on Dmoesin movement through biochemical characterization. The membrane and soluble fractions of head extracts from flies expressing Dmoesin-WT-GFP, Dmoesin-T559A-GFP, and Dmoesin-T559D-GFP were fractionated by SDS-PAGE and analyzed by Western blots using anti-GFP antibodies (Fig. 8 A). Although illumination reduced the Dmoesin level in the membrane fraction and concomitantly increased Dmoesin levels in the cytosol of Dmoesin-WT-GFP, the distribution of phosphorylation-defective Dmoesin mutants was unmodified by light (Fig. 8, A and B). As expected, the major fraction of Dmoesin-T559A-GFP was restricted to the soluble fraction, whereas the Dmoesin-T559D-GFP appeared in both the membrane-associated and the cytosol fractions (Fig. 8).

Together, these results demonstrate that the phosphorylation/dephosphorylation reactions of T559 regulate light-induced subcellular movement of Dmoesin in photoreceptors. In dark-raised flies, Dmoesin binds to the channels in the rhabdomere and illumination induces both Dmoesin dephosphorylation and relocation to the photoreceptor cytoplasm. Consistent with this interpretation, mutations impairing T559 phosphorylation either prevent (*T559A*) or reduce (*T559D*) association of Dmoesin with the rhabdomere. These mutations totally block intracellular redistribution of the protein in response to light.

Estimation of Dmoesin dynamics was obtained from Western blot analysis in which the reduction of Dmoesin levels in the membrane fraction and increase in the soluble fraction were measured after increasing durations of illumination (Fig. S2, available online at <http://www.jcb.org/cgi/content/full/jcb.200503014/DC1>). After 5 min of illumination, a significant fraction of Dmoesin was still detectable in the membranes. After 10, 30, and 60 min of light exposure, the level of Dmoesin further decreased in the membranes and increased in the soluble fraction (Fig. S2). Thus, the time scale of Dmoesin movement from the rhabdomere is similar to the time scale of TRPL translocation (Bähner et al., 2002).

Impaired phosphorylation of Dmoesin leads to degeneration of photoreceptor cells during prolonged illumination

Together with the redistribution of signaling molecules, we examined if the light-induced movement of Dmoesin was important for the physiology of photoreceptors. We examined the effects of Dmoesin mutations that impair T559 phosphorylation on the retinal structure after extended exposure to light. As prolonged illumination produces toxic effects in photoreceptors expressing GFP (unpublished data), we used transgenic lines carrying the same mutations, with the exception of Dmoesin, which was tagged with the *myc* epitope instead of GFP (Speck et al., 2003).

Newly eclosed flies expressing WT, T559A, or T559D Dmoesin proteins were subjected to constant light for 2, 4, and 7 d, and the ultrastructure of the ommatidia was analyzed by transmission electron microscopy. Ommatidia of flies expressing Dmoesin-WT-*myc* were indistinguishable from WT controls and presented a well organized ommatidium after 7-d illumination (Fig. 9, A and B). In contrast, the retinae of flies expressing T559A Dmoesin-*myc* were abnormal and revealed the initial stages of degeneration (Rubinstein et al., 1989) after only 2-d illumination (Fig. 9, E and F). Although the ommatidia of the illuminated T559D mutants appeared normal after 2-d illumination, slight degeneration was visible after 4-d illumination and a significant degeneration appeared after 7-d illumination (Fig. 9, C and D). Control experiments in which flies that expressed either WT or mutant Dmoesin forms were kept in the dark and did not show any sign of retinal degeneration (unpublished data).

These data suggest that the dynamic regulation of Dmoesin phosphorylation is critical for photoreceptor viability upon illumination. The slower degeneration of T559D mutants suggests that the presence of “active” Dmoesin in the rhabdomere is probably necessary, but not sufficient, to prevent degeneration during prolonged illumination and that dynamic phosphorylation/dephosphorylation reactions are required to prevent degeneration.

Discussion

Since their initial discovery in the 1980s, the ERM family of proteins has been implicated in numerous aspects of the control of actin organization (for review see Bretscher et al., 2002). Interestingly, ERM proteins are activated by signaling pathways to bridge actin filaments to membrane receptors and channels, thereby constituting a signal-dependent regulation of the cytoskeleton–membrane interface. However, functional analysis of ERM proteins during vertebrate development has been hampered by the functional redundancy of the three ERM paralogs. As their genome contains a unique ERM gene, *Caenorhabditis elegans* and *D. melanogaster* have become alternative model systems that recently revealed the important developmental roles of ERM proteins (Polesello and Payre, 2004). Several lines of evidence implicate Dmoesin in the regulation of the actin cytoskeleton throughout the stages of fly development (Jankovics et al., 2002; Polesello et al., 2002; Speck et al., 2003), including eye development (Karagiannis and Ready, 2004). This study extends the functional analysis of ERM proteins to investigate their role in the physiology of mature eyes. Our results provide novel data on the mechanism by which illumination may initiate reorganization of the cytoskeleton and suggest that the light-induced regulation of Dmoesin distribution is required to protect illuminated photoreceptors from degeneration.

Light- and phosphorylation-dependent interaction of Dmoesin with the light-activated channels TRP and TRPL

Illumination of *D. melanogaster* photoreceptor cells induces multiple molecular responses, which are initiated in the rhabdomere. Actin has been reported to undergo light-induced reorganization in both squid (Tsukita and Matsumoto, 1988) and *D. melanogaster*

photoreceptors (Kosloff et al., 2003), thus showing that light-sensitive cytoskeletal rearrangements are a common phenomenon. However, it remains unclear how illumination can modify the intracellular distribution of both signaling and cytoskeletal molecules. As a step toward understanding the molecular mechanisms that underlie these aspects of the light response in *D. melanogaster* photoreceptors, we analyzed the potential role of Dmoesin in this process.

