Bidirectional regulation of fragile X mental retardation protein phosphorylation controls rhodopsin homoeostasis

Xiao Wang\(^1\), Yawen Mu\(^1\), Mengshi Sun\(^1\), and Junhai Han\(^{1,2,*}\)

\(^1\) Institute of Life Sciences, The Key Laboratory of Developmental Genes and Human Disease, Southeast University, Nanjing 210096, China
\(^2\) Co-Innovation Center of Neuroregeneration, Nantong University, Nantong 226001, China

* Correspondence to: Junhai Han, E-mail: junhaihan@seu.edu.cn

Homoeostatic regulation of the light sensor, rhodopsin, is critical for the maintenance of light sensitivity and survival of photoreceptors. The major fly rhodopsin, Rh\(^1\), undergoes light-induced endocytosis and degradation, but its protein and mRNA levels remain constant during light/dark cycles. It is not clear how translation of Rh\(^1\) is regulated. Here, we show that adult photoreceptors maintain a constant, abundant quantity of ninaE mRNA, which encodes Rh\(^1\). We demonstrate that the Fmr\(^1\) protein associates with ninaE mRNA and represses its translation. Further, light exposure triggers a calcium-dependent dephosphorylation of Fmr\(^1\), which relieves suppression of Rh\(^1\) translation. We demonstrate that Mts, the catalytic subunit of protein phosphatase 2A (PP2A), mediates light-induced Fmr\(^1\) dephosphorylation in a regulatory B subunit of PP2A (CKa)-dependent manner. Finally, we show that blocking light-induced Rh\(^1\) translation results in reduced light sensitivity. Our results reveal the molecular mechanism of Rh\(^1\) homoeostasis and physiological consequence of Rh\(^1\) dysregulation.

**Keywords:** G protein-coupled receptor, rhodopsin, fragile X mental retardation protein, dephosphorylation, calcium, protein phosphatase 2A

---

### Introduction

The G protein-coupled receptors called rhodopsins are light sensors that are evolutionarily conserved in invertebrates and vertebrates (Palczewski and Saari, 1997; Montell, 1999; Hardie and Raghu, 2001). Rh\(^1\), the major rhodopsin in *Drosophila*, localizes to the rhabdomeres, the light-sensory organelles of photoreceptor cells. These organelles consist of numerous tightly packed microvilli and contain the phototransduction machinery (Montell, 1999). Rh\(^1\) protein levels are constant during normal light/dark cycles, which is critical for maintaining the high sensitivity to light of photoreceptors (Johnson and Pak, 1986; Hartman et al., 2001; Han et al., 2007; Tian et al., 2013). Pulse-expressed Rh\(^1\) has been shown to exist for longer than 8 days (Acharya et al., 2004), demonstrating that Rh\(^1\) is stable under normal condition. Conversely, ninaE mRNA, which encodes Rh\(^1\), is abundant in adult photoreceptors (Kumar and Ready, 1995; Sheng et al., 1997) and constant during a 12-h light/dark cycle (Hartman et al., 2001), indicating that Rh\(^1\) synthesis is repressed under normal condition. How ninaE mRNA translation is suppressed remains unclear.

In fly phototransduction, photon absorption causes the photoisomerization of chromophores, resulting in the formation of activated metarhodopsin. Metarhodopsin subsequently activates heterotrimeric G proteins and phospholipase C (PLC). In turn, the activation of PLC leads to transient receptor potential (TRP) and transient receptor potential-like (TRPL) channels opening and extracellular Ca\(^{2+}\) influx (Montell and Rubin, 1989; Ranganathan et al., 1991; Phillips et al., 1992). In contrast to fly phototransduction, activation of rhodopsins in vertebrate rods and cones leads to an increased activity of a cyclic guanosine monophosphate (cGMP) phosphodiesterase, which subsequently reduces cGMP levels and closes the cGMP-gated channels (Fu and Yau, 2007). Upon light exposure, a small fraction of activated Rh\(^1\) is internalized and degraded under normal physiological conditions, which prevents photoreceptor hyperactivity and excessive calcium influx (Lee and Montell, 2004; Satoh and Ready, 2005; Han et al., 2007). However, Rh\(^1\) protein levels remain constant through 12-h light/dark cycles (Hartman et al., 2001), suggesting that light exposure promotes Rh\(^1\) synthesis to compensate for its loss by endocytosis and degradation (Xiong and Bellen, 2013). Consistent with this model, the calmodulin-binding protein related to a Rab3 GDP/GTP exchange protein (Crag) has been shown to function in the...
transport of Rh1-bearing post-Golgi vesicles to rhabdomeres upon light stimulation. Loss of Crag results in light-induced accumulation of newly synthesized Rh1 in the cytoplasm of photoreceptors and eventually causes photoreceptor degeneration (Xiong et al., 2012). However, how light exposure promotes Rh1 translation and synthesis is still unknown.

