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Genetic code expansion, click chemistry, and light-activated PI3K reveal details of membrane protein trafficking downstream of receptor tyrosine kinases

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Abstract

Ligands such as insulin, epidermal growth factor, platelet derived growth factor, and nerve growth factor (NGF) initiate signals at the cell membrane by binding to receptor tyrosine kinases (RTKs). Along with G-protein coupled receptors, RTKs are the main platforms for transducing extracellular signals into intracellular signals. Studying RTK signaling has been a challenge, however, due to the multiple signaling pathways to which RTKs typically are coupled, including MAP/ERK, PLCγ, and Class 1A phosphoinositide 3-kinases (PI3K). The multi-pronged RTK signaling has been a barrier to isolating the effects of any one downstream pathway. Here, we used optogenetic activation of PI3K to decouple its activation from other RTK signaling pathways. In this context, we used genetic code expansion to introduce a click chemistry noncanonical amino acid into the extracellular side of membrane proteins. Applying a cell-impermeant click chemistry fluorophore allowed us to visualize delivery of membrane proteins to the plasma membrane in real time. Using these approaches, we demonstrate that activation of PI3K, without activating other pathways downstream of RTK signaling, is sufficient to traffic the TRPV1 ion channels and insulin receptors to the plasma membrane.

eLife assessment

This study develops a new and **important** method for dissecting out two overlapping cell signaling pathways, phosphoinositide signaling and membrane protein trafficking. The combination of two state-of-the-art spectroscopic techniques provides **compelling** evidence for a reciprocal influence between an enzyme and a channel. The work will be of interest to the broader cell biology, biophysics and biochemistry communities.

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Introduction

Receptor tyrosine kinases activate phosphoinositide 3-kinase

Receptor tyrosine kinases (RTKs) are the second largest class of cell surface receptors, with 58 members in the human genome (Robinson et al., 2000). Binding of agonists, typically polypeptides such as growth factors, to their extracellular amino-terminal domains results in autophosphorylation of their intracellular carboxy-terminal domains (Lemmon and Schlessinger, 2010). The autophosphorylated receptors then signal to activate phospholipase Cγ, mitogen activated protein kinase, and phosphoinositide 3-kinase (PI3K). The complex web of signals downstream of RTKs underlies their importance in a cell regulation and dysregulation but also makes studying the roles and dynamics of any particular branch of their signaling pathways challenging.

The Class IA phosphoinositide 3-kinases (PI3K) coupled to RTKs are obligate heterodimers composed of regulatory p85 and catalytic p110 subunits (**Figure 1A**) (Geering et al., 2007). The p85 regulatory subunit contains two SH2 domains (N-SH2 and C-SH2) separated by an inter-SH2 domain (iSH2). The p110 catalytic subunit interacts with the entire N-SH2-iSH2-C-SH2 region of PI3K, with the N-SH2 acting as an autoinhibitory domain to inhibit catalysis. For the well studying RTK tropomyosin receptor kinase A (TrkA), binding of its agonist nerve growth factor (NGF) triggers autophosphorylation of a pair of tyrosines. Phospho-TrkA then binds to the PI3K N-SH2. This has the dual effect of recruiting PI3K to the PM (Thorpe et al., 2015; Ziemba et al., 2016) and causing a conformational change in p85 to relieve auto-inhibition of p110 by the N-SH2 (Zhang et al., 2020). Activation of the RTK insulin receptor (InsR) by its agonist, insulin, also involves autophosphorylation of tyrosines on its intracellular domain and subsequent activation of PI3K; however, in the case of InsR, various insulin receptor substrate adaptor proteins link InsR to PI3K (Ye et al., 2017; Saltiel, 2021). PI3K bound directly or indirectly to activated RTKs on the membrane then phosphorylates phosphoinositide 4,5-bisphosphate ($PI(4,5)P_2$) to generate phosphoinositide 3,4,5-trisphosphate ($PI(3,4,5)P_3$). $PI(3,4,5)P_3$ is a well-recognized signal for membrane fusion in organisms ranging from *dictyostelium* (Nichols et al., 2015) to humans (Hawkins et al., 2006; Hawkins and Stephens, 2015). Indeed, $PI(3,4,5)P_3$-triggered membrane fusion mediates tumor cell motility, with PI3K acting as an oncoprotein and its corresponding phosphatase, PTEN, as a tumor suppressor (Hawkins et al., 2006).