In dark-raised flies, Dmoesin interacts with both the TRP and TRPL channels, as evidenced by reciprocal coimmunoprecipitation experiments. In contrast, virtually no Dmoesin–TRP and –TRPL complexes are coimmunoprecipitated from illuminated eyes, thus providing strong evidence for Dmoesin binding to the photoreceptor-specific channels primarily in the dark. Furthermore, our results show that light induces dissociation of Dmoesin from TRP and TRPL channels followed by movement of Dmoesin from the rhabdomere membranes to the cytoplasm. As there is increasing evidence to suggest that functions of invertebrate TRPs are conserved in their mammalian counterparts, our findings might provide new insights for characterizing vertebrate TRP functions. Interestingly, TRPC3 is part of a multimolecular signaling complex containing Ezrin, PLC β 1, and G α q/11 that is involved in Ca²⁺-mediated regulation of channel activity and cytoskeletal reorganization (Lockwich et al., 2001). In addition, it has been shown that the ERM adaptor EBP50/Na⁺/H⁺ exchanger regulatory factor associates with PLC β , TRPC4, and TRPC5 and regulates channel activity and sub-cellular localization (Tang et al., 2000; Mery et al., 2002; Obukhov and Nowycky, 2004). Altogether, these data strongly suggest that TRP–ERM interactions are an evolutionarily conserved mechanism with important functional properties. Our ability to modify Dmoesin binding to TRPs *in vivo* using illumination should constitute an invaluable tool for investigating the molecular mechanisms regulating this interaction.

In this study, the critical role of T559 phosphorylation on Dmoesin activation (Polesello et al., 2002; Speck et al., 2003) was extended through the demonstration that dissociation of the Dmoesin from the channel proteins upon illumination depends on T559 dephosphorylation. Accordingly, specific antibodies for the phosphorylated T559 form of Dmoesin immunoprecipitated the TRP channel of dark-raised flies, but not of illuminated flies. Moreover, monospecific TRP antibody immuno-precipitated the phosphorylated form of Dmoesin only in dark-raised flies. These results strongly suggest that only the phosphorylated form of Dmoesin binds TRP (Fig. 10). This finding further suggests that light induces dephosphorylation of Dmoesin, leading to dissociation of Dmoesin from the channel proteins, followed by its movement to the cell body.

Using WT and transgenic flies that express Dmoesin-GFP fusion proteins, we directly visualized the light-induced movement of Dmoesin from the rhabdomere to the cell body, through confocal imaging of fixed and living retinæ. The critical role of T559 phosphorylation on light-induced Dmoesin movement *in vivo* was further demonstrated through the use of two mutant forms of Dmoesin, in which T559 was replaced by alanine or aspartate residues. The fact that light-activated movement of Dmoesin was blocked in the T559A mutant that remains localized primarily to the soluble fraction of the cell body strongly supports the conclusion that phosphorylation of T559 is crucial for binding of Dmoesin to the channel proteins. Although the T559A mutation kept Dmoesin in its inactive cytosolic state, the T559D phosphomimetic mutation was expected to keep Dmoesin constitutively active. Although some T559D Dmoesin was also found in the cytosol, we indeed found a significant fraction of T559D Dmoesin in the membrane fraction that remains associated with the rhabdomeres after illumination. In addition, we found that both T559A and T559D mutations blocked the light-dependent movement of Dmoesin.

How could nontrafficking forms of Dmoesin (Dmoesin T559A and T559D) lead to light-induced retinal degeneration when expressed in an otherwise WT background? T559 phosphomutants of the Dmoesin protein have been shown to perturb the role of endogenous Dmoesin in actin organization and Oskar localization during oogenesis (Polesello et al., 2002). We found consistently that expression of DmoesinT559A-GFP and DmoesinT559D-GFP impairs the ability of endogenous Dmoesin to move upon illumination (unpublished data). As ERM proteins are capable of homotypic interaction (usually as dimers; Bretscher et al., 2002), Dmoesin T559A and Dmoesin T559D can titrate either WT Dmoesin or other functional partners. Therefore, the light-induced degeneration observed upon Dmoesin T559A and Dmoesin T559D expression can be explained by this reduction of endogenous Dmoesin traffic.

Altogether, these findings indicate that the rhabdomeric localization of Dmoesin requires its open (active) state, which is achieved either by phosphorylation or by the T559D phosphomimetic mutation. Our results, further, support that light-induced dephosphorylation triggers the movement of Dmoesin to the cytosol, and when this reaction is impaired by mutations, the light dependent movement of Dmoesin is blocked.

The implications of light-induced Dmoesin movement on translocation of signaling molecules, cytoarchitectural changes, and maintenance of the photoreceptor cells

Recent studies have demonstrated reversible light-induced reorganization of the actin cytoskeleton of the microvilli (Kosloff et al., 2003) and translocation of the TRPL channel (Bähner et al., 2002) from the rhabdomere to the cell body in time scales comparable to that of light-induced Dmoesin movement. Therefore, the light-induced movement of Dmoesin is likely involved in the control of the aforementioned processes.