Regulation of protein translation requires the presence and regulation of RNA-binding proteins that manage the process, such as the protein encoded by the fragile X mental retardation 1 gene (fmr1). Fmr1. Fmr1 normally functions as a pan-neuronal RNA-binding protein that associates with several mRNAs and represses their translation (Ashley et al., 1993; Brown et al., 2001; O’Donnell and Warren, 2002; Ascano et al., 2012). Loss of Fmr1 upregulates the translation of these target mRNAs (Zhang et al., 2001; Lee et al., 2003; Lu et al., 2004). Fmr1 includes three RNA-binding domains, one RGG domain, and two heterogeneous nuclear ribonucleoprotein (hnRNP) K homology domains (KH domains) (Ashley et al., 1993; Siomi et al., 1993). Consistent with its influence on regulating protein synthesis, the majority of Fmr1 in the cell is associated with polyribosomes (Tamanini et al., 1996; Stefani et al., 2004; Darnell et al., 2011; Chen et al., 2014). Post-translational modifications (phosphorylation in particular) have been shown to influence the translation state of Fmr1-associated polyribosomes (Ceman et al., 2003). Ribosomal run-off assays have suggested that unphosphorylated Fmr1 associates with actively translating ribosomes, while phosphorylated Fmr1 is found in stalled ribosomes (Ceman et al., 2003).

Here, we show that rhodopsin translation is bi-directionally controlled by a molecular switch: Fmr1 phosphorylation and dephosphorylation. We show that Fmr1 associates with ninaE mRNA and represses their translation under normal condition. Light exposure promotes rhodopsin translation via Mts-mediated Fmr1 dephosphorylation. We reveal that blocking light-induced Rh1 translation results in reduced light sensitivity. Taken together, our results demonstrate the molecular mechanism of Rh1 homoeostasis, which is essential for the visual function.

Results

Adult photoreceptors contain abundant and constant ninaE mRNA

Massive amounts of ninaE mRNA are transcribed during the late pupal stage to synthesize high amounts of Rh1 in photoreceptors (Kumar and Ready, 1995; Sheng et al., 1997). However, quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis showed that adult flies contain a comparable amount of ninaE mRNA with the late-stage pupae (Figure 1A). Consistent with the previous result of developmental northern analysis of ninaE transcription (Kumar and Ready, 1995), sequencing analysis confirmed that adult flies and late-stage pupae have identical ninaE mRNA sequences, implying that the presence of ninaE mRNA in late-stage pupae might be used for Rh1 translation in adult photoreceptors. Interestingly, we found that adult flies maintain a constant level of Rh1 under conditions of constant darkness and during 12-h light/dark cycles (Figure 1B and C), consistent with previous observations (Hartman et al., 2001; Han et al., 2007; Tian et al., 2013). Given that pulse-expressed Rh1 can exist for more than 8 days (Acharya et al., 2004), Rh1 does not seem to undergo rapid cycling under normal condition. Thus, the above observations strongly indicate that Rh1 translation in adult photoreceptors is strictly controlled under normal condition.

To explore how Rh1 translation is controlled in adult flies, we investigated the subcellular distribution of ninaE mRNA in adult photoreceptors. In situ hybridization experiments revealed that ninaE mRNA was predominately localized in the cytoplasm of adult photoreceptors (Figure 1D). To further visualize the distribution of ninaE mRNA in the translational machinery, we isolated the post-mitochondrial supernatant fractions from wild-type fly head extracts and separated messenger ribonucleoprotein particles (mRNPs), ribosomal subunits, monoribosomes, and polyribosomes using linear sucrose density gradient fractionation. The sedimentation of translational components was assessed by the absorption at 254 nm. Interestingly, ninaE mRNA was detected primarily in the fractions containing 80S monoribosomes (~35.5%) and polyribosomes (~35.2%, Figure 1E). These results indicate that ninaE mRNA translation is repressed in the translational machinery under normal condition.

Fmr1 associates with ninaE mRNA and represses its translation

Fmr1 associates with ribosomes and many different mRNAs to repress their translation in vivo (Feng et al., 1997; Zhang et al., 2001; Lee et al., 2003; Reeve et al., 2005; Chen et al., 2014; Wan et al., 2015). Thus, we hypothesized that Fmr1 associates with ninaE mRNA to repress the translation. To test this hypothesis, we conducted immunostaining experiments to examine whether Fmr1 is expressed in photoreceptor cells. We found that Fmr1 was highly expressed in photoreceptor cells and predominantly localized in the cytoplasm (Figure 2A). Next, we conducted co-immunoprecipitation experiments to identify which mRNA associate with Fmr1 in vivo. For this experiment, the mRNA of chickadee, the gene encoding profilin, was used as a positive control (Reeve et al., 2005), while α-tubulin mRNA was used as a negative control (Lee et al., 2003). Using anti-Fmr1 antibody, we immunoprecipitated mRNAs from the lysates derived from wild-type adult fly heads. RT-PCR analyses revealed that ninaE and chickadee mRNAs were readily detected in the total RNA and the Fmr1 antibody-immunoprecipitated RNA, whereas α-tubulin mRNA was rarely detected (Figure 2B). In contrast, arr2 mRNA, which encodes a key regulator in photoreceptors, was only weakly detected (Figure 2B). These results demonstrate that Fmr1 associates with ninaE mRNA in vivo.

