Phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] hydrolysis by phospholipase C (PLC) is a conserved mechanism of signalling. Given the low abundance of PI(4,5)P₂, its hydrolysis needs to be coupled to resynthesis to ensure continued PLC activity; however, the mechanism by which depletion is coupled to resynthesis remains unknown. PI(4,5)P₂ synthesis is catalyzed by the phosphorylation of phosphatidylinositol 4 phosphate (PI₄P) by phosphatidylinositol 4 phosphate 5 kinase (PIPK5). In Drosophila photoreceptors, photon absorption is transduced into PLC activity and during this process, PI(4,5)P₂ is resynthesized by a PIP5K. However, the mechanism by which PIP5K activity is coupled to PI(4,5)P₂ hydrolysis is unknown. In this study, we identify a unique isoform dPIP5K₁, that is both necessary and sufficient to mediate PI(4,5)P₂ synthesis during phototransduction. Depletion of PNUT, a non-redundant subunit of the septin family, enhances dPIP5K₁ activity in vitro and PI(4,5)P₂ resynthesis in vivo; co-depletion of dPIP5K₁ reverses the enhanced rate of PI(4,5)P₂ resynthesis in vivo. Thus, our work defines a septin-mediated mechanism through which PIP5K activity is coupled to PLC-mediated PI(4,5)P₂ hydrolysis.

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Introduction

In many eukaryotic cells the binding of extracellular ligands to plasma membrane receptors is transduced by the activation of phospholipase C (PLC). PLC hydrolyzes the membrane lipid phosphatidylinositol 4, 5-bisphosphate [PI(4,5)P₂] on the inner leaflet of the plasma membrane to generate inositol 1,4, 5 trisphosphate (IP₃) and diacylglycerol (DAG), both of which encode information leading to cellular responses (Berridge, 2009). PI(4,5)P₂ is present in low amounts at the plasma membrane and during the high rates of PLC activation seen during cell surface receptor activation, PI(4,5)P₂ at the plasma membrane would be rapidly depleted leading to loss of signalling sensitivity. However, PI(4,5)P₂ levels at the plasma membrane are relatively stable and the depletion of this lipid during cell PLC activation is typically only transient. Thus, the utilization of PI(4,5)P₂ by PLC is coupled to the synthesis of this lipid; in eukaryotes, the principal route of PI(4,5)P₂ synthesis is through the sequential phosphorylation of phosphatidylinositol (PI) at positions 4 and 5 by phosphatidylinositol 4 kinase (Nakatsu et al, 2012; Balakrishnan et al, 2018) and phosphatidylinositol 4 phosphate 5-kinase (Stephens et al, 1991; Chakrabarti et al, 2015) (reviewed in Kolay et al [2016]). However, for these enzymes to resynthesize PI(4,5)P₂ in response to its utilization by PLC activity, it is necessary that plasma membrane PI(4,5)P₂ levels are sensed and any drop in the levels of this lipid be communicated to the enzymatic machinery that regulates its synthesis.

Drosophila photoreceptors are an influential model for the analysis of G-protein–coupled PLC signalling (Raghu et al, 2012). In these cells, the absorption of light by rhodopsin in photoreceptors is transduced into ion channel activity through G-protein–coupled PLC activation leading to the hydrolysis of PI(4,5)P₂. As part of their visual ecology, Drosophila photoreceptors experience bright light illumination leading to high rates of PLC activation (Fain et al, 2010), yet fly photoreceptors do not undergo rapid inactivation and must therefore have mechanisms to sustain PI(4,5)P₂ levels during high rates of PLC activity experienced during bright light illumination. Previous studies have shown that in photoreceptors, during illumination, PI(4,5)P₂ resynthesis is regulated by a number of molecules including the phosphatidylinositol transfer protein RDGB (Yadav et al, 2015) and PI4KIIIα that generates PI₄P (Balakrishnan et al, 2018; Liu et al, 2018). Photoreceptors also express two genes encoding PIP5K activity that is needed to generate PI(4,5)P₂ from PI₄P, sktl (Hassan et al, 1998) and dPIP5K (Chakrabarti et al, 2015). Although dPIP5K null photoreceptors continue to express sktl, they show defects in both the electrical response to light and PI(4,5)P₂ turnover suggesting that this gene plays an indispensable role in generating a pool of PI(4,5)P₂ that is required for normal phototransduction (Chakrabarti et al, 2015). However, the mechanism by which dPIP5K activity is regulated during light induced PLC activity in photoreceptors remains unknown.

Septins are a group of evolutionarily conserved, GTP-binding proteins first discovered in yeast but now known to be widely distributed in the animal kingdom (Cao et al, 2009). Septins are part
of a multiprotein complex composed of multiple classes of sub-units; the complex is assembled at the inner leaflet of the plasma membrane using monomers and each class of monomer is composed of multiple members (Fung et al, 2014). They have been implicated in many processes including cell polarity, mitotic spindle positioning and cytokinesis. At the sub-cellular level, septins have been implicated in cytoskeletal organization (Mostowy & Cossart, 2012) through the formation of polymeric, filamentous structures. They have also been studied in the context of plasma membrane compartmentalization through a barrier function, and a role for septins in regulating store operated calcium influx at the plasma membrane has also been reported (Sharma et al, 2013). In mammals, the septin gene family is expanded to encode up to 13 members (Nishihama et al, 2011) divided into four classes and septin oligomers usually include members from each class. In addition to protein interactions between the monomers, septins have also been shown to interact with many cellular proteins and through this mechanism regulate sub-cellular processes (Neubauer & Zieger, 2017). In organisms such as Drosophila, the septin gene family is less diverse; for example, the Sept7 subgroup is encoded by a single gene, pnut (Neufeld & Rubin, 1994). Null mutations in pnut are homozygous lethal during development; however clonal analysis as well as studies of pnut<sup>fl</sup>/+ in Drosophila have implicated Pnut function in cytoskeletal regulation of cell shape and development in many tissues (Adam et al, 2000; Menon & Gaestel, 2015) and also in the regulation of store operated calcium influx (Deb et al, 2016). Importantly, septin monomers have been shown to bind P(4,5)P<sub>2</sub> (Zhang et al, 1999) through an N-terminal phosphoinositide-binding domain. This P(4,5)P<sub>2</sub> binding has been shown to modulate the activity of this class of proteins (Tanaka-Takiguchi et al, 2009; Bertin et al, 2010). Thus, septins are a class of proteins localized to the inner leaflet of the plasma membrane and bind P(4,5)P<sub>2</sub>. Given that PLC activity also hydrolyzes P(4,5)P<sub>2</sub> at the inner leaflet of the plasma membrane, and some septins have been shown to regulate calcium influx downstream of PLC signalling (Sharma et al, 2013), septins are suitable candidates for the regulation of P(4,5)P<sub>2</sub> resynthesis. However, to date there is no report of the regulation of P(4,5)P<sub>2</sub> levels at the plasma membrane by septins.