Interestingly, genetic elimination of either signaling protein PLC β (*norpA*) or TRP prevents the light-induced movement of Dmoesin. These mutations are known to either block (*norpA*), or to strongly reduce (*trp*), the light-induced Ca²⁺ entry into the photoreceptor cells (Hardie and Minke, 1992; Peretz et al., 1994a). The effect of light on Dmoesin movement could thus be mediated via Ca²⁺-induced dephosphorylation of Dmoesin; e.g., by activation of a Ca²⁺-dependent phosphatase. PLC β -mediated hydrolysis of PIP₂ (which is highly enriched in rhabdomere membranes) might also participate in the release of Dmoesin into the cytoplasm upon illumination, as several works have shown the positive effect of PIP₂ binding on ERM protein activation, membrane localization, and binding to their partners (Niggli et al., 1995; Barret et al., 2000; Fievet et al., 2004). The data we accumulated in this study indicate the existence of a tight link between light reception and the Dmoesin-mediated reorganization of the rhabdomere cytoarchitecture.

Although the elucidation of the full spectrum of the physiological functions of the light-induced Dmoesin movement now awaits further works, we would like to suggest that these light-induced changes are necessary for the functional maintenance of photoreceptor cells. Photoreceptors are vulnerable cells because of their prolonged interaction with light (Kirschfeld and Franceschini, 1977). The peculiar organization of the rhabdomere in the form of very long (and tightly packed) microvilli makes it difficult for housekeeping mechanisms to operate in the rhabdomere. Light-activated reorganization of actin, along with cytoarchitectural changes, may allow the housekeeping function to operate and/or to participate in the down-regulation of signaling mechanisms triggered by light reception.

Materials and methods

Fly strains and genetics

D. melanogaster of the following strains were used: WT Oregon-R *w*; *trp*^{P343} (Scott et al., 1997) and *norpA*^{P24} (Pearn et al., 1996) null mutants for the TRP channel and eye-specific PLC β , respectively (both obtained from W.L. Pak, Purdue University, West Lafayette, IN); *trpl*³⁰² (Niemeyer et al., 1996) and *inaD*¹ (obtained from C.S. Zuker, University of California, San Diego, San Diego, CA); and *UAS Dmoesin-WT-GFP*, *UAS Dmoesin-T559A-GFP*, *UAS Dmoesin-T559D-GFP* (Polesello et al., 2002), *UAS Dmoesin-WT-myc*, *UAS Dmoesin-T559A-myc*, and *UAS Dmoesin-T559D-myc* (Speck et al., 2003). Dmoesin variants encoded in the transgenic lines were expressed using the Gal4/UAS targeted expression (Brand and Perrimon, 1993) using the *Rh1-Gal4* driver line (obtained from C. Desplan, New York University, New York, NY).

Light-dependent localization of Dmoesin

Flies were raised in complete darkness from the first instar larval stage to eclosion. For illumination experiments, live flies were placed in a transparent dish with reflective aluminum foil at the bottom and subjected to illumination with blue light (18 W fluorescent lamp with a wide band filter [1 mm; model BG 28; Schott]) for various durations at 22°C. Illumination with white light of the same intensity produced similar results. After illumination, the flies were moved to 4°C in the dark and the fly heads were promptly dissected. Three to five flies were used for each lane of the Western blots. Fly heads were homogenized in a hypotonic buffer (50 mM Hepes, pH 7, 300 mM NaCl, 3 mM MgCl, 10% vol/vol glycerol, protease inhibitor [Sigma-Aldrich], phosphatase inhibitor [Sigma-Aldrich], and 1 μ M caliculin A [Calbiochem]). Membrane and cytosolic fractions were separated by centrifugation at 15,800 *g* for 15 min, at 4°C. The pellet was washed and centrifuged again, and the supernatants were combined. Ultracentrifugation at 150,000 *g* for 30 min did not substantially change the distribution of Dmoesin between the fractions. Protein samples ran on 10% SDS-PAGE and were subjected to Western blotting using anti-Dmoesin or anti-GFP polyclonal antibodies. Quantification of the gels was performed using the BAS-1000 system (Fujifilm Worldwide) with TINA version 2.0 software.

Immunoprecipitation

Frozen heads (500–2,000) of dark-raised or illuminated flies were homogenized in 200 μ l of buffer containing 50 mM Hepes, pH 7.6, 150 mM NaCl, 3 mM MgCl₂, 10% glycerol, and protease and phosphatase inhibitors (Sigma-Aldrich). The homogenate was centrifuged at 100 *g* for 5 min to remove chitin materials. Membranes were isolated by centrifugation at 20,000 *g* for 30 min at 4°C and resuspended to a final equivalent of 200 μ l. Membrane proteins were extracted by incubating the membranes with 1% Triton X-100 and 500 mM NaCl for 1 h and centrifuging at 20,000 *g* for 30 min. For immunoprecipitations, protein A beads were incubated first with the relevant antibody (α TRP, α TRPL, α GFP, α INAD, or α Dmoesin) overnight at 4°C. The membrane extracts (of WT, *inaD*, *trp*, *norpA*, and *trpl* mutants) were incubated with the relevant antibody crosslinked to protein A beads (vol 20–100 μ l) in 200 μ l of total volume overnight at 4°C. The beads were washed in the Triton X-100 washing buffer (0.1% Triton X-100, 100 mM NaCl, and 50 mM Tris-HCl, pH 8.0). Immunoprecipitated proteins were eluted from protein A–agarose beads with 50 μ l of 1 \times SDS-PAGE loading buffer and subsequently analyzed by Western blot.

Immunoblots were incubated with primary antibodies in blocking buffer at the following dilutions: monoclonal mouse α TRP (Pollock et al., 1995) and mAb83F36 (obtained from S. Benzer, California Institute of Technology, Pasadena, CA), 1:2,000; rabbit α Dmoesin (obtained from D. Keihart, Duke University, Durham, NC), 1:2,000; rabbit α INAD, 1:1,000;

rabbit α -Phospho-ERM (Cell Signaling Technology), 1:2,000; mouse α TRPL, α Rh1, and α Choptin (Hybridoma Bank), 1:1,000. Immunoreactive bands were visualized by chemiluminescence reaction (obtained from Biological Industries), using HRP-conjugated goat anti-rabbit and anti-mouse as secondary antibodies (Jackson ImmunoResearch Laboratories).