Fmr1 has been shown to have dose-dependent function in vertebrates and invertebrates (Peier et al., 2000; Morales et al., 2002; Menon et al., 2004; Reeve et al., 2005). To provide further evidence that Fmr1 associates with ninaE mRNA and represses its translation in vivo, we genetically manipulated Fmr1 protein levels and measured Rh1 expression. Two fmr1 mutant heterozygotes, fmr1<sup>1113M/+</sup> and fmr1<sup>1550M/+</sup>, both with reduced Fmr1 protein levels, showed significantly increased Rh1 levels (Figure 2C). We also generated Rh1-Gal4/++; tubulin-Gal80<sup>Δ</sup>/UAS-Fmr1 flies
and examined the potential role of Fmr1 overexpression in Rh1 translation suppression. In these flies, Fmr1 overexpression was suppressed during all development stages by the ubiquitous expression of temperature-sensitive Gal80ts and selectively induced by exposure to a temperature of 30°C upon onset of adulthood. We entrained the adult flies under a 12-h light/dark cycle, which enables light-induced Rh1 internalization and degradation. After a 6-day treatment, Rh1-Gal4/+; tubulin-Gal80ts/UAS-Fmr1 flies showed lower Rh1 protein levels than control flies (Figure 2D), while the ninaE mRNA levels were normal (Figure 2E). Taken together, these data demonstrate that Fmr1 associates with ninaE mRNA and represses Rh1 translation in adult photoreceptors.

**Light exposure promotes Rh1 synthesis in adults**

Upon light exposure, a small portion of activated Rh1 undergoes internalization and degradation (Satoh and Ready, 2005; Han et al., 2007). However, adult flies show constant Rh1 protein levels when exposed to 12-h light/dark cycles (Hartman et al., 2001; Acharya et al., 2004; Han et al., 2007; Xiong and Bellen, 2013). Thus, Rh1 is thought to be synthesized to maintain constant levels during light exposure. Given that the turnover rate of Rh1 is much higher in the light than in the dark, light stimulation seems to promote Rh1 synthesis to compensate for the loss of Rh1. Because Rh1 is abundant and undergoes light-induced endocytosis and degradation, it is difficult to distinguish the newly synthesized from mature Rh1 to measure
light exposure-induced Rh₁ translation. To compare Rh₁ synthesis rates under light and dark conditions, we generated GMR-Gal₄/+;tubulin-Gal80ts/UAS-Rh₁-GFP flies and completed pulse stimulation by exposing adult flies to a temperature of 30°C for 30 min. This treatment enabled sufficient mRNA transcription for subsequent Rh₁-GFP translation. In these flies, Rh₁-GFP represents the newly synthesized Rh₁, which can be distinguished from mature Rh₁. Western blot analysis revealed that Rh₁-GFP was synthesized rapidly in the light, but underwent slow synthesis in the dark (Figure 3A). These data demonstrate that light stimulation promotes Rh₁ translation. We excluded the possibility that light exposure promotes Rh₁-GFP translation through the elevated transcription of Rh₁-GFP mRNA, because qRT-PCR analysis showed that its level was comparable between dark-entrained and light-exposed flies (Figure 3B).

**Light exposure promotes Fmr1 dephosphorylation**

Fmr1 is post-translationally phosphorylated, and Fmr1 dephosphorylation is an essential step for the dissociation of the Fmr1–ribosome complex, thus activating mRNA translation (Darnell et al., 2011; Chen et al., 2014). Therefore, we wondered whether light exposure promotes Fmr1 dephosphorylation, which subsequently dissociates the Fmr1–ribosome complex and activates ninaE mRNA translation. To test this hypothesis, we investigated the distribution of phosphorylated Fmr1 in the translational machinery. Due to the absence of the specific antibody that recognizes phosphorylated Drosophila Fmr1, we separated phosphorylated Fmr1 and unphosphorylated Fmr1 using Phos-tag sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Hosokawa et al., 2015). By using calf intestinal alkaline phosphatase (CIP) treatment, we identified the protein band that corresponds to phosphorylated Fmr1, which migrated slowly relative to unphosphorylated Fmr1 and disappeared upon CIP treatment (Figure 4A). Both unphosphorylated and phosphorylated Fmr1 were detected primarily in the fractions containing mRNPs, ribosomal subunits, and 80S monoribosomes (Figure 4B). Although unphosphorylated Fmr1 was present in the fractions containing polyribosomes, phosphorylated Fmr1 was absent from these fractions (Figure 4B). These results confirm that Fmr1 dephosphorylation is correlated with the dissociation of the Fmr1–ribosome complex.
Hardie and Raghu,