In this study, we identify an isoform of dPIP5K, dPIP5K<sup>L</sup> that is enriched in the eye and is both necessary and sufficient to support P(4,5)P<sub>2</sub> synthesis during PLC signalling and a normal response to light. Depletion of Pnut, a member of the septin family, enhances dPIP5K<sup>L</sup>-mediated synthesis of P(4,5)P<sub>2</sub> during PLC signalling in photoreceptors. Together, our findings define Pnut as a regulator of P(4,5)P<sub>2</sub> resynthesis during G-protein–coupled PLC signalling in vivo.

Results

Identification of dPIP5K<sup>L</sup>, a novel eye enriched isoform of dPIP5K

dPIP5K is an eye enriched PIP5K which produces P(4,5)P<sub>2</sub> required for normal phototransduction (Chakrabarti et al, 2015). In a loss of function mutant of dPIP5K (dPIP5K<sup>fl</sup>) the electrical response to light is severely reduced. dPIP5K has multiple predicted splice variants (Flybase-version FB2021_06: http://fb2021_06.flybase.org/) (Figs S1A and 1A). We observed that the reduced light response could be rescued by re-expressing all the isoforms of dPIP5K using a bacterial artificial chromosome rescue construct (Venken et al, 2006) for dPIP5K (dPIP5K<sup>BAC</sup>; see the Materials and Methods section for fly details). However, re-expression of a single isoform (dPIP5K<sup>L</sup>) was unable to restore the light response (Fig 1B and C). Based on the differential rescue and given that the dPIP5K gene is alternatively spliced, it seemed possible that a splice variant other than dPIP5K<sup>L</sup> must be required to support P(4,5)P<sub>2</sub> resynthesis during phototransduction. To identify such an isoform, we reverse transcribed total RNA extracted from 1-d old adult fly retina using gene specific primers, designed based on the genomic sequence of the dPIP5K locus. Sequence analysis of the amplified cDNA product revealed a novel isoform (Full sequence in Fig S1B), distinct from the already known isoform dPIP5K<sup>L</sup>. This transcript encoded a unique C-terminus of 273 amino acids previously not reported for existing isoforms. Therefore, we named this longer isoform as dPIP5K<sup>S</sup> and the known shorter isoform as dPIP5K<sup>L</sup> (Figs 1A and D and S1). Through a comparison of RNA extracted from wild-type and so<sup>0</sup> mutant (without eyes) heads, we also found that the dPIP5K<sup>L</sup> transcript is enriched in the eye (Fig 1E). Protein blots from Drosophila S2R+ cells transfected with dPIP5K<sup>L</sup> show the expression of a protein with the predicted molecular mass of 100 kDa (Fig 1F).

Previously we have reported that dPIP5K is localized to the microvillar plasma membrane, using an anti-dPIP5K antibody (Chakrabarti et al, 2015). That antibody was raised against a common region of both dPIP5K<sup>C</sup> and dPIP5K<sup>L</sup> and hence could not be used to detect specific isoforms by immunofluorescence. To detect the localization of each isoform, we generated epitope-tagged constructs for each of them (dPIP5K<sup>C</sup>::HA and dPIP5K<sup>L</sup>::GFP). When reconstituted individually into dPIP5K<sup>BAC</sup> (a protein null allelic) photoreceptors, we found that dPIP5K<sup>L</sup> localizes to the plasma membrane at the base of the microvilli (Fig 1G), whereas dPIP5K<sup>C</sup> was distributed throughout the cell body of photoreceptors, away from the microvillar membrane (Fig 1H). These observations prompted us to study if dPIP5K<sup>L</sup>, with its unique C terminal domain, could be relevant to phototransduction.

dPIP5K<sup>L</sup> encodes a polypeptide with PIP5K activity in vitro

To understand the functions of dPIP5K<sup>C</sup> and dPIP5K<sup>L</sup>, we measured their enzymatic activities. PIP5K enzymes use PI4P as a substrate and generate P(4,5)P<sub>2</sub>, Drosophila S2R+ cells do not express dPIP5K. For the enzymatic assay, we used the cell lysate from Drosophila S2R+ cells transiently expressing either dPIP5K<sup>L</sup> or dPIP5K<sup>C</sup> (Fig 2A). Using these lysates, we set up an in vitro kinase assay to quantify the extent of P(4,5)P<sub>2</sub> produced by the conversion of the exogenously supplied substrate 374 PI4P to P(4,5)P<sub>2</sub>. The synthetic substrate and product of unique molecular mass was detected and quantified by LC–MS/MS. The extent of enzymatic conversion was measured by the response ratio which is derived by dividing the area under the curve (AUC) of synthetic P(4,5)P<sub>2</sub> signal by the AUC of synthetic PI4P signal. Under these in vitro assay conditions, we observed that the response ratio of dPIP5K<sup>L</sup> transfected lysates was significantly higher than that from untransfected cell lysates,
signifying a high PIP5K activity (Fig 2B). Also, dPIP5K\(^\text{S}\) was catalytically inactive in vitro; the response ratio was not different between dPIP5K\(^{\text{S}}\) transfected cell lysates and those from untransfected controls (Fig 2B). The baseline PIP5K activity seen in untransfected cell lysates most likely arises from the endogenous PIP5K expressed in these cells encoded by \(\text{sstd}\) (Hassan et al, 1998), the only other gene in the Drosophila genome that encodes a PIP5K enzyme. We also confirmed that the PIP5K activity of dPIP5K\(^{\text{S}}\) is due to its kinase domain as a catalytically dead version of dPIP5K\(^{\text{S}}\) (dPIP5K\(^{\text{K189A}}\)) did not elicit a significantly different response ratio compared with untransfected controls (Fig 2C and D). Thus, we conclude that dPIP5K\(^{\text{S}}\) has a high PIP5K activity in vitro whereas dPIP5K\(^{\text{S}}\) by itself does not exhibit PIP5K activity.

dPIP5K\(^{\text{L}}\) is sufficient to sustain phototransduction

To study the functional relevance of dPIP5K\(^{\text{L}}\), we tested if it could rescue the defects in the electrical response to light in the eye (measured using an electoretinogram-ERG) seen in dPIP5K\(^{\text{S}}\). We expressed dPIP5K\(^{\text{L}}\) in dPIP5K\(^{\text{S}}\). We observed that most of the flies eclosed with eyes having morphological defects, hence could not be used for ERG recordings; this phenotype is likely due to altered developmental events and was dependent on the kinase activity of dPIP5K\(^{\text{L}}\) as it was not seen on expression of dPIP5K\(^{\text{K189A}}\) (Fig S2A). However, on rearing the cross at 18°C during development, we could obtain some escapers with normal eye morphology. Western blot analysis of lysates from normal eye morphology.
the eyes of such animals showed the selective re-expression of the dPIP5K1 isoform (Fig 2E). ERG recordings from these flies showed that expressing dPIP5KL alone in dPIP5K18 flies completely rescues both the amplitude as well as transients in the ERG (Fig 2F and G). We also observed that when expressed in dPIP5K18, dPIP5KL-K189A, which is catalytically inactive (Figs 2E and S2B), was unable to rescue the ERG phenotype (Fig 2F and G). We found that SKTL, the other PIP5K, failed to rescue the ERG phenotype (Fig S2C). These findings demonstrate that dPIP5K1 is sufficient to sustain a normal electrical response to light, a function that is dependent on its kinase activity.