EM

Fly heads were separated, bisected longitudinally, and fixed for 12 h in a solution of 1.5% PFA and 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The heads were washed three times in the same phosphate buffer and dehydrated in graded aqueous ethanol concentrations of up to 90%. After fixation, eyes were post-fixed with 1% osmium tetroxide for 4 h, dehydrated in ethanol and propylene oxide, and embedded in Epon. Thin sections were cut and stained with saturated aqueous uranyl acetate and lead citrate. Sections were observed with a Tecnai-12 transmission electron microscope (FEI) and photographed with a Mega-view II charge-coupled device camera (Philips).

Fluorescent confocal microscopy

Live retinæ from dark-raised or illuminated transgenic flies were isolated from the cornea and brain and kept in Ringer's solution as described previously (Peretz et al., 1994b). Optical sections of single ommatidia were visualized using the Fluoview confocal microscope (model 200 IX70; Olympus) using a LUM Plano Fl 60 \times , 0.9 NA, water objective. Optical sections were recorded from the upper region of the ommatidia, at a depth of 6–10 μ m from the tip of the ommatidium. Autofluorescence and GFP fluorescence were recorded sequentially using laser excitation wavelengths of 568 and 488 nm, respectively. Pictures are merged sequential images obtained by 568- followed by 488-nm excitation lights, on separate channels.

Immunocytochemistry

Dissected eyes of *yw D. melanogaster* or *norpA^{P24}* mutant in *w* background were fixed in 2% PFA in PBS (175 mM NaCl, 8 mM Na₂HPO₄, and 1.8 mM NaH₂PO₄, pH 7.2) for 1 h at RT, and then washed two times in 0.1 M phosphate buffer (0.1 M Na₂HPO₄ and 0.1 M NaH₂PO₄). This was followed by three washes in 10% sucrose and two washes in 25% sucrose for 15 min each. Eyes were then infiltrated with 50% sucrose overnight at 4°C, cryofixed in melting pentane, and sectioned at 10 μ m thickness in a cryostat (Mikrom Laborgeräte GmbH) at –25°C. The cryosections were incubated in 2% PFA in PBS for 10 min, washed two times in PBS, and then blocked in 1% BSA and 0.3% Triton X-100 in PBS (PBS-T) for 2 h at RT. The sections were incubated with α -Dmoesin diluted 1:2,000 or α -phospho-ERM diluted 1:50 in PBS-T overnight at 4°C. The sections were subsequently washed three times in PBS and were incubated with a Cy5-coupled secondary goat anti-rabbit antibody (Dianova) and rhodamin-coupled phalloidin (Sigma-Aldrich) in 0.5% fish gelatine and 0.1% ovalbumin in PBS for at least 4 h at RT. The sections were finally washed three times in PBS, mounted in Mowiol 4.88 (Polyscience), and examined with a confocal laser-scanning microscope (LSM-SP).

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Abbreviations used in this paper

EBP50

ezrin binding phosphoprotein 50

ERM	ezrin-radixin-moesin
INAD	inactivation-no-afterpotential D
NIS	non-immune serum
PDZ	PSD95/DlgA/ZO-1 homology
PIP₂	phosphatidylinositol 4,5-bisphosphate
T559	threonine 559
TRP	transient receptor potential
TRPL	TRP-like
WT	wild-type

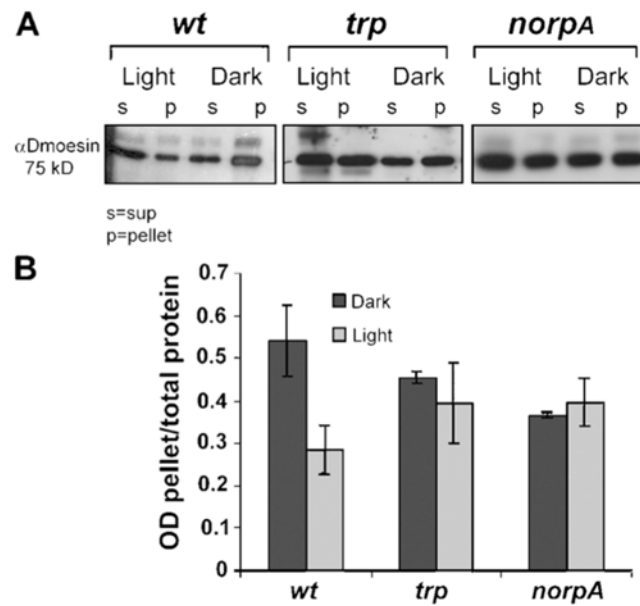


Figure 1. Protein blot showing light-dependent movement of Dmoesin from the membrane to the cytosol

(A) Membrane-associated (pellet) and soluble (sup) protein fractions were separated by high-speed centrifugation and processed for Western blotting with α Dmoesin antibodies. Head extracts were prepared from dark-raised (Dark) or illuminated (Light) flies of the following genotypes: WT, trp^{P343} (*trp*), and $norpA^{P24}$ (*norpA*). Although illumination induces redistribution of Dmoesin from membranes to the cytosol in WT flies, inactivation of either TRP or NORPA blocks the light-dependent movement of Dmoesin. (B) The histogram plots Dmoesin levels in the pellet divided by the total amount of Dmoesin present in extracts from WT, *trp*, and *norpA* heads, as revealed from repeated experiments done in similar conditions to those in A. $P < 0.01$; $n = 5$. The error bars are SEM.