Ceman et al.,

Tian et al.,

Narayanan et al.,

Montell,

Hardie,

Figure 3 Light exposure promotes Rh1 translation. (A) Upper panel: Rh1-GFP expression levels under dark and light exposure conditions. A 30-min heat shock was applied immediately before time point 0. Lower panel: quantification of Rh1-GFP protein levels for the corresponding times and conditions. Two-way ANOVA test. (B) Quantification of relative Rh1-GFP mRNA levels after 60 min of darkness or light exposure. Two-tailed student’s t-test.

Next, we monitored the level of phosphorylated Fmr1 upon light stimulation. Approximately 50% of Fmr1 was phosphorylated in dark-adapted retinai (Figure 4C and D). Interestingly, 30 min of light stimulation significantly decreased the amount of phosphorylated Fmr1, and prolonged light stimulation (60 min) reduced the amount further, accompanied with a reduction in the total Fmr1 level (Figure 4C and D). Phosphorylated Fmr1 and total Fmr1 levels were partially recovered after 3 h of light exposure (Figure 4C and D). Given that Fmr1 mRNA is a target of Fmr1 protein (Didiot et al., 2008; Ascano et al., 2012), the recovery of Fmr1 protein might be due to the relieved suppression of its own translation. The dynamic state of Fmr1 phosphorylation was consistent with the time course of light-induced Rh1 translation (Figure 3A). These results demonstrate that light stimulation promotes Fmr1 dephosphorylation, and suggest that dephosphorylated Fmr1 dissociates the Fmr1–ribosome complex, thus activating Rh1 translation.

Drosophila Fmr1 has been shown to be primarily phosphorylated at a highly conserved serine residue (S406), which is corresponding to Ser499 of human Fmr1 (Siomi et al., 2002). Moreover, this phosphorylation may influence the translation state of Fmr1-associated polyribosomes (Ceman et al., 2003; Muddashetty et al., 2011). To examine whether the residue (S406) is the phosphorylation site of Fmr1 in fly photoreceptor cells, we mutated Ser406 to Ala in the context of full-length Drosophila Fmr1 and conducted an in vitro phosphorylation assay through an incubation of purified Fmr1 proteins with the extract of photoreceptor cells. Convincingly, a MBP-Fmr1 mutant (S406A) was not phosphorylated, whereas wild-type Fmr1 was phosphorylated (Figure 4E). This result indicates that Ser406 is the primary phosphorylation site of fly Fmr1 in photoreceptor cells.

Light-induced Fmr1 dephosphorylation is dependent on the activation of phototransduction cascades

It is well known that light stimulation triggers the activation of phototransduction cascades, and prolonged light stimulation induces Rh1 endocytosis (Montell, 1999; Hardie and Raghu, 2001; Satoh and Ready, 2005). We asked how light stimulation triggers Fmr1 dephosphorylation. First, we investigated whether light-induced Rh1 endocytosis promoted Fmr1 dephosphorylation. We blocked Rh1 endocytosis by expressing a temperature-sensitive, dominant-negative form of dynamin, shibire (Shi) (Chen et al., 1991; van der Bliek and Meyerowitz, 1991). We found that expression of Shi in photoreceptors failed to suppress the light-induced Fmr1 dephosphorylation at a restrictive temperature of 31°C (Figure 5A). This observation excludes the possibility that the endocytic Rh1 induces Fmr1 dephosphorylation.

Next, we examined whether light-induced Fmr1 dephosphorylation was dependent on the activation of phototransduction cascades. In Drosophila, the norpA gene encodes PLC, a key component of phototransduction cascades that leads to the opening of TRP and TRPL ion channels (Hardie and Raghu, 2001). In contrast to wild-type flies, norpA mutant flies showed a constant level of phosphorylated Fmr1 upon light exposure (Figure 5B), indicating that activation of phototransduction cascades is critical for Fmr1 dephosphorylation. To further validate the role of phototransduction activation in Fmr1 dephosphorylation and Rh1 translation, we conducted a pulse expression experiment, using Rh1-GFP in the norpA mutant background. Convincingly, the rate of light-induced Rh1-GFP expression was significantly reduced in norpA mutant relative to wild-type flies (Figure 5C). These results demonstrate that light-induced Fmr1 dephosphorylation and subsequent Rh1 translation are dependent on the activation of the phototransduction cascades.