dPIP5K1 is essential for normal phototransduction

Next, we checked whether dPIP5K1 is essential for phototransduction. To answer this question, we designed short hairpin constructs that would specifically target and knockdown dPIP5KL by targeting the exon coding for the unique C terminus region of dPIP5KL (Ni et al, 2009; Perkins et al, 2015). To test the effectiveness of these hairpin constructs, a Western blot was performed from Drosophila S2R+ cells co-transfected with dPIP5K1 and each of the hairpin constructs to check for dPIP5KL protein knockdown. Of the three hairpins we designed, construct 1 showed the highest level of...
knockdown (Fig S3A) and hence we generated transgenic fly lines with it. We used two different eye specific enhancers that differ in terms of strength and timing of expression (GMR-expression starts third larval instar onwards and Rh1 expression starts 70% pupal development onwards) to deplete dPIP5K\textsuperscript{i} transcript (Fig S3B) and this protein isoform was also depleted (Fig 3A). The transcript level of dPIP5K\textsuperscript{i} was not affected (Fig S3C). We observed that with increasing degree of dPIP5K\textsuperscript{d} depletion, the amplitude of the ERG response diminishes (Fig 3B and C). Furthermore, upon knockdown of Rh1 enhancer, which starts expressing from 70% pupal development, about 70% of the eclosed flies showed a lower ERG amplitude when compared with controls but with normal on/off transients (that report normal synaptic transmission), implying that the diminished amplitude upon depletion of dPIP5K\textsuperscript{i} is not a downstream consequence of altered synaptic transmission but rather the protein is required to sustain a normal receptor potential during the light response. These experiments demonstrate that dPIP5K\textsuperscript{i} is both necessary and sufficient to sustain a normal electrical response to light.

Light-dependent retinal degeneration, or collapse of the apical photoreceptor membrane post development in adult flies, is a phenotype frequently associated with changes in gene products that are a part of phototransduction (reviewed in Raghu et al (2012)). When all isoforms of dPIP5K (Rh1>dPIP5K\textsuperscript{NSV}) are overexpressed in photoreceptors using dPIP5K\textsuperscript{NSV} (Toba et al, 1999), a strong light dependent retinal degeneration is seen (Fig 3D and E). To test the contribution of each isoform to this phenotype, in this study, we overexpressed them individually and found that overexpression of dPIP5K\textsuperscript{i} (Rh1>dPIP5K\textsuperscript{i}) led to light dependent retinal degeneration (Fig 3D and E), whereas overexpression of dPIP5K\textsuperscript{d} did not result in this phenotype (Rh1>dPIP5K\textsuperscript{d}) (Fig 3D and E). The ability of dPIP5K\textsuperscript{i} overexpression to trigger retinal degeneration was dependent on kinase activity of the enzyme because overexpression of dPIP5K\textsuperscript{i,K189A}, a kinase dead version, did not show any degeneration (Fig 3D and E).

The heterotrimeric G-protein α subunit, Gq, is essential for PLCβ activity which in turn is required for phototransduction (Scott et al, 1995). Expression of a constitutively active form of Gq (Ac\textsubscript{Gq}) hyperstimulates phospholipase Cβ leading to excessive electrical activity and triggering retinal degeneration (Lee et al, 1994). If dPIP5K\textsuperscript{i} generates the PI(4,5)P\textsubscript{2} pool required for PLCβ activity, then one might predict that depletion of this isoform, thus reducing the availability of PI(4,5)P\textsubscript{2} required for PLCβ activity, might suppress the effects of Ac\textsubscript{Gq} expression. Consistent with this, when Ac\textsubscript{Gq} (Ratnaparkhi et al, 2002) was expressed in photoreceptors (Rh1>Ac\textsubscript{Gq}), we found that when reared in light, these photoreceptors underwent retinal degeneration (Fig 3F), and this retinal degeneration was slowed when dPIP5K\textsuperscript{i} was depleted in photoreceptors (Fig 3F).

dPIP5K\textsuperscript{i} is necessary and sufficient to support PI(4,5)P\textsubscript{2} turnover during phototransduction

dPIP5K\textsuperscript{i} and dPIP5K\textsuperscript{d} showed a differential ability to rescue the electrical response to light, a process that depends critically on normal PI(4,5)P\textsubscript{2} turnover during phototransduction. It has previously been reported that in the loss-of-function mutant of dPIP5K\textsuperscript{d}, the kinetics of PI(4,5)P\textsubscript{2} turnover is severely impaired (Chakrabarti et al, 2015). To test if this differential rescue of the ERG amplitude by dPIP5K\textsuperscript{i} and dPIP5K\textsuperscript{d} correlated with their ability to support PI(4,5)P\textsubscript{2} turnover, we measured PI(4,5)P\textsubscript{2} levels at the microvillar membrane in vivo, using a PI(4,5)P\textsubscript{2} reporter, PH-PLC\textsubscript{ε}:GFP, whose fluorescence intensity is a read out for PI(4,5)P\textsubscript{2} levels at the microvillar plasma membrane (see the Materials and Methods section for details). When dPIP5K\textsuperscript{i} was selectively expressed in dPIP5K\textsuperscript{d}, the delayed kinetics of PI(4,5)P\textsubscript{2} resynthesis in this mutant was rescued; by contrast selective expression of dPIP5K\textsubscript{i,K189A} or dPIP5K\textsuperscript{d} failed to do so (Fig 3G and H). Moreover, upon overexpression of dPIP5K\textsuperscript{i} in otherwise wild-type flies, the kinetics of PI(4,5)P\textsubscript{2} re-synthesis was accelerated; this was dependent on the kinase activity of dPIP5K\textsuperscript{i} because overexpression of dPIP5K\textsuperscript{i,K189A} (Rh1> dPIP5K\textsuperscript{i,K189A}) did not accelerate the resynthesis of PI(4,5)P\textsubscript{2} levels (Fig 3H and J). These observations indicate that dPIP5K\textsuperscript{i} is both necessary and sufficient to control PI(4,5)P\textsubscript{2} resynthesis after light-induced PLCβ activity.