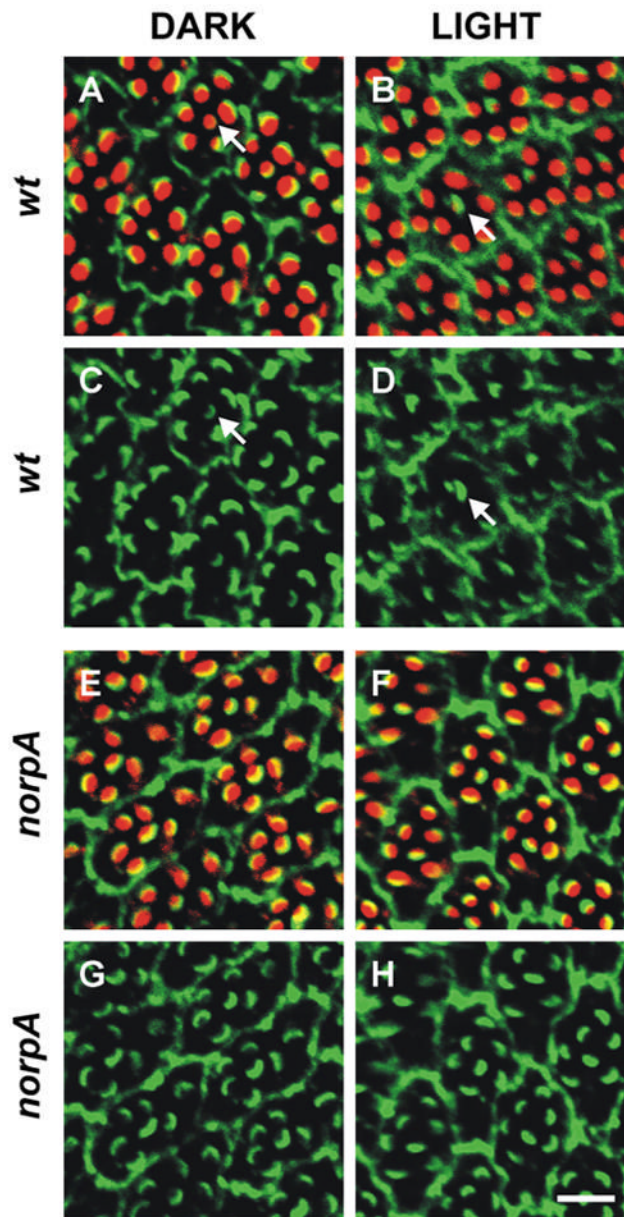


Figure 2. Light-dependent movement of Dmoesin in the photoreceptor cell of WT *D. melanogaster* retina

***D. melanogaster* retina**

(A–D) Cross sections through dark-raised and illuminated *wt* flies (Schott BG28 blue light for 1 h). Flies were double labeled with rhodamin-coupled phalloidin (red) and α Dmoesin (1:2,000 dilution). Primary Dmoesin antibody was detected by a Cy5-coupled secondary antibody (green; C and D). The overlay of both markers appears yellow in some ommatidia (A and B). Arrows indicate R7 cells. (E and F) The same experiment as in A–D was performed with *norpA*^{P24} mutant. Bar, 8 μ m.

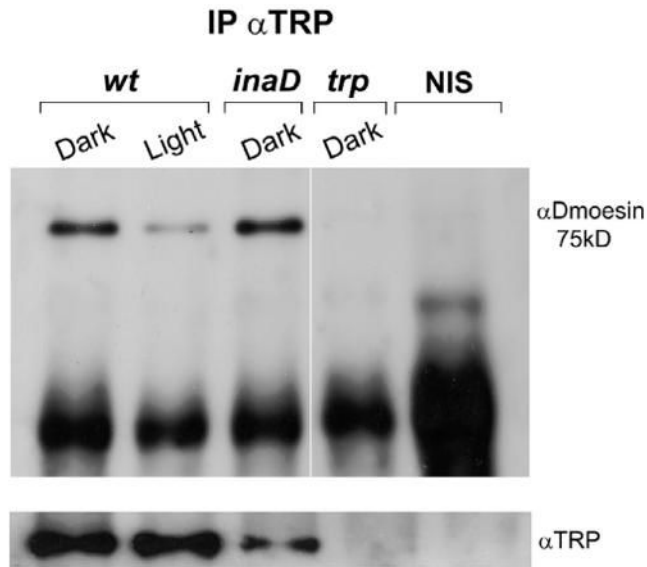


Figure 3. TRP interacts with Dmoesin only in dark-raised flies

Coimmunoprecipitation of TRP and Dmoesin. Protein extracts from heads of dark-raised and illuminated fly strains of WT, *inaD*¹, and *trp*^{P343} mutant flies were immunoprecipitated using α TRP or NIS and fractionated by SDS-PAGE. Western blot was probed with α Dmoesin. (bottom) Western blot of the same head extracts probed with antibodies against α TRP. Bradford analysis indicated that roughly equal amounts of proteins were loaded on each lane ($n = 12$).