Activation of the phototransduction cascades opens TRP and TRPL channels and induces calcium influx, which evokes diverse intracellular responses (Hardie, 2001; Wang and Montell, 2007). We generated trpl;trp double-mutant and found that trpl;trp double-mutant flies showed a constant level of phosphorylated Fmr1 upon light exposure (Figure 5D). These results clarify that light-induced Fmr1 dephosphorylation is depended on the opening of TRP and TRPL channels. Next, we asked whether an elevated intracellular Ca2+ level promotes Fmr1 dephosphorylation. To test this hypothesis, we examined the dephosphorylation of Fmr1 by supplying the isolated ommatidia with the calcium ionophore, ionomycin. Interestingly, 5 μM ionomycin was sufficient to trigger Fmr1 dephosphorylation within 30 min in the dark (Figure 5E). Taken together, these data demonstrate that activation of the phototransduction cascade-induced Ca2+ influx triggers Fmr1 dephosphorylation.

Mts dephosphorylates Fmr1 in photoreceptors

Protein phosphatase 2A (PP2A) has been shown to mediate Fmr1 dephosphorylation in mammals (Narayanan et al., 2007; Muddashetty et al., 2011; Tian et al., 2012). To test whether PP2A mediates the light-induced Fmr1 dephosphorylation in flies, we monitored the activity of PP2A during light exposure. Interestingly, upon light exposure, the activity of PP2A was significantly increased within 30 min, and returned to the baseline
within 60 min (Figure 6A), agreeing with the dynamic of light-induced Fmr1 dephosphorylation. These results suggest that fly PP2A mediates the light-induced Fmr1 dephosphorylation.

PP2A is a major Ser/Thr phosphatase in all eukaryotic cells, which predominantly exists as a heterotrimeric complex consisting of a catalytic C subunit, a structural A subunit, and variable regulatory B subunits (Lechward et al., 2001; Shi, 2009). Because microtubule star (mts) encodes the major PP2A catalytic subunit in Drosophila, we investigated whether Mts mediates light-induced Fmr1 dephosphorylation in photoreceptors. Interestingly, photoreceptor-specific knockdown of mts largely suppressed light-induced Fmr1 dephosphorylation (Figure 6B and C). In addition, we found that Mts co-immunoprecipitated with Fmr1 in vivo (Figure 6D). The efficiency of Fmr1 immunoprecipitation by anti-Mts antibody was significantly increased after 30 min of light stimulation, but returned to the baseline after 60 min of light stimulation (Figure 6E). These results are consistent with the observed dynamics of light-induced PP2A activation (Figures 4C and 6A). These observations strongly suggest that light-enhanced Mts activation mediates Fmr1 dephosphorylation.

To examine the role of Mts activation in light-induced Rh1 translation, we generated Rh1-Gal4/+; tubulin-Gal80Δ/UAS-mts RNAi flies and measured their Rh1 levels. These flies have two important phenotypes. First, adult flies express mts RNAi after exposure to a temperature of 30°C. Second, light-induced Rh1 internalization and degradation is activated by the entrainment of the flies in a 12-h light/dark cycle. After a 6-day entrainment, Rh1-Gal4/+; tubulin-Gal80Δ/UAS -mts-RNAi flies showed a significant reduction in Rh1 levels (Figure 6F). These observations indicate that Mts mediates light-induced Rh1 translation in photoreceptors.