Sep7 depletion alters the light response and PI(4,5)P\textsubscript{2} turnover

A previous study has implicated septin function in the regulation of PI(4,5)P\textsubscript{2} turnover during G-protein–coupled PLCβ signalling at the plasma membrane (Sharma et al, 2013). To examine if septins have any role in regulating PI(4,5)P\textsubscript{2} turnover during phototransduction, we performed an RNAi depletion based screen against all Drosophila septins looking for an effect on the amplitude of the electrical response to light. From this study, we found that knockdown of the sole class 7 septin in Drosophila (pnuts) (Figs 4C and 4D) led to an enhanced ERG amplitude (Fig 4A and B). Null mutants of pnut (pnut\textsuperscript{DN}) have previously been generated; homoyzgous pnut\textsuperscript{DN} mutants are not viable as adult flies and homoyzgous mosaic eyes of this allele impact eye development (Neufeld & Rubin, 1994) and preclude analysis of signalling in homoyzgous adult photoreceptors. However, in Western blots from Drosophila heads, pnut\textsuperscript{DP}/+ flies show circa 50% of the protein levels as compared with wild-type controls (Figs 4C and 4A) and photoreceptors are morphologically normal. We found that on stimulation with equal intensities of light, pnut\textsuperscript{DN}/+ flies have an enhanced ERG amplitude compared with controls (Fig 4D and E). Thus, depletion of PNUT results in an altered electrical response to light. We also measured PI(4,5)P\textsubscript{2} turnover in PNUT-depleted photoreceptors; resting levels of PI(4,5)P\textsubscript{2} were no different between pnut\textsuperscript{DN}/+ and controls (Fig 4F) however, after PLCβ activation by illumination, PI(4,5)P\textsubscript{2} levels recovered much faster in pnut\textsuperscript{DN}/+ compared with controls (Fig 4G).

Septin monomers have been shown to bind PI(4,5)P\textsubscript{2} (Zhang et al, 1999) and this binding can modulate the activity of this class of proteins (Tanaka-Takiguchi et al, 2009; Bertin et al, 2010) raising the attractive possibility that they may form the molecular link between PLCβ activity and PI(4,5)P\textsubscript{2} turnover at the plasma membrane. We incubated nitrocellulose membrane spotted with increasing concentrations of PI(4,5)P\textsubscript{2} with extracts of S2R+ cells overexpressing PNUT (Fig 4H). We found that PNUT shows a concentration-dependent binding to PI(4,5)P\textsubscript{2} (Fig 4I). Thus, PNUT
Figure 3. The kinase activity of dPIP5K1 is essential for phototransduction.

(A) Western blot from 1-d old fly retinae showing the degree of knockdown of dPIP5K (100 kD, topmost band, marked by arrowhead) in the mentioned genotypes upon expression of an RNAi to this isoform; blot was probed with dPIP5K antibody. Tubulin at 55 kD is used as loading control. All the crosses for RNAi were reared in 25°C incubators except for GMR>dPIP5K-RNAi@29, which was reared at 29°C, to enhance the efficacy of the RNAi.

(B) Representative ERG trace from 1-d-old flies of the indicated genotypes. The duration of the stimulus (a 2 s flash of green light) is shown. Y-axis shows response amplitude in mV, X-axis shows duration of the recording (s).

(C) Quantification of the peak amplitude for ERG response in each of the mentioned genotypes. Y-axis represents peak amplitude and X-axis shows the genotypes. Each
can bind PI(4,5)P2, and depletion of PNUT enhances PI(4,5)P2 turnover during PLCβ signalling in photoreceptors.

**PNUT regulates PI(4,5)P2 synthesis via dPIP5K1 and downstream of RDGB function**

During PLCβ signalling in Drosophila photoreceptors, PI(4,5)P2 is replenished through the sequential phosphorylation of PI by Ptk1 (Balakrishnan et al., 2018) and dPIP5K (Chakrabarti et al., 2015); the PI required as substrate is provided at the plasma membrane by the lipid transfer protein RDGB (Yadav et al., 2015; Raghu et al., 2021). To uncover the step of the phototransduction cycle that could be regulated by PNUT, we tested the effect of PNUT depletion in photoreceptors that were also depleted for RDGB. Previous work has shown that in the strong hypomorph rdgBΔ, there is a reduction in the electrical response to light, light dependent retinal degeneration and delayed PI(4,5)P2 resynthesis (Yadav et al., 2015). In rdgBΔ; pnutΔ/+ , although the basal PI(4,5)P2 levels were similar to controls (Fig 5A and B), the rate of PI(4,5)P2 resynthesis and the light dependent retinal degeneration were same as seen in rdgBΔ flies (Figs 5A and SSD and E). The ERG amplitude in rdgBΔ; pnutΔ/+ was only marginally improved compared with rdgBΔ alone (Fig S5C). Thus, intact RDGB function is required for the enhanced PI(4,5)P2 resynthesis seen in pnutΔ/+.

Next, we tested if dPIP5K1 depletion can impact the consequences of PNUT depletion. When dPIP5K1 depletion was performed in photoreceptors that were also pnutΔ/+ , the enhanced ERG amplitude of pnutΔ/+ was suppressed (Fig 5B). To test the relationship of this genetic interaction seen at the level of the ERG amplitude to PI(4,5)P2 turnover, we measured PI(4,5)P2 turnover kinetics comparing pnutΔ/+ with dPIP5K1 knockdown. We found that depletion of dPIP5K1 reversed the enhanced PI(4,5)P2 turnover kinetics seen in pnutΔ/+ flies (Fig 5C). Thus, the enhanced PI(4,5)P2 turnover kinetics seen in pnutΔ/+ requires normal levels of dPIP5K1.

To understand the mechanism of the genetic interaction between pnut and dPIP5K1 during PLCβ signalling, we studied the localization of these proteins in Drosophila S2R+ cells; in these cells, endogenous PNUT was found at the plasma membrane and when transfected into these cells, dPIP5K1 was also found at this location (Fig 5D). Because our genetic data (Fig 5B and C) suggest a negative interaction between pnut and dPIP5K1, we tested if dPIP5K1 activity might be altered in PNUT depleted cells. For this, we performed an in vitro kinase assay from Drosophila S2R+ cells expressing dPIP5K1Δ, where PNUT had been depleted using dsRNA. The degree of dsRNA induced depletion of PNUT protein was quantified (Figs 5F and S5F and G). Under these conditions, the activity of dPIP5K1Δ was significantly increased as compared with control cells (treated with dsRNA against GFP) (Fig S5E).

**Discussion**

The activation of PLC is a widespread mechanism of signalling triggered when plasma membrane receptors bind their cognate ligands. Given that the substrate of PLC, PI(4,5)P2, is present at the plasma membrane in low amounts, PLC activation can rapidly deplete this lipid leading to loss of signalling function in cells. This situation may arise in settings such as neurons where GPCRs activate PLC at high rates or T-cell receptor activation where sustained activation of PLC occurs over a long period of time. To avoid the depletion of PI(4,5)P2 leading to loss of signalling, it is essential that the resynthesis of this lipid is coupled with its consumption by PLC activity. To achieve this, conceptually, cells require a mechanism that couples PLC activation to the enzymatic machinery for synthesis so as to restore PI(4,5)P2 levels at the plasma membrane.

In eukaryotic cells, the synthesis of PI(4,5)P2 is primarily mediated by the PIP5K class of enzymes (reviewed in Kolay et al. 2016) and in some cells pools of PI(4,5)P2 specifically used during PLC signalling are synthesized by PIP5K (Chakrabarti et al., 2015). How is the activity of PIP5K controlled to trigger PI(4,5)P2 resynthesis? In this study we report a splice variant of dPIP5K (dPIP5KΔ), that is both necessary and sufficient to support PI(4,5)P2 turnover during Drosophila phototransduction, a process in which the plasma membrane of photoreceptors experiences high rates of PLC activation. Although the dPIP5K gene is alternately spliced to give two isoforms, dPIP5K1 and dPIP5K2, only dPIP5K1 was enzymatically active in vitro. Consistent with its function during phototransduction, we found that dPIP5K1 expression is enriched in Drosophila photoreceptors and the protein localizes to the base of the microvillus membrane, close to the sub-cellular site of phototransduction. A number of findings reported here strongly indicate that the kinase activity of dPIP5KΔ is required for its ability to support PI(4,5)P2 synthesis for PLC signalling in vivo: (i) both the reduced electrical...
response and the delayed kinetics of PI(4,5)P₂ resynthesis of dPIP5K null mutants can be rescued by a wild-type dPIP5Kα transgene but not a point mutant which is kinase dead; (ii) overexpression of wild-type but not kinase-dead dPIP5Kα results in light dependent retinal degeneration; and (iii) retinal degeneration triggered by constitutive activation of PLCβ can be rescued by depletion of dPIP5Kα. By contrast, although also contains a kinase domain, dPIP5Kδ did not show kinase activity in vitro and was not able to support PLC signalling in Drosophila photoreceptors in vivo. The function of dPIP5Kδ remains to be determined. However, given that the principal difference between dPIP5Kδ and dPIP5Kα is the C-terminal extension of the longer isoform, it would seem reasonable that this unique C-terminal region of 273 amino acids will be important in the control of enzyme activity and ability to support function in vivo. A conceptually similar observation has been reported in the case of a mammalian PIP5Kγ isoform, where the binding of FERM domain of talin to the C-terminal region of the enzyme has been reported to link this isoform to ongoing integrin signalling (Di Paolo et al., 2002; Ling et al., 2002).

For dPIP5Kα to support PI(4,5)P₂ synthesis, the enzyme needs to be regulated during light induced PLC activation. In a search for molecules that can mediate this regulation, we found that...
depletion of PNUT, the only septin 7 ortholog in Drosophila, results in an enhanced light response and accelerates the resynthesis of PI(4,5)P2 at the microvillar plasma membrane after PLC activation. These observations are consistent with a role of PNUT in regulating PI(4,5)P2 synthesis after PLC activation. We also found that the enhanced rate of PI(4,5)P2 resynthesis seen on PNUT depletion was abolished in rdgB mutants. Because the activity of RDGB (phosphatidylinositol transfer protein) supplies PI at the plasma membrane for PI(4,5)P2 synthesis (Yadav et al, 2015), this finding suggests that the enhanced PI(4,5)P2 synthesis seen in pnutXP/+ requires ongoing RDGB function and PNUT likely works on a step of PI(4,5)P2 resynthesis downstream of RDGB.

Figure 5. PNUT regulates PI(4,5)P2 synthesis via dPIP5KL and downstream of RDGB function. (A, C) Graphs quantifying the recovery kinetics of fluorescent pseudopupil with time in flies expressing the PI(4,5)P2 reporter PH-PLCδ::GFP. Y-axis represents the mean fluorescence intensity of the pseudopupil image expressed as percentage of the intensity of first image acquired, X-axis represents the red light illumination period in minutes (n ~ 7 flies for A, 10 flies for C). Error bars represent SEM. (B) Quantification of the peak amplitude for ERG response in each of the mentioned genotypes. Y-axis represents peak amplitude and X-axis shows the genotypes. Each data point represents an individual fly tested (n ~ 10 flies). Error bars represent SEM. (D) Representative confocal images of S2R+ cells transfected with GFP::PI(4,5)P2. Localization of endogenous PNUT is detected using the anti–PNUT antibody. GFP::PI(4,5)P2 is detected using the anti GFP antibody. Scale bar: 5 μm. (E) Response ratio of product/substrate [37:4-PI(4,5)P2:37:4-PI4P] in an in vitro kinase assay with cell free lysate prepared from S2R+ cells transfected with dPIP5KL, and treated either with control dsRNA against GFP (dPIP5KL+ds_GFP), or with dsRNA against PNUT (dPIP5KL+ds_Pnut) (n ~ 5 biological replicates). This experiment was repeated twice. Error bars represent SEM. (F) Western blots showing expression of PNUT from S2R+ cell extract transfected with dPIP5KL, and treated with dsRNA either against PNUT or control GFP. PNUT is detected at 60 kD. Knockdown of PNUT can be seen in ds_PNUT treated cells (dPIP5KL+ds_Pnut) as compared with control ds_GFP treated cells (dPIP5KL+ds_GFP). dPIP5KL (at 100 kD) is expressed equivalently in both cases. Tubulin at 55 kD is used as loading control. Data information: XY plots and scatter dot plots with mean ± SEM are shown. Statistical tests: (E) two tailed unpaired t test with Welch correction. (B) One-way ANOVA with post hoc Tukey’s multiple pairwise comparisons. (A, C) Two-way ANOVA grouped analysis with Bonferroni’s post multiple-comparisons tests to compare means. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. The statistical significance for the following data pairs is represented on graph (C) pnutXP/+ versus pnutXP/+; Rh1 dPIP5KL-RNAi.

Figure 6. Model for PNUT-mediated regulation of PI(4,5)P2 synthesis during G-protein–coupled PLC signalling in Drosophila photoreceptors. Towards left, is a cartoon showing cross section of a single Drosophila photoreceptor cell with rhabdomere (the apical plasma membrane, PM), sub-microvillar cisternae (SMC) which is a modified smooth ER compartment and cell body. The area indicated by the red box is expanded to show the biochemical reactions involved in the resynthesis of PI(4,5)P2. Protein and lipid components are depicted according to the compartment in which they are distributed. During phototransduction, upon light stimulation, activated PLC (PLCβ) hydrolyses the PI(4,5)P2 produced by dPIP5KL, depleting the PI(4,5)P2 at the plasma membrane. PNUT acts as a negative regulator of dPIP5KL activity, thus regulating the levels of PI(4,5)P2 present at the microvillar PM. This illustration was created with http://BioRender.com
downstream of RDGB during the synthesis of PI(4,5)P₂, the synthesis of Ptd4P by Ptd4KIIa and the phosphorylation of Ptd4P by PIP5K to generate PI(4,5)P₂. Our analysis revealed that the accelerated PI(4,5)P₂ resynthesis seen in pnut<sup>+/−</sup> could be rescued by depletion of dPIP5K<sup>1</sup>, consistent with the idea that PNUT regulates PI(4,5)P₂ resynthesis through a mechanism that requires PIP5K activity. We observed that in Drosophila S2R+ cells, both PNUT and dPIP5K<sup>1</sup> are localized to the plasma membrane, consistent with a role for PNUT dependent regulation of PIP5K function. Although PNUT and dPIP5K<sup>1</sup> co-localized at the plasma membrane, we were not able to demonstrate a direct protein–protein interaction between PNUT and dPIP5K<sup>1</sup> (data not shown). Thus, the regulation of dPIP5K<sup>1</sup> by PNUT may be indirect through the function of additional molecules. Many proteins interacting with septins have been described (Neubauer & Zieger, 2017). Indeed in Drosophila, PNUT has been shown to interact with septin interacting protein 1 (SIP1) (Shih et al, 2002) and an analysis of the role of such molecules in regulating PLC signalling will likely be useful in understanding regulation of PI(4,5)P₂ resynthesis by septins.

Given that the enhanced rate of PI(4,5)P₂ resynthesis on depletion of PNUT (pnut<sup>+/−</sup>) is suppressed on depletion of dPIP5K<sup>1</sup>, it seems likely that PNUT is a negative regulator of dPIP5K<sup>1</sup>. In principle PNUT could negatively regulate dPIP5K<sup>1</sup> in distinct ways: (i) PNUT might directly inhibit enzyme activity of dPIP5K<sup>1</sup>; and (ii) depletion of PNUT might improve the accessibility of the substrate Ptd4P to dPIP5K<sup>1</sup>. We found that the in vitro activity of dPIP5K<sup>1</sup> in the test tube was enhanced when provided with exogenous lipid substrate and a cell lysate depleted of PNUT and containing dPIP5K<sup>1</sup>. Because the activity on exogenously added Ptd4P was enhanced in PNUT-depleted lysates, it is possible that PNUT regulates dPIP5K<sup>1</sup> activity directly. However, it cannot be ruled out that in vivo, loss of PNUT may also improve the accessibility of dPIP5K<sup>1</sup> to Ptd4P. Previous studies have presented experimental evidence for septin 2 (Hu et al, 2010) and septin 7 (Ewers et al, 2014) in limiting protein diffusion, but a role for septins in modulating PI(4,5)P₂ resynthesis through a mechanism that requires PIP5K activity. We demonstrated a direct protein–protein interaction between PNUT and dPIP5K<sup>1</sup> (data not shown). Thus, the regulation of dPIP5K<sup>1</sup> by PNUT may be indirect through the function of additional molecules. Many proteins interacting with septins have been described (Neubauer & Zieger, 2017). Indeed in Drosophila, PNUT has been shown to interact with septin interacting protein 1 (SIP1) (Shih et al, 2002) and an analysis of the role of such molecules in regulating PLC signalling will likely be useful in understanding regulation of PI(4,5)P₂ resynthesis by septins.

### Materials and Methods

#### Fly stocks

Fly stocks were maintained in 25°C laboratory incubators with 50% relative humidity and no internal illumination. All flies were raised on standard corn meal media containing 1.5% yeast. For all the constant light experiments, flies were grown in an incubator with constant illumination from a white light source for the required time periods.

The wild type used was Red Oregon-R (ROR). Gal4 UAS system was used for targeted expression of transgenic constructs. The following fly alleles and insertions obtained for the experiments are described here: so<sup>P</sup> (#4287; Bloomington Stock), dPIP5K<sup>CG1</sup> over-expression line GS200386, with 1X UAS (DGRC-Kyoto), dPIP5K<sup>7B</sup> (Chakrabarti et al, 2015), dPIP5K<sup>L</sup>, dPIP5K<sup>K<sub>ATRA</sub></sup>, dPIP5K<sup>K<sub>RNAi</sub>−</sup> and dPIP5K<sup>K<sub>Δ</sub>−<sub>HA</sub></sup> and dPIP5K<sup>K<sub>Δ</sub>−<sub>GFP</sub></sup> (generated during this study), pnut<sup>+/−</sup>, UAS-AcGFLy-A (Gaiti Hasan, NCBs), PNUT<sup>RNAi</sup> (VDRC: v11791). For the generation of dPIP5K<sup>Bac</sup> fly, a bacterial artificial chromosome (BAC) clone encompassing dPIP5K, CH321-03B05 (in attr-B-Pacman-CmR vector) was obtained from [pacman] resource (Venken et al, 2006). This BAC clone was 57.178 Kb long and included the dPIP5K gene with extended 5’ and 3’ regions having the promoter and the regulatory regions of the gene present in 5’ UTR. The presence of dPIP5K in the clone was verified by PCR and this clone containing plasmid was isolated. The isolated plasmid after PCR verification was microinjected into embryos and inserted on third chromosome via ΦC31 integration into the VK00033 attP docking site (BDSC #32543) to generate the dPIP5K genomic transgene.

#### RNA extraction and Q-PCR

RNA extraction was done from 1-d-old Drosophila retinai or heads using TRIzol reagent (Invitrogen). After this, purified RNA was treated with amplification grade DNase I (Invitrogen). Reverse transcription was done using SuperScript II RNase H−Reverse Transcriptase (Invitrogen) and random hexamers (Applied Biosystems). Quantitative PCR (Q-PCR) was performed with the Applied Biosystems 7500 Fast Real-Time PCR instrument. Primers were designed at the exon-exon junction following the parameters recommended for Q-PCR. Transcript levels of the ribosomal protein 49 (RP49) were used for normalization across samples. Three separate samples were collected from each genotype, and duplicate measures of each sample were conducted to ensure the consistency of the data. The following primer pairs were used:

**RP49**

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**Sktl**

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**dIP5K**

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**dIP5K<sub>L</sub>**

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**dIP5K<sub>K<sub>Δ</sub>−<sub>HA</sub></sub>**

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**Cloning of dIP5K<sub>L</sub>**

Total RNA was isolated from fly retinae using the Trizol reagent, following which the RNA was treated with DNase I amplification grade for digestion of genomic DNA. Full length cDNA clones were obtained using primer specific to the 3’ end of dIP5K<sub>L</sub> and then cloned into an intermediate vector: pCR-XL-TOPO (TOPO XL PCR Cloning Kit; Invitrogen) and finally subcloned into fly expression vector.
vector pUAST-attB using Not1 and Xho1 restriction sites. Transgenic flies were obtained upon microinjection. dIP5KL\(^{K189A}\) (kinase dead) was obtained by doing a site directed mutagenesis at Lysine (K)189 position in dIP5KL. The following primer pairs were used:

To clone dIP5KL\(^{K189A}\) into pUAST-attB:

- UAS dIP5KL\(^{K189A}\) Not1_fwd: GCGGCCGCATGGCCTCCGGCGAT
- UAS dIP5KL\(^{K189A}\) Xho1_rev: GAGAGACTGAGCATATTATT

To generate dIP5KL\(^{K189A}\) kinase dead mutant:

- dIP5KL\(^{K189A}\) fwd: ACGAGTTGTCATAGCGACGTGCAACACAA
- dIP5KL\(^{K189A}\) rev: ACGAGTTGTCATAGCGACGTGCAACACAA

**Generation of dIP5KL-RNAi flies**

The Transgenic RNAi Project (TRiP) guidelines were used to synthesize shRNA constructs against dIP5KL, which were then cloned into Walium 20 vector. Transgenic flies were obtained upon microinjection of the constructs. The following primer pair was used:

- dIP5KL-RNAi fwd: CTAGCAGTGCTCTAGAACCATACGCTATGCTTGAATA
- dIP5KL-RNAi rev: AATTCGCGCGTCCTAGAAGACCTCTACCTATGCTTGAATA

**Electroretinogram (ERG)**

Flies were briefly put on ice to anaesthetize and immobilized at the end of a disposable pipette tip such that the head protruded outward. Recordings were done using glass microelectrodes filled with 0.8% wt/vol NaCl solution. Voltage changes were recorded between electrode placed at the surface of the eye and the ground electrode placed on the thorax. Before recording, flies were dark adapted for 5 min. Recordings were done with 2–s flashes of green light stimulus, with 10 stimuli (flashes) per recording and 15 s of recovery time between two flashes of light. Green light stimulus was delivered from a LED light source within 5 mm of the fly’s eye through a fibre optic guide. Calibrated neutral density filters were used to vary the intensity of the light source during intensity response experiments. Voltage changes were amplified using a DAM50 amplifier (SYS-DAM50; WPI) and recorded using pCLAMP 10.7 (Molecular Devices). Analysis of traces was performed using Clampt 10.7. Analysed results were plotted using GraphPad Prism software.

**Pseudopupil assay**

To monitor PI(4,5)P2 dynamics in live flies, transgenic flies expressing PH-PLC::GFP [PI(4,5)P2 biosensor] were anaesthetized and immobilized at the end of a pipette tip using a drop of colorless nail varnish and fixed by clay on the stage of an Olympus IX71 microscope. The fluorescent deep pseudopupil (DPP, a virtual image that sums rhabdomeric fluorescence from ~20 to 40 adjacent ommatidia) (Franceschini et al., 1981; Chakrabarti et al., 2015) was focused and imaged using a 10× objective. Time-lapse images were taken by exciting GFP using a 90 ms flash of blue light and collecting emitted fluorescence. The program used for this purpose was created in Micromanager. Following preparation, flies were adapted in red light for 6 min after which the eye was stimulated with a 90 ms flash of blue light (\(\lambda_{max}=488\) nm). The blue light used to excite GFP was also the stimulus to rapidly convert most of the rhodopsin (R) to metarhodopsin (M), thus activating the phototransduction cascade and triggering depletion of rhabdomeric PI(4,5)P2. Between the blue light stimulations, photoreceptors were exposed to long wavelength (red – \(\lambda_{max}=660\) nm) light that reconverts M to R. The recovery in DPP fluorescence intensity with time indicates translocation of the probe from cytoplasm to rhabdomere membrane upon PI(4,5)P2 resynthesis. The DPP intensity was measured using ImageJ from NIH. Cross-sectional areas of rhabdomeres of R1–R6 photoreceptors were measured and the mean intensity values per unit area were calculated. For quantification of recovery, the fluorescence value of the second image was subtracted from all images and this value for the first image was set as 100. The fluorescence value of subsequent images (i.e., n-second) were normalized to the value for first image.

**Optical neutralization**

To study retinal degeneration, flies of the desired age and reared under the required experimental conditions were decapitated and the heads fixed on a glass slide using a drop of colorless nail polish. Imaging of the photoreceptors was done using a 40× oil immersion objective lens (BX43; Olympus). Images were acquired by using CellSens software. For light adaptation, newly eclosed flies were transferred to incubators programmed with constant light settings.

**Scoring retinal degeneration**

To calculate a quantitative index of degeneration, 50 ommatidia from five different flies of each genotype were counted for each time point. The central photoreceptor, which is UV sensitive, hence did not show any light dependent retinal degeneration, was used as the reference and the rest of the photoreceptors were scored. Each intact rhabdomere was assigned a score of 1, and each degenerated rhabdomere was counted as 0. Hence, the maximum number is 7 and minimum is 1 (representing R7, which is insensitive to white light) Thus, control photoreceptors will have a score of 7 and the genotypes that undergo degeneration will have a score from 1 to 7. Analysed results were plotted using GraphPad Prism software.

**Western blotting**

Heads or retinae from 1-d-old flies were harvested and crushed in 2× SDS–PAGE sample buffer followed by boiling at 95°C for 5 min. For protein sample preparation from S2R+ cells, 48 h post transfection, cells were harvested, washed twice with PBS and lysed in 2× SDS–PAGE sample buffer. The genomic DNA was sheared using an insulin syringe following by boiling the samples at 95°C for 5 min. Protein extracts were separated using SDS–PAGE and electroblotted onto nitrocellulose filter membrane (Hybond-C Extra; [GE Healthcare]), using semidy transfer apparatus (Bio–Rad). Next, the membrane was blocked in 5% Blotto (sc-2325; Santa Cruz Biotechnology) in PBS with 0.1% Tween 20 (Sigma-Aldrich) (PBST) for 2 h at room temperature. Primary antibody incubation was done overnight at 4°C using the appropriate antibody dilutions. The
following antibodies were used: anti-α-tubulin (1:4,000, DSHB [E7c]), anti dPIPSK (1:1,000, lab generated), anti GFP (12,000, SC [B2]), anti HA (1:1,000, CST [6E2]), and anti-PNUT (1:250, DSHB [4C9H4-c]). Following this, the membrane was washed thrice in PBST for 10 min each and incubated with 1:1,000 dilutions of appropriate secondary antibody coupled to horseradish peroxidase (Jackson ImmunoResearch Laboratories) at room temperature for 2 h. Next, the membrane was washed thrice in PBST for 10 min each, developed with ECL reagent (GE Healthcare) and imaged in LAS 400 instrument (GE Healthcare).

Immunohistochemistry

For Immunohistochemistry, retinae from 1-d old flies were dissected in chilled PBS, followed by fixation in 4% paraformaldehyde in PBS with 1 mg/ml saponin at room temperature for 30 min on orbital shaker. Fixed retinae were washed three times in PBTx (1× PBS + 0.3% Triton X-100) for 10 min each. The sample was then blocked using 5% FBS in PBTx for 2 h at room temperature on shaker, after which the sample was incubated with primary antibody in blocking solution overnight at 4°C on a shaker. The following antibodies were used: anti-GFP (1:5,000, Abcam [ab13970]) and anti-HA (1:50, CST [6E2]). Appropriate secondary antibodies conjugated with a fluorophore were used at 1:300 dilutions (Alexa Fluor 488/568/633 IgG [Molecular Probes]) and incubated for 4 h at room temperature. Wherever required, during the incubation with secondary antibody, Alexa Fluor 568-phalloidin (1:200; Invitrogen [A12380]) was also added to the tissues to stain the F-actin. After three washes in PBTx, samples were mounted in 70% glycerol in PBS + 0.3% Triton X-100. Fixed retinae were washed three times in PBTx (1× PBS with 1 mg/ml saponin at room temperature for 30 min on orbital shaker. Fixed retinae were washed three times in PBTx (1× PBS), followed by permeabilization with 0.37% Igepal (Sigma-Aldrich) in 1× M1 for 13 min and then blocked for 1 h at room temperature in 1× M1 containing 5% FBS and 2 mg/ml BSA. After blocking, the cells were incubated with primary antibodies in

Cell culture, transfections and dsRNA treatment

Drosophila S2R+ cells stably expressing ActGal4 were maintained on Schneider’s media supplemented with 10% non heat inactivated FBS and contained antibiotics-streptomycin and penicillin (Schneider’s Complete Media, SCM). Cell transfections were carried out using Effectene (301425; QIAGEN) and 200 μl of 10 mM Tris-Cl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 50 mM NaF, 0.27M sucrose, and 0.1% 2-mercaptoethanol with freshly added protease inhibitor and phosphatase inhibitor cocktail (Roche). Protein equivalent to 10 μg was estimated by Bradford assay.

Lipid kinase assay

Cell lysate preparation

S2R+ cells expressing the desired constructs were dislodged from plates, harvested by centrifugation at 1,675g for 5 min in a table top centrifuge. Then they were washed twice with PBS, and lysed with lysis buffer (50 mM Tris-Cl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 50 mM NaF, 0.27M sucrose, and 0.1% 2-mercaptoethanol) with freshly added protease inhibitor and phosphatase inhibitor cocktail (Roche). Protein equivalent to 10 μg was estimated by Bradford assay.

Micelle formation and kinase assay

600 pmoles of 37:4 PI4P (LM1901; Avanti) was dried in LoBind 1.5 ml Eppendorf tubes along with 20 μl 0.5 mM Phosphatidylserine (Sigma-Aldrich) and 200 μl of CHCl3 in vacuum for 20 min at 94°C. After drying of lipids, 50 μl of 10 mM Tris–HCl (pH 7.4) was added to each tube and sonicated at room temperature for 3 min. Next, 50 μl of 2× kinase assay buffer containing 40 μM ATP (Roche) was added to the tubes and 10 μg equivalent of corresponding lysate was added. Immediately after this, the tubes were transferred to a shaking incubator at 30°C for 30 min for the kinase reaction to happen. The reaction was stopped by adding 125 μl of 2.4N HCl to the reaction tubes. Then 250 μl each of CHCl3 and methanol were added. After this, the tubes were vortexed for 2 min and centrifuged at 1,000g for 5 min to allow a clean phase separation to happen. The upper phase was punctured and the lower organic phase was transferred to a fresh LoBind 1.5 ml Eppendorf. To this tube, 500 μl of Lower Phase Wash Solution (LPWS: methanol/1 M hydrochloric acid/choroflorin in a ratio of 235/245/15 [vol/vol/vol]) was added. It was vortexed for 2 min and clean phase separation was obtained by spinning tubes at 1,000g for 5 min. Again, the lower organic phase was transferred to a fresh LoBind 1.5-ml Eppendorf tube.

Derivatization and LC MS/MS

The organic phase obtained after lipid extraction was directly subjected to derivatization using 2M TMS-diazomethane (Acros). 50 μl TMS-diazomethane was added to each tube and vortexed gently for 10 min at 670g. The reaction was neutralized by 10 μl of glacial acetic acid. The samples were dried, and reconstituted in 200 μl of methanol. LC MS/MS run method was used as described in Ghosh et al (2019). The MRMs transitions for 37:4 PI4P was used 995.5/613.5 and for 37:4 PI(4,5)P2 was 1,103.5/613.5. The area under the peaks for individual lipid species was extracted using MultiQuant software. Numerical analysis was performed in Microsoft Excel and the graphs were plotted using GraphPad Prism software.

Immunofluorescence from S2R+ cells

48 h post transfection with dPIPSKΔ::GFP, cells were fixed in 2.5% PFA for 20 min. Upon fixation, cells were washed thrice with PBTx (1× PBS + 0.3% Triton X-100) for 10 min each, permeabilized with 0.37% Igepal (Sigma-Aldrich) in 1× M1 for 13 min and then blocked for 1 h at room temperature in 1× M1 containing 5% FBS and 2 mg/ml BSA. Post blocking, the cells were incubated with primary antibodies in

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blocking solution overnight at 4°C. The following antibodies were used: anti-GFP (15,000; Abcam [ab13970]) and anti-PNUT (1:250; DSHB [4C9H4]). Next morning, the cells were washed thoroughly, and incubated with the appropriate secondary antibodies conjugated with fluorophore at 1:300 dilutions (Alexa Fluor 488/568/633 IgG, [Molecular Probes]) and incubated at room temperature for 2 h. The cells were then washed and imaged on an Olympus FV3000 confocal microscope using Plan-Apochromat 60×, NA 1.4 objective (Olympus). Image analysis was performed using ImageJ from NIH.

Lipid-binding assay

48 h post transfection, S2R+ cells expressing UAS-PNUT were harvested and washed twice with PBS. Then the cells were sheared in fat blot buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl) using an insulin syringe. Strips of nitrocellulose membranes (Hybond-C Extra; [GE Healthcare]) were spotted with increasing picomoles of PI4P and PI(4,5)P2 (Echelon lipids [P-4016 and P-4516]). The spotted membranes were dried for 1 h and blocked using 5% blotto (HiMedia) in fat blot buffer for 1 h 30 min at room temperature. Then, the strips were incubated overnight at 4°C with the cell lysate. Next morning, the membranes were washed extensively five times with fat blot buffer with 0.1% tween and then incubated with anti PNUT (1:250; DSHB [4C9H4]) for 3 h at room temperature. The membranes were then probed with HRP-conjugated anti mouse secondary antibody (1:10,000; Jackson Immunochemicals). Binding was detected using ECL (GE Healthcare) on a LAS4000 instrument.

Statistical analysis

All statistical analyses were performed in GraphPad Prism 10 software. Unpaired two-tailed $t$ test with Welch correction, one-way ANOVA, followed by Tukey’s multiple-comparisons test or two-way ANOVA grouped analysis with Bonferroni’s post multiple comparisons were carried out where applicable.

Supplementary Information

Supplementary Information is available at https://doi.org/10.26508/lsa.202101293 vol 5 | no 6 | e202101293.

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Author Contributions

A Kumari: resources, formal analysis, validation, investigation, methodology, and writing—original draft, review, and editing.

A Ghosh: formal analysis, methodology, and writing—original draft, review, and editing.

S Kolay: formal analysis.

P Raghu: conceptualization, supervision, project administration, and writing—original draft, review, and editing.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

References

Regulation of PLC signalling by septins

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