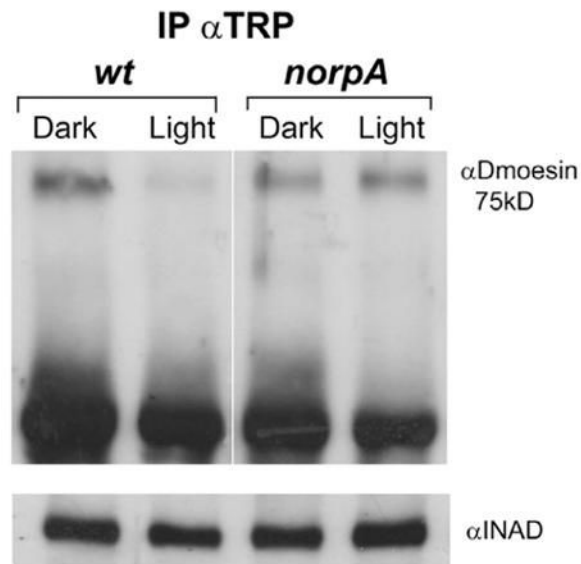


Figure 4. Dissociation of the Dmoesin–TRP complex depends on activation of the phototransduction cascade by light

Coimmunoprecipitation of TRP and Dmoesin from *D. melanogaster* photoreceptors cells. Protein extracts from heads of WT and *norpA*^{P24} mutant flies were immunoprecipitated using α TRP as indicated, and analyzed in Western blot with α Dmoesin. The *norpA* mutation prevented dissociation of the Dmoesin–TRP complex by light. (bottom) Western blot of the same head extracts probed with α INAD revealed that equivalent amounts of INAD, which interacts with TRP, are present in all samples ($n = 4$).

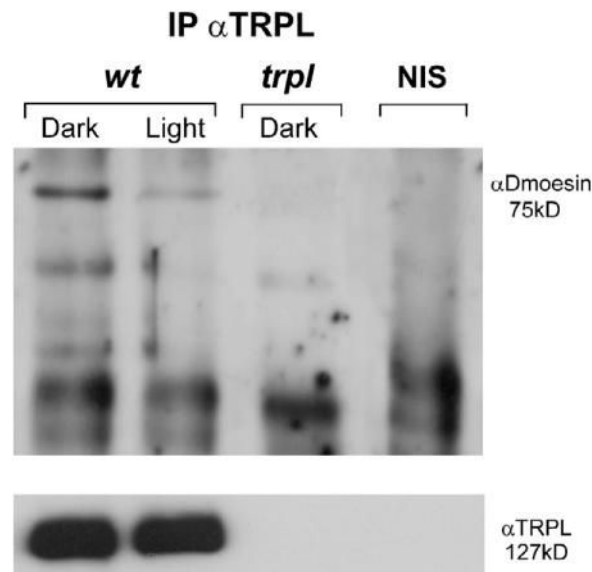


Figure 5. TRPL interacts with Dmoesin only in dark-raised flies

Coimmuno-precipitation of TRPL and Dmoesin from *D. melanogaster* photoreceptor cells. Immune complexes obtained from heads of dark-raised and illuminated fly strains including WT and *trpl*³⁰² null mutant (*trpl*) flies using α TRPL or NIS were fractionated by SDS-PAGE, and the Western blot was probed with α Dmoesin. (bottom) Western blot of the same head extracts probed with α TRPL ($n = 5$).

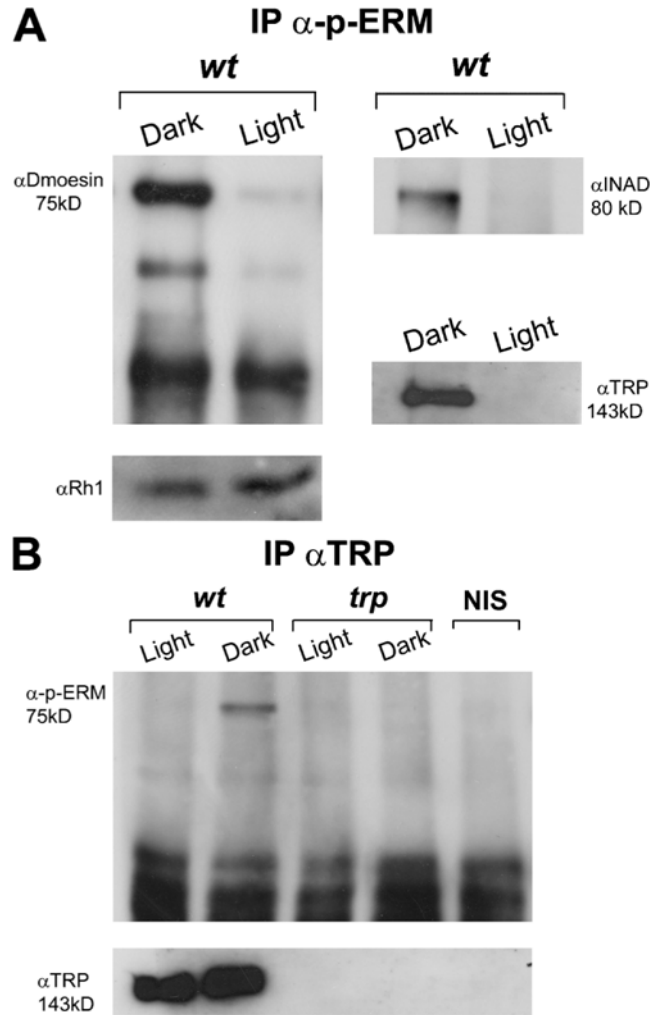


Figure 6. Light- and phosphorylation-dependent interactions between Dmoesin and the TRP channel

(A) Immunoprecipitation of *D. melanogaster* head extracts using α -phospho-ERM. Extracts were prepared from fly heads of dark-raised and illuminated WT flies and protein complexes were probed with α Dmoesin (left) and with α INAD or α TRP (right) in a separate experiment. (bottom) Western blot analysis of the same head extracts probed with the major rhodopsin, α Rh1. To detect TRP and INAD proteins in the immune complex, a threefold larger amount of head extracts were used ($n = 5$). (B) The experiments in A were repeated exactly, except that α TRP was used for the immunoprecipitation from head extracts of WT and *trp*^{P343} mutant, and protein complexes were probed with α -phospho-ERM. (bottom) Western blot of the same head extracts probed with α TRP. The two right lanes are Western blots from WT and *trp*^{P343} head extract probed with α -phospho-ERM ($n = 4$).

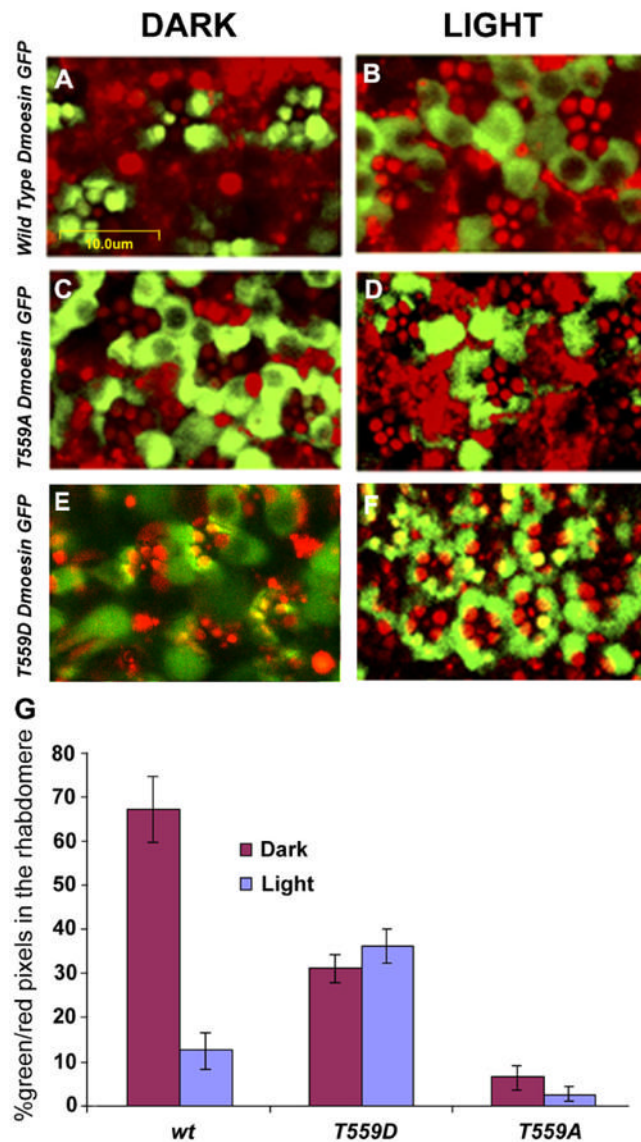


Figure 7. Light- and phosphorylation-dependent movement of Dmoesin from the rhabdomere to the cell body

(A–F) Intracellular distribution of Dmoesin-GFP protein fusions, as observed in confocal micrograph cross sections of living *D. melanogaster* retinæ of the transgenic lines: *UAS Dmoesin-WT-GFP* (A and B), *UAS Dmoesin-T559A -GFP* (C and D), and *UAS Dmoesin-T559D-GFP* (E and F). Green indicates GFP fluorescence. The strong autofluorescence of the rhabdomeres (red) allows localizing Dmoesin distribution with respect to photoreceptor compartments. Dark-raised flies were kept in obscurity (A, C, and E) or submitted to blue light illumination (B, D, and F). In flies expressing Dmoesin-WT-GFP (A and B), the fluorescent protein moves from the rhabdomere and cortical actin regions to the cell body of photoreceptors in response to light. Almost all Dmoesin-T559A-GFP (C and D) proteins accumulate outside of the rhabdomeres, independently of the illumination regime, and a significant fraction of Dmoesin-T559D-GFP (E and F) was observed in the rhabdomeres and cortical actin regions regardless of illumination regime. (G) The histogram plots the ratio of the number of green (GFP) to red (autofluorescence) pixels in the rhabdomere and cortical actin regions, as defined

by the area that displays autofluorescence. $P < 0.01$; $n = 20$ for each fly strain. The error bars are SEM.

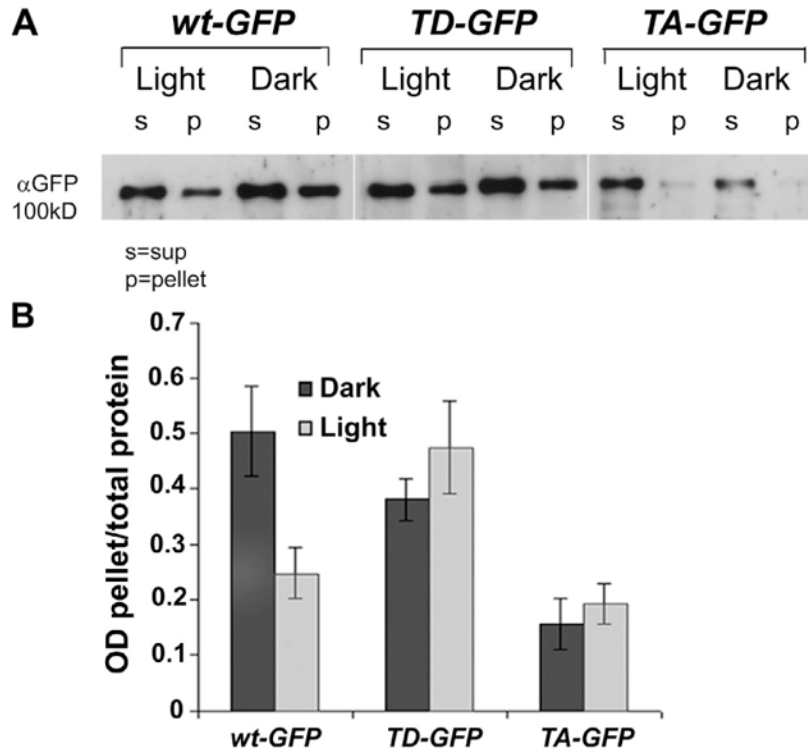


Figure 8. The light-dependent movement of Dmoesin from the membrane to the cytosol is blocked in the *Dmoesin-T559A* and *Dmoesin-T559D* mutants

(A) Western blot analysis of Dmoesin distribution in membrane-bound or -soluble fractions of *D. melanogaster* head protein extracts. Membrane and soluble proteins extracted from dark-raised and illuminated Dmoesin-GFP transgenic lines were Western blotted using α GFP. Extracts were prepared from the same fly strains as Fig. 7, as indicated. (B) The histogram plots the ratio of membrane-bound to total Dmoesin signals from replicate experiments similar to that shown in A. Although illumination halves levels of WT Dmoesin in association with membranes ($P < 0.01$; $n = 3$), no significant modification of Dmoesin distribution is provoked by illumination of Dmoesin-T559A-GFP and Dmoesin-T559D-GFP. The error bars are SEM.

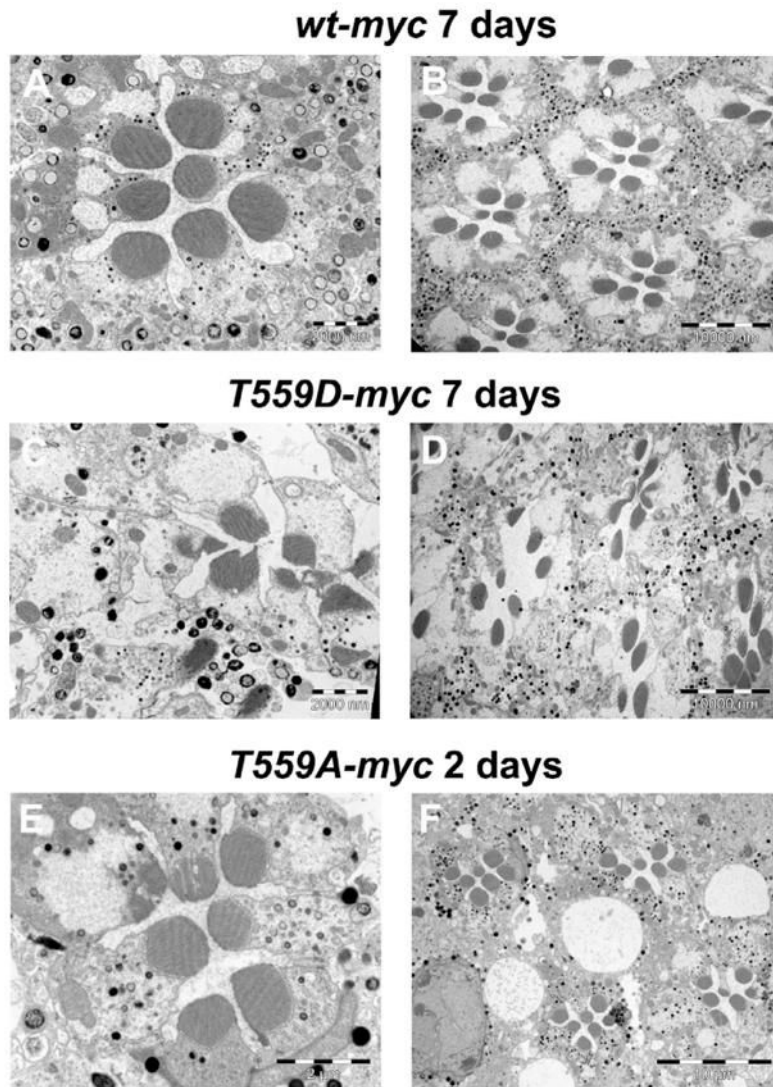


Figure 9. Impaired phosphorylation of Dmoesin leads to degeneration of photoreceptor cells during prolonged illumination

Electron micrographs showing the ultrastructural organization of ommatidia after 2- or 7-d-long white illumination. Eye sections from *D. melanogaster* expressing Dmoesin-WT-myc (A and B), Dmoesin-T559D-myc (C and D), and Dmoesin-T559A-myc (E and F).

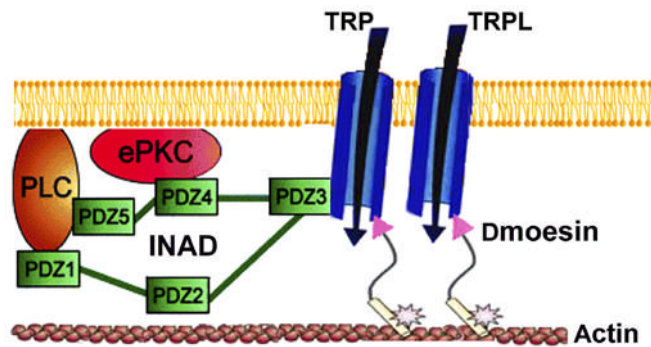


Figure 10. A scheme that summarizes the subcellular organization of Dmoesin and the major signaling proteins in the rhabdomeric membrane
 TRP anchors the INAD signaling complex, which includes PLC and eyePKC (ePKC), to the plasma membrane via the PDZ3 domain of INAD. The NH₂-terminal region of Dmoesin molecules (arrowheads) bind either directly or through a PDZ adaptor protein to the TRP and TRPL channels, whereas the COOH-terminal region is bound to the actin cytoskeleton. The phosphorylated site of T559 is indicated by an asterisk.