RTK/PI3K signaling increases sensitivity to painful stimuli via TRPV1 ion channels

Increased sensitivity to painful stimuli in injured and inflamed tissue is due to adaptations in the peripheral and central nervous systems (McMahan et al., 2013). For peripheral sensitization, G-protein coupled receptors and RTKs signal through multiple pathways to produce cellular changes across multiple time scales. NGF is an inflammatory mediator released from leukocytes, including mast cells, eosinophils, macrophages, and lymphocytes, in response to tissue injury and inflammation (Freund-Michel and Frossard, 2008; Reis et al., 2022). Paradoxically, in addition to its role increasing sensitivity to painful stimuli, NGF also is critical for wound healing (Ebadi et al., 1997; Lambiase et al., 2000; Bonini et al., 2002). Thus, strategies to block NGF-mediated pain sensitization have the unfortunate consequence of interfering with post-injury recovery.

One of the mechanisms by which NGF increases sensitivity to pain involves a PI3K-induced increase in the number of pain-transducing TRPV1 ion channels in the plasma membrane (PM) of sensory neurons (Bonnington and McNaughton, 2003; Stein et al., 2006; Zhu and Oxford, 2007). We recently discovered reciprocal regulation between TRPV1 and PI3K. We found that

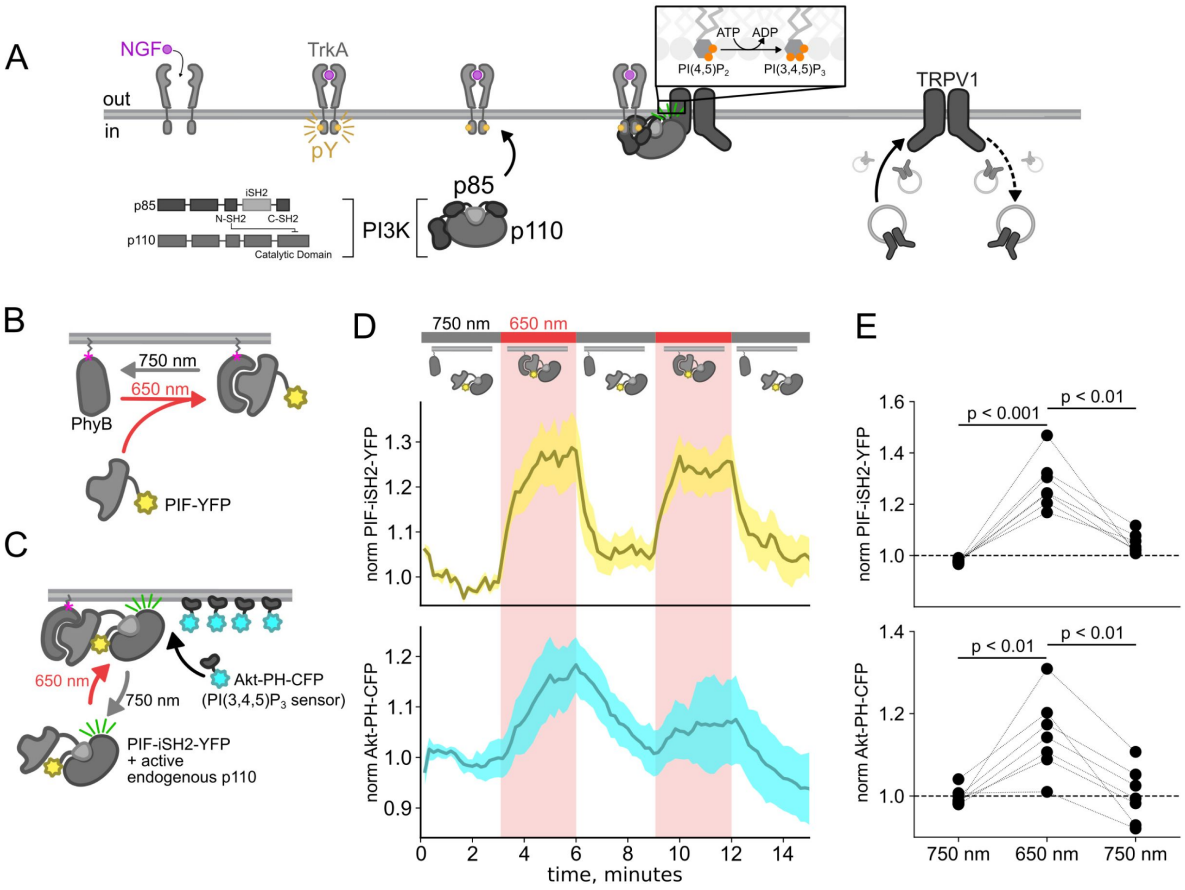

Figure 1

Using the PhyB/PIF system to activate PI3K with light

The OptoPI3K system reversibly activates PI3K to generate PI(3,4,5)P_3 at the PM. (A) Diagram of PI3K subunits and domains illustrating the regulatory p85 and catalytic p110 subunits. iSH2 domain in p85 subunit interacts with p110. Binding of NGF to TrkA receptor triggers the translocation of PI3K to the PM, phosphorylation of PI(4,5)P_2 to PI(3,4,5)P_3 and fusion of TRPV1-containing vesicles with the PM. (B,C) Schematic diagram for OptoPI3K system using PIF-iSH2-YFP. PhyB-mCherry is tethered to the PM using CAAX lipidation (magenta star). The iSH2 domain of p85 is fused to PIF so that translocation of PIF-iSH2-YFP, together with endogenous p110, to the PM promotes PI(3,4,5)P_3 synthesis upon 650 nm light. (D) Monitoring PIF-iSH2-YFP translocation to and from the PM with 650 nm and 750 nm light, respectively (top, yellow). Synthesis of PI(3,4,5)P_3 follows PIF-iSH2-YFP translocation to the PM, as indicated by the localization of the PI(3,4,5)P_3 probe Akt-PH-CFP (bottom, sky blue). F-11 cells transiently expressing PhyB-mCherry-CAAX, PIF-iSH2-YFP, and Akt-PH-CFP were illuminated with 750 nm or 650 nm light as indicated with the upper bar. Collected traces of PIF-iSH2-YFP and Akt-PH-CFP normalized to the initial baselines during the first episode of 750 nm illumination. The black line indicates the mean of the data and the colored envelope represents the standard error of the mean (n = 8). Because of the very low density of PI(3,4,5)P_3 present in the PM even in light- or NGF-stimulated cells (Auger et al., 1989), we used total internal reflection fluorescence (TIRF) microscopy to measure PI(3,4,5)P_3 density instead of confocal microscopy. TIRF illumination decreases exponentially with distance from the coverslip, selectively illuminating and exciting fluorophores within ~150 nm of the PM (Lakowicz, 2006; Mattheyses and Axelrod, 2006). (E) Scatter plot of PIF-iSH2-YFP and Akt-PH-CFP fluorescence for individual cells. Each point represents the 20 s average for 750 nm (2.66 - 3 min), 650 nm (5.66 - 6 min), and 750 nm (8.66 - 9 min). Translocation of both PIF-iSH2-YFP and Akt-PH-CFP is reversible.

the NGF-stimulated rise in the PI3K product PI(3,4,5)P_3 is much greater in cells transiently transfected with TRPV1 than in control cells without TRPV1, i.e., TRPV1 enhances NGF-mediated PI3K activity (Stratiievska et al., 2018). Interestingly, TRPV1 interacts directly with PI3K (p85α and p85β subunits; **Figure 1A**) via its amino-terminal ankyrin repeat domain (ARD) (Stein et al., 2006) and the ARD is sufficient to increase NGF-induced PI3K activity (Stratiievska et al., 2018). Based on these findings, we propose that TRPV1 regulates NGF-induced PI3K activity via this direct interaction.

Insulin/PI3K signaling

Activation of InsR by insulin signals via PI3K to activate a powerful protein kinase, Akt, which in turn regulates multiple cellular processes involved in metabolism (Saltiel, 2021). The PI3K/Akt pathway is thus of great interest in understanding obesity and type 2 diabetes. Insulin-dependent recycling of InsR has also been extensively studied (Knutson, 1991; Chen et al., 2019). Ligand-simulated InsR endocytosis is followed by either targeting to lysosomes for degradation or recycling endosomes for reinsertion into the PM. This return to the PM is not fully understood, but it is believed to involve small Rab family GTPases (Iraburu et al., 2021). Importantly, pharmacological inhibition of PI3K has been shown to inhibit InsR trafficking to the PM, indicating that PI3K activity is necessary for InsR homeostasis (Sasaoka et al., 1999).