Figure 4 Light exposure promotes Fmr1 dephosphorylation. (A) Identification of phosphorylated Fmr1 band in Phos-tag SDS-PAGE. p-Fmr1 represents the phosphorylated Fmr1 band, whereas Fmr1 indicates the unphosphorylated Fmr1 band. (B) Unphosphorylated Fmr1, but not phosphorylated Fmr1, co-sedimented with polyribosomes. Upper panel: the absorption profile of a linear sucrose gradient at 254 nm, with the sedimentation and major ribosomal peaks indicated. Lower panel: the distribution of phosphorylated and unphosphorylated Fmr1 corresponding to the sedimentation profile. Fraction numbers are indicated below the corresponding lanes. (C) Phos-tag SDS-PAGE showing phosphorylated Fmr1 level in the retinae during light exposure is presented. Dark-adapted adult flies were exposed to room light (750 lux) at the indicated times and retinae were isolated. (D) Quantification of relative total Fmr1 and phosphorylated Fmr1 protein levels at each time. One-way ANOVA test. (E) In vitro phosphorylation assay showing the phosphorylation of MBP-Fmr1, but not MBP-Fmr1 mutant (S406A). The lower panel shows the loading of MBP-Fmr1 and MBP-Fmr1 mutant. The phosphorylation of MBP-Fmr1 was validated by CIP treatment.
Figure 5 Fmr1 dephosphorylation is dependent on the activation of phototransduction. (A) Phos-tag SDS-PAGE shows phosphorylated Fmr1 levels in UAS-ShiΔ/Δ; R1-Gal4/+ flies upon light exposure at restrictive (31°C) and non-restrictive temperatures (23°C). The lower panel shows quantification of relative phosphorylated Fmr1 levels. (B) Phosphorylated Fmr1 levels in wild-type and norpA mutant retinae upon light exposure. The lower panel shows quantification of relative phosphorylated Fmr1 levels. (C) Rh1-GFP expression in wild-type and norpA mutant flies upon light exposure. The lower panel shows quantification of Rh1-GFP protein levels. (D) Phosphorylated Fmr1 levels in trpl/trp double-mutant retinae upon light exposure. The right panel shows quantification of relative phosphorylated Fmr1 levels. (E) Phosphorylated Fmr1 level in isolated ommatidia after ionomycin treatment. Isolated wild-type ommatidia were incubated with 5 μM ionomycin under dark conditions, and were collected for Phos-tag SDS-PAGE at the indicated times. The right panel shows quantification of relative phosphorylated Fmr1 levels. One-way ANOVA test in A, B, E. Two-way ANOVA test in C. Two-tailed student’s t-test in D.

CKα mediates light-induced Mts activation

We sought to address how light exposure enhances the activity of Mts. First, we excluded the possibility that light exposure increases Mts expression, as qRT-PCR and western blotting showed that mts mRNA and Mts protein levels were constant during light exposure (Figure 7A and B). Next, we investigated whether light-induced Mts activation was mediated by any regulatory B subunits of PP2A, which are thought to control substrate specificity, activity, and intracellular distribution of PP2A holoenzymes (Virshup, 2000; Janssens and Goris, 2001; Lechward et al., 2001; Shi, 2009). Among those B subunits, members of the PR72 family contain two calcium-binding sites formed by EF hand motif, and have been shown to mediate calcium activation of PP2A (Ahn et al., 2007; Park et al., 2013; Wlodarchak et al., 2013). However, depletion of the fly homolog of RP72, CG4733, exhibited normal light-induced Mts activation and normal light-promoted Fmr1 dephosphorylation (Figure 7C and D). These results indicate that CG4733 does not mediate the light-induced Mts activation in photoreceptor cells.

We looked for other B subunit families that respond to Ca²⁺. Striatin, a member of the PR10 family, has been shown to associate with PP2A and calmodulin and regulate PP2A activity in Ca²⁺-dependent manner (Moreno et al., 2000; Ribeiro et al., 2010). Interestingly, we found that photoreceptor-specific depletions of CKα, the fly striatin, repressed light-induced Mts activation and Fmr1 dephosphorylation (Figure 7E and F), indicating that CKα mediates the light-induced signalling via this pathway in photoreceptor cells.

Blocking light-induced Rh1 translation reduces light sensitivity of photoreceptors

To explore the physiological role of Rh1 homoeostasis, we examined whether light-induced Rh1 translation is essential for light sensitivity of the fly photoreceptor. Sensitivity is determined largely by two factors: the density of rhodopsin molecules on the rhabdomeral membrane and the number of available phototransduction components (Johnson and Pak, 1986; Han et al., 2007). By conducting electroretinogram recordings, we compared the sensitivity of photoreceptors after entraining 1-day-old flies under either constant dark or 12-h light condition. After 12-h light exposure, wild-type flies showed a comparable light sensitivity as that without light exposure (Figure 8A and B), confirming that an intrinsic control mechanism is in place for Rh1 homoeostasis. In contrast, both Fmr1-overexpressing and Mts depletion flies resulted in a statistically significant reduction in light sensitivity (Figure 8A and B). These results are consistent with the previous observations that impaired Rh1 production leads to reduced light sensitivity (Han et al., 2007; Xiong et al., 2012).

In addition, we investigated whether the excessive Rh1 production has any effects on the physiological functions of photoreceptors. Although 1-day-old fmr1Δ112M/+ flies expressed
excessive Rh1 proteins (Figures 2C and 8C) and displayed the enlarged rhabdomere (Figure 8D), 14-day-old \textit{fmr}1\textsuperscript{Δ113M/+} flies showed significantly reduced Rh1 level (Figure 8C) and collapsed rhabdomere membrane (Figure 8D, arrows), suggesting that excessive Rh1 production might finally damage the survival and function of photoreceptors.

Taken together, our results reveal a molecular mechanism of Rh1 homoeostasis (Figure 8E). We propose that Fmr1 associates with \textit{ninaE} mRNA and ribosomes to repress Rh1 translation under normal condition. Upon light stimulation, Ca\textsuperscript{2+}-induced Mts activation dephosphorylates Fmr1, which subsequently dissociates the Fmr1–ribosome complex and relieves the suppression of Rh1 translation. Moreover, we show that Rh1 homoeostasis is essential for light sensitivity and photoreceptor survival.

**Discussion**

The \textit{Drosophila} eye is an ideal model to study rhodopsin biology and photoreceptor degeneration to further the understanding of retinitis pigmentosa (RP) pathology (Wang and Montell, 2007). Although the cycle of rhodopsin production and turnover has been extensively studied, the transcriptional and translational regulation of rhodopsin is still an area of active research (Wang and Montell, 2007; Xiong and Bellen, 2013). Here, we demonstrate that bidirectional regulation of Fmr1 phosphorylation controls rhodopsin homoeostasis. Our results reveal a
novel intrinsic mechanism for dysregulated Rh1.

In this study, we show that ninaE mRNA associates with Fmr1, a protein that contains three RNA-binding domains and functions as a conserved translational repressor in different species (Siomi et al., 1993; Feng et al., 1995; Grosset et al., 2000; Zhang et al., 2001). Moreover, we show that overexpression of Fmr1 represses Rh1 translation, whereas depletion of Fmr1 enhances Rh1 translation, consistent with the role of Fmr1 in translational repression (Siomi et al., 1993; Zhang et al., 2001). These results provide solid evidence that the translation of abundant ninaE mRNA in adult flies is tightly controlled by Fmr1.

Translation can be regulated at three different points: initiation, elongation, and termination (Groppo and Richter, 2009). Fmr1 may regulate translation initiation through its interactions with the cap-binding translation factor eIF4E and cytoplasmic Fmr1-interacting protein 1 (Napoli et al., 2008; Ronesi et al., 2012), as well as miRNAs (Jin et al., 2004; Edbauer et al., 2010; Muddashetty et al., 2011). In addition, Fmr1 may bind to ribosomes directly, thereby stalling polysomes and regulating at the elongation step (Corbin et al., 1997; Chen et al., 2014). Our gradient fractionation experiment reveals that ninaE mRNA predominantly exists in the fractions containing 80S monoribosomes and polyribosomes. Consistent with a previous report (Wan et al., 2015), our result illuminates that fly Fmr1 also exists in the fractions containing 80S monoribosomes and polyribosomes. These data suggest that Fmr1 regulates ninaE mRNA translation by stalling polyribosomes during elongation (Corbin et al., 1997; Chen et al., 2014).

Because Rh1 shows a constant protein level under 12-h light/dark cycles and Rh1 turnover is much faster in the light than in the dark, it has been proposed that light stimulation promotes Rh1 production (Xiong and Bellen, 2013). Using Rh1-GFP as a reporter, we provide solid evidence that light exposure promotes Rh1-GFP production. Convincingly, Crag and Rab11 have been shown to mediate Rh1 transport to rhabdomere membranes upon light stimulation and Ca2+ influx (Xiong et al., 2012). These data strongly support the hypothesis that light exposure promotes Rh1 production.

Post-translational modifications are known to regulate activity-dependent protein synthesis (Routenberg and Rekart, 2005). Fmr1 undergoes phosphorylation at a highly conserved serine residue (S499), which may influence the translation state of Fmr1-associated polyribosomes (Ceman et al., 2003; Muddashetty et al., 2011). Casein kinase II (CKII) has been shown to phosphorylate Fmr1 at a highly conserved serine residue (S406) in Drosophila (Siomi et al., 2002). Because CKII is a constitutively active kinase and its activation is not directly regulated by calcium (Hathaway and Traugh, 1982; Olsten and Litchfield, 2004), it is
reasonable to speculate that some of Fmr1 should be present in its phosphorylated form under normal condition. Our Phos-tag SDS-PAGE results show that ~50% of Fmr1 in photoreceptors is phosphorylated under normal condition. Our mutation experiments reveal that Ser406 is the primary phosphorylation site of Fmr1 in fly photoreceptor cells. Furthermore, our gradient fractionation experiment reveals that phosphorylated Fmr1 exists in the fractions containing 80S monoribosomes, but is rarely detected in the fractions containing polyribosomes, supporting the previous observation that the phosphorylated FMRP is associated with stalled polyribosomes (Ceman et al., 2003). Thus, it is possible that Fmr1 dephosphorylation subsequently dissociates the Fmr1—ribosome complex and relieves the suppression of Rh1 translation.

In contrast to human FMRP, the phosphorylation status of fly Fmr1 also influences its association with specific mRNAs in vivo (Siomi et al., 2002). Therefore, we can not completely exclude a role of Fmr1 dephosphorylation in its dissociation from ninaE.
mRNA, which may also relieve the suppression of Rh1 translation. Indeed, our results show that fly Fmr1 is rarely detected in the fractions containing heavy polyribosomes. There must exist additional molecular machineries to restrict ninaE mRNA translation in the fractions containing heavy polyribosomes, which needs to be further investigated.

In this study, we show that light stimulation induces a transient reduction in phosphorylated Fmr1 and total Fmr1 levels in photoreceptor cells. The transient reduction of total Fmr1 could be due to its degradation, because Fmr1 dephosphorylation can enhance its ubiquitination and degradation (Hou et al., 2006; Nalavadi et al., 2012). Recovery of total Fmr1 protein levels might be attributed to the synthesis of Fmr1, because Fmr1 targets its own mRNA (Didiot et al., 2008; Ascano et al., 2012).

Once Fmr1 is synthesized, it may be phosphorylated by constitutively active CKII (Hathaway and Traugh, 1982; Olsten and Litchfield, 2004). Thus, Fmr1 undergoes regulation upon light stimulation, in which light exposure induces Fmr1 dephosphorylation and degradation, and reduced Fmr1 levels trigger Fmr1 translation to restore protein levels.

Light stimulation triggers the activation of phototransduction cascades and Ca^{2+} influx (Montell, 1999; Hardie and Raghu, 2001), whereas prolonged light stimulation induces the ectodysis of activated Rh1 (Satoh and Ready, 2005). We reveal that light-induced Ca^{2+} influx, but not endocytosed Rh1, promotes Fmr1 dephosphorylation and subsequent Rh1 translation. Nevertheless, we also reveal that light induces Fmr1 dephosphorylation by Ca^{2+} activation of Mts, consistent with the role of PP2A in mediating FMRF dephosphorylation in mammals (Narayanan et al., 2007; Muddashetty et al., 2011). Importantly, we show that Ca^{2+}-induced activation of Mts in photoreceptor cells are mediated by CKa, which has been shown to associate with Mts in vivo (Ribeiro et al., 2010). Taken together, our results show a dual-layer feedback regulation model for Rh1 homeostasis. First, Fmr1 undergoes a homeostatic regulation during light exposure, and second, bidirectional regulation of Fmr1 phosphorylation controls Rh1 homeostasis.

**Materials and methods**

**Fly genetics and light treatment**

Wild-type flies (strain w^{1189}) were maintained on standard medium in the dark at 23°C, 60%–80% relative humidity, and transferred to new vials (with food) before light treatment. To induce Rh1 expression and Fmr1 dephosphorylation, the flies were exposed to room light (750 lux, from regular fluorescent tubes) for the indicated lengths of time. To avoid age-dependent retinal degeneration in some flies, all flies used for light-induced Rh1-GFP expression and light-induced Fmr1 dephosphorylation were examined at 1–2 days of age, except those indicated in Figure 8C and D.

**Phos-tag SDS-PAGE**

Phos-tag SDS-PAGE was performed as previously described (Kinoshita et al., 2006). Briefly, 6% acrylamide gel was mixed with 100 μM Phos-tag acrylamide (AAL-107, Wako) and 200 μM MnCl₂. Proteins were resolved by electrophoresis, and the gel was washed with blotting buffer containing 10 mM EDTA for 1 h to chelate Mn^{2+}. Proteins were transferred to PVDF membrane and used for blotting as described above. The signals were detected with ECL Prime Western Detection Reagents (GE Healthcare). Signal intensities of phosphorylated and unphosphorylated Fmr1 were normalized to fly head loadings that were assessed by staining membranes using Coomassie blue. Data from three independent experiments were averaged.

More detailed experimental procedures are described in Supplementary material.

**Statistics**

The quantitative data are presented as mean ± SEM with scatter plots to indicate the individual data points. The two-tailed student’s t-test, one-way ANOVA test, and two-way ANOVA test were used to compare data from different genotypes or conditions. Statistical significance was set as: *P* < 0.05, **P** < 0.01, ***P* < 0.001; ns, no significance, *P > 0.05.

**Supplementary material**

Supplementary material is available at Journal of Molecular Cell Biology online.

**Acknowledgements**

We thank Dr Craig Montell (University of California, Santa Barbara) for p[UAS::Rh1-GFP] transgenic flies and anti-PLC antibody, the Bloomington Stock Center and Tsinghua Fly Center for providing flies, Dr Hong-Sheng Li (University of Massachusetts Medical School) for anti-Arr2 antibody, and members of the Han laboratory for critical comments on the manuscript.

**Funding**

This work was supported by grants from the Ministry of Science and Technology of China (2014CB942803 and 2012CB517903), the Excellent Youth Foundation of Jiangsu Province of China (BK20140024), and the Fundamental Research Funds for the Central Universities to J.H.

**Conflict of interest:** none declared.

**References**