Approaches to studying membrane protein trafficking

The properties of conventional fluorescent labels have posed a significant barrier to understanding trafficking of membrane proteins, including TRPV1. Previous studies have used large tags to label the channel protein, typically a fluorescent protein (e.g., GFP) fused to the intracellular N- or C-terminus. Fusion to fluorescent proteins carries a number of disadvantages. Fluorescent proteins are relatively dim and are susceptible to bleaching. We and others have previously shown that the intracellular termini participate in critical protein-protein interactions, some of which are involved in channel trafficking (Morenilla-Palao et al., 2004; Stein et al., 2006; Jeske et al., 2008; Camprubi-Robles et al., 2009; Jeske et al., 2009; Xing et al., 2012; Gregorio-Teruel et al., 2015; Mathivanan et al., 2016; Meng et al., 2016). The presence of large fluorescent proteins could perturb these interactions (Tsien, 1998; Montecinos-Franjola et al., 2020). TRPV1 is expressed heavily in intracellular membranes, making it nearly impossible to optically isolate just those channels on the PM (Liu et al., 2003; Gallego-Sandín et al., 2009). Fluorescent anti-TRPV1 antibodies have been used to label extracellular epitopes to distinguish these populations (e.g., (Meng et al., 2016; Nakazawa et al., 2021)), but antibodies have the same problem of large size, and we have found that antibodies that recognize extracellular regions of TRPV1 are not very specific.

Click chemistry offers a rapid, specific, and flexible method for labeling of proteins in living cells (Lang and Chin, 2014; Nikić et al., 2015; Kenry and Liu, 2019). We have previously incorporated click chemistry-compatible noncanonical amino acids (ncAAs) into amino acid positions in the cytoplasm of mammalian cells and shown we can achieve highly efficient and rapid labeling when introducing the appropriate click chemistry probe into the medium (Jang et al., 2020; Jana et al., 2023). This approach requires co-expression of the target gene with an amber stop codon (TAG) with a plasmid encoding an evolved aminoacyl tRNA synthetase that is orthogonal to mammalian cells, together with its cognate tRNA (Chin, 2014; Uttamapinant et al., 2015). An additional plasmid encoding a dominant negative form of eukaryotic dominant negative elongation release factor can be expressed to increase efficiency of ncAA incorporation (Schmied et al., 2014). Incorporating a click chemistry ncAA into an extracellular position in a membrane protein and then applying a membrane-impermeant click chemistry-conjugated fluorophore would allow specific labeling of protein at the surface, even for those proteins that localize to both the surface and intracellular membranes (Gregory et al., 2016; Mateos-Gil et al., 2016; Neubert et al., 2018; Ojima et al., 2021). The recent development of click chemistry

ncAAs and fluorescent probes that react very rapidly (>10^4 $M^{-1}s^{-1}$) make this an attractive approach for tracking membrane protein trafficking in real time (Peng and Hang, 2016; Row and Prescher, 2018; Meineke et al., 2020; Jana et al., 2023).

In this work, we leverage optogenetics to activate PI3K with light, genetic code expansion to incorporate a click chemistry ncAA, and a new, membrane-impermeant click chemistry-conjugated fluorophore to interrogate the mechanism by which NGF induces trafficking of TRPV1 to the PM. By isolating the PI3K pathway downstream of NGF from the other pathways coupled to TrkA (PLCγ and MAP/ERK), we demonstrate that PI3K activity is sufficient to increase TRPV1 trafficking to the PM. We apply the same approach to InsR, to demonstrate that our new approach is of general use for different types of membrane proteins, and show that activation of PI3K is sufficient to increase InsR trafficking to the PM. Given the importance of InsR recycling in the development of insulin resistance, understanding the role of PI3K in regulating InsR expression on the PM is critical.

Results

Here, we used the previously published phytochrome B (PhyB)/phytochrome interacting factor (PIF) system to activate and deactivate PI3K with light (Levskaya et al., 2009; Toettcher et al., 2011). Uncoupling PI3K from activation of TrkA allowed us to study the effects of PI3K on membrane protein trafficking without activating other signaling pathways downstream of TrkA. To apply the PhyB/PIF system to control PI3K activity, we fused the inter-SH2 domain (iSH2) of the p85 regulatory subunit of PI3K to PIF (**Figure 1B**). It has previously been shown that the iSH2 domain associates with the endogenous p110 catalytic subunit of PI3K (Klippel et al., 1993). Because this heterodimer is missing the autoinhibitory domains of p85, it is constitutively active, so that PI3K phosphorylates its substrate, $PI(4,5)P_2$ to make $PI(3,4,5)P_3$ whenever the PIF-iSH2-YFP is driven to the membrane (**Figure 1C**) (Suh et al., 2006; Idevall-Hagren et al., 2012). The iSH2 domain has been previously expressed as a fusion protein with PIF and has been shown to reversibly translocate to the PM and activate PI3K in response to light (**Figure 1 – figure supplement 1**) (Levskaya et al., 2009; Toettcher et al., 2011).

Using a CFP-labelled pleckstrin homology domain from the enzyme Akt (Akt-PH-CFP), which specifically binds the $PI(3,4,5)P_3$ product of PI3K (Lemmon, 2008), as a probe for $PI(3,4,5)P_3$, we confirmed that the PhyB/PIF-iSH2 machinery could be used to rapidly generate $PI(3,4,5)P_3$ in response to 650 nm light (**Figure 1E-F**). In response to 750 nm light, PIF-iSH2-YFP was released from the membrane and the levels of $PI(3,4,5)P_3$ returned to baseline, presumably due to the activity of the endogenous $PI(3,4,5)P_3$ phosphatase PTEN. Similar experiment using GRP1-PH-CFP, another $PI(3,4,5)P_3$ probe yielded the same results (Figure 1-figure supplement 2).

Activation of PI3K, without other pathways downstream of NGF/TrkA, is sufficient to drive trafficking of TRPV1 to the PM

We and others have previously demonstrated that activation of the RTK TrkA by nerve growth factor leads to increased trafficking of the ion channel TRPV1 to the PM (Zhang et al., 2005; Stein et al., 2006; Zhu and Oxford, 2007; Stratiievska et al., 2018) This increase in surface expression is associated with increased sensitivity to painful stimuli sensed by TRPV1. As shown in **Figure 2A-B**, the NGF-induced trafficking of TRPV1 follows the increase in PM $PI(3,4,5)P_3$ levels. Although TrkA couples with PLCγ and the MAP/ERK pathways in addition to PI3K, significant evidence indicates that PI3K activation is *necessary* to NGF-induced trafficking of TRPV1 to the PM (Zhang et al., 2005; Stein et al., 2006; Zhu and Oxford, 2007; Stratiievska et al., 2018).

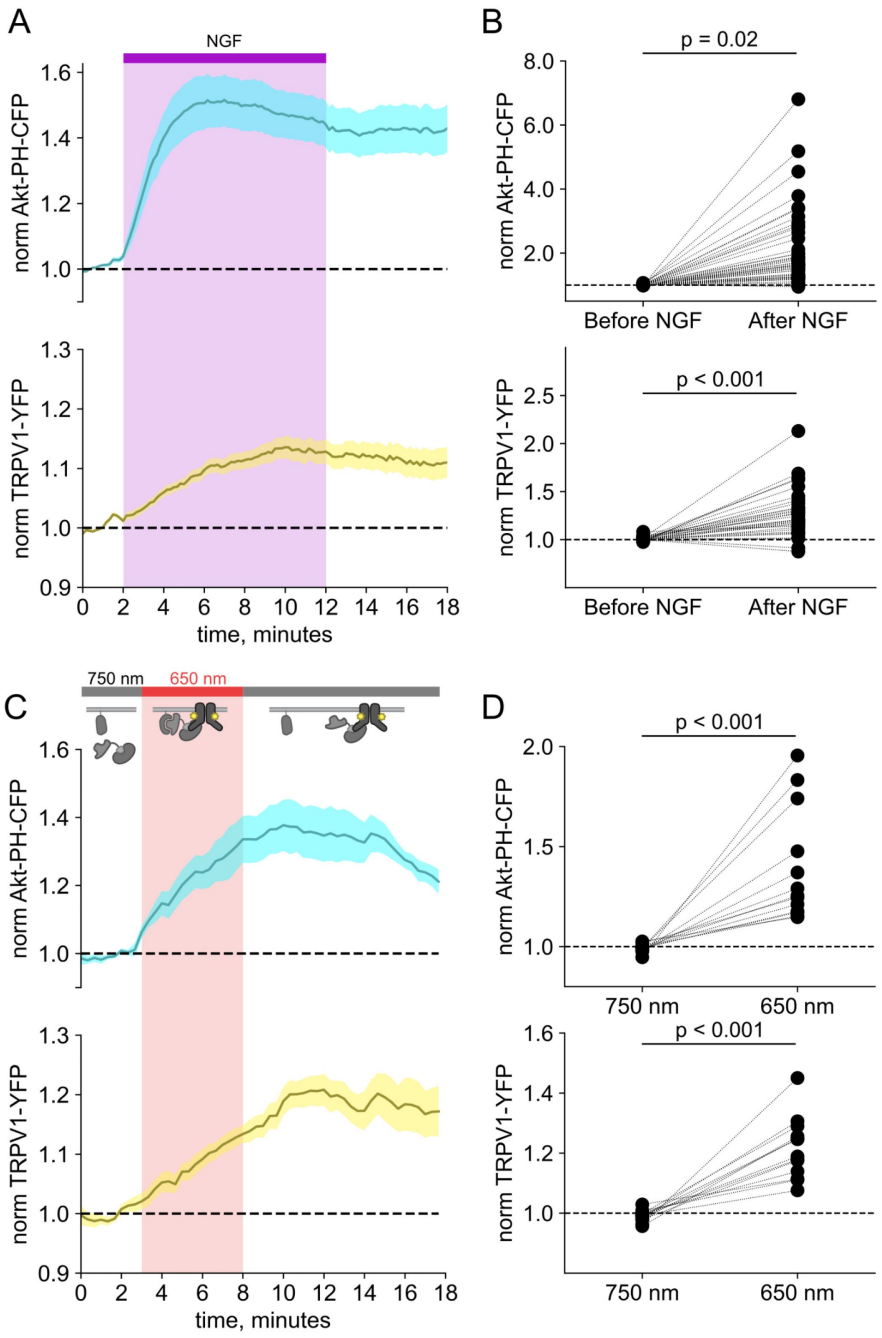

Figure 2.

Activation of PI3K with light is sufficient to induce trafficking of TRPV1 to the PM.

Simultaneous TIRF measurement of PI(3,4,5)P_3 (cyan) and TRPV1 (yellow) in the PM in response to either (A) NGF or (C) light. (A) F-11 cells were transfected with TrkA/p75NTR, Akt-PH-CFP, and TRPV1-YFP. NGF (100 ng/mL) was applied during the times indicated by the bar/shading. Plotted are the PM-associated fluorescence in Akt-PH-CFP (top, cyan) and TRPV1-YFP (bottom, yellow) within the cell footprints. Data are reproduced from (Stratiievska et al., 2018) (C) F-11 cells transfected with PhyB-mCherry-CAAX, PIF-ISH2 (without a fluorescent tag), Akt-PH-CFP, and TRPV1-YFP were illuminated with 750 nm or 650 nm light as indicated. Color scheme as in (A), with line indicating the mean and envelope indicated the standard error of the mean (n = 13 for Akt-PH-CFP; n = 16 for TRPV1-YFP). Note the poor or irreversible increase of PM PI(3,4,5)P_3 in the PM. Inset cartoons depict the model for retention of iSH2 at the PM via binding to TRPV1. (B, D) Scatter plots of for individual cells. Each point represents the 20 s average for before (0.83 – 1.17 min) and after NGF (14.8 – 15.2 min) or for before (1.31 – 1.63 min) and after 650 nm (10.8 -11.2 min).

We used PhyB with PIF-iSH2 to test the hypothesis that PI3K activity is *sufficient* to drive trafficking of TRPV1 to the PM. Together with PhyB, PIF-iSH2 (without a fluorescent tag) and Akt-PH-CFP, we expressed TRPV1 fused to YFP. Stimulation of cells with 650 nm light gave the expected rise in PM-associated Akt-PH-CFP fluorescence and, importantly, a rise in PM-associated TRPV1-YFP fluorescence (**Figure 2C-D**). The light-induced rise in PM $PI(3,4,5)P_3$ and TRPV1 qualitatively resembled those induced by NGF. Because PLCγ and the MAP/ERK were not directly activated by the 650 nm light, we conclude that activation of the PI3K pathway is sufficient to cause TRPV1 trafficking to the PM.

TRPV1 prevents dissociation of PIF-iSH2-YFP from the membrane in response to 750 nm light

In monitoring localization of PIF-iSH2-YFP in light-activated PI3K experiments in TRPV1-expressing cells, we found that PIF-iSH2-YFP no longer translocated to the cytoplasm in response to 750 nm light (**Figure 2B** and **Figure 2 – figure supplement 1A-B**). Indeed, the small amount of mCherry excitation light used to identify PhyB-mCherry positive cells seemed to be sufficient to cause a slow accumulation of PIF-iSH2-YFP at the PM even during continuous exposure of the cell to 750 nm light (**Figure 2 – figure supplement 1C-D**). Not surprisingly, with PIF-iSH2-YFP accumulating at the PM during imaging of TRPV1-expressing cells, the 650 nm light-induced increase in plasma-membrane associated Akt-PH-CFP did not reverse upon exposure to 750 nm light (**Figure 2B** and **Figure 2 – figure supplement 1A-B**). TRPV1 expression had no effect on a version of PIF-YFP that lacked the iSH2 domain (**Figure 2 – figure supplement 2**), indicating that irreversible localization to the PM required both the iSH2 fragment of PI3K and TRPV1. Expression of the related TRPM4 ion channels, which do not include an ankyrin repeat domain, also had no effect on 750 nm light-induced dissociation of PIF-iSH2-YFP from the PM (**Figure 2 – figure supplement 3**), indicating that this effect is specific to TRPV1. We have previously shown that the ankyrin repeat domain of TRPV1 interacts directly with the p85 subunit of PI3K from which iSH2 is derived (Stein et al., 2006). We speculate that the PIF-iSH2-YFP in TRPV1-expressing cells might not dissociate from the PM when stimulated with 750 nm light because it encounters TRPV1 at the PM and binds to it, remaining bound to TRPV1 even after releasing PhyB.

Resolving changes in surface expression requires improved approaches

TIRF microscopy was needed to resolve the NGF- and light-induced increase in PM $PI(3,4,5)P_3$ levels but is a poor approach for distinguishing membrane protein localization in the PM from that in intracellular compartments (Stratiievska et al., 2018). For membrane proteins with very high endoplasmic reticulum localization, like TRPV1 (Tominaga et al., 1998), this makes any changes in fluorescence intensity measured with TIRF an underestimate of true changes in surface localization.

To circumvent contamination of TIRF signals on the PM with fluorescence from intracellular compartments, we implemented an inverse-electron-demand Diels–Alder cycloaddition click chemistry approach to selectively label PM localized proteins (Arsić et al., 2022). For this application, we used genetic code expansion to incorporate a noncanonical amino acid at an extracellular site (Neubert et al., 2018; Bessa-Neto et al., 2021; Kuhlemann et al., 2021). We used Tet3-Bu (**Figure 3A**), a tetrazine-containing amino acid which can be efficiently labelled with a cyclopropane-fused strained trans-cyclooctene (sTCO) with no detectable off-target reactivity within the time scale of our experiments (see below) (Jang et al., 2020; Jana et al., 2023). Our goal was to express membrane proteins incorporating Tet3-Bu at an extracellular site measure labeling with a membrane-impermeant sTCO-coupled dye (**Figure 3B**) under control conditions and then again after activation of PI3K with either NGF or 650 nm light.

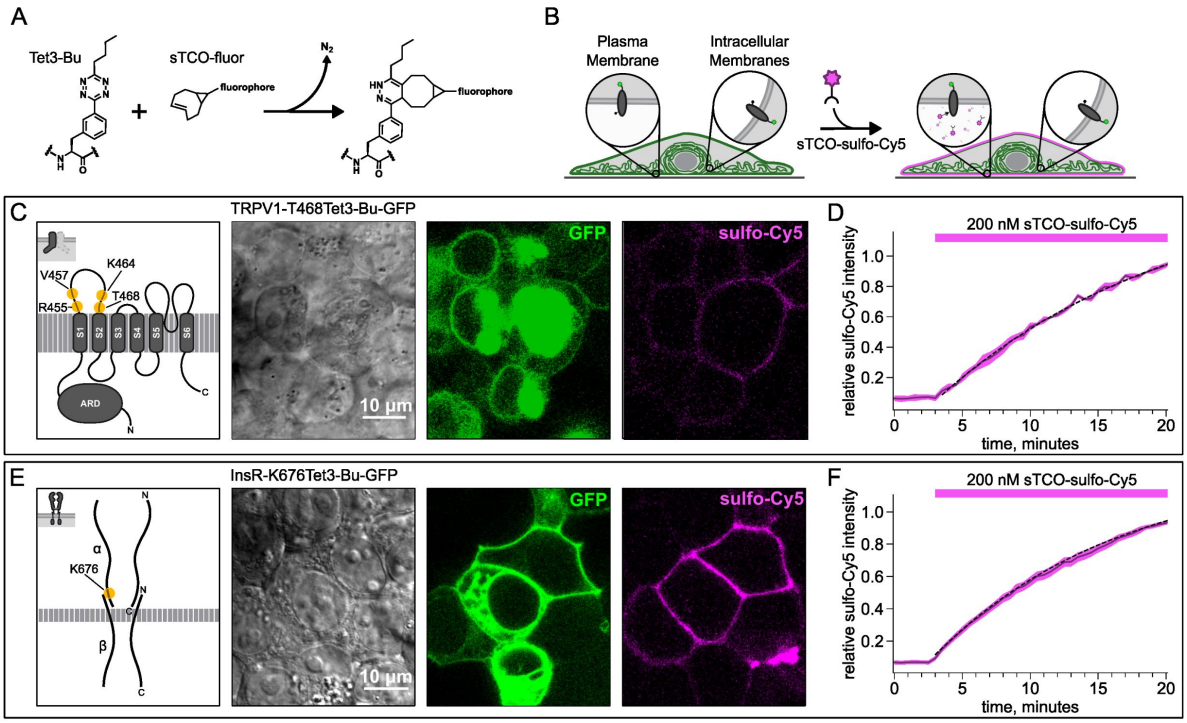

Figure 3.

Labeling the TRPV1 and InsR with membrane-impermeant sTCO-Cy5.

Confocal imaging illustrates the labeling of membrane proteins incorporating the ncAA Tet3-Bu with sTCO-sulfo-Cy5 in HEK293T/17 cells. The membrane impermeable dye labeled only the proteins on the PM. (A) Schematic of the reaction between Tet3-Bu and sTCO-conjugated dyes. (B) Cartoon representing the selective labeling of membrane proteins incorporating Tet3-Bu at an extracellular site with membrane-impermeant sTCO-sulfo-Cy5. (C & E) Confocal images of HEK293T/17 cells expressing (C) TRPV1-468Tet3-Bu-GFP or (E) InsR-676Tet3-Bu-GFP. GFP fluorescence reflects expression of the proteins in the confocal volume across the field of view. Initially the cells did not show any detectable Cy5 fluorescence but after incubation of several minutes of 200 nM sTCO-sulfo-Cy5 showed Cy5 fluorescence at the PM. The Cy 5 images shown for (C) TRPV1-Tet3-Bu and (E) InsR-Tet3-Bu were obtained at the end of the experiment (20 minutes). (D & F) The graphs summarize the Cy5 fluorescence at the PM in (D) TRPV1-Tet3-Bu-GFP or (F) InsR-Tet3-Bu-GFP expressing cells. Solid traces represent the mean and envelopes the standard error of the mean (n = 3 for TRPV1 and n = 11 for InsR). Dashed traces represent a fit to the mean with a single exponential (tau = 17.8 s for TRPV1; tau = 13.8 s for InsR). Fits to the individual time courses for all the cells gave a mean of 18.2 s for TRPV1 (± 2.2 s) and 19.3 s for InsR (±4.0 s).

We tested our click chemistry/noncanonical amino acid approach using TRPV1 and the insulin receptor (InsR), a model RTK we and others have previously used in studies with different noncanonical amino acids (Nikić et al., 2015; Jones et al., 2021). For both proteins, we used amber codon suppression to site-specifically incorporate Tet3-Bu (Jang et al., 2020) into an extracellular loop (T468Tet3-Bu for TRPV1 and Tet3-BuK676 for InsR). This involved introducing a TAG stop codon at the selected site within the coding sequence that would place the noncanonical amino acid on the extracellular side of the PM. We then co-expressed one plasmid encoding either TRPV1-T468TAG or InsR-K676TAG fused to GFP, a second plasmid encoding an aminoacyl tRNA synthetase evolved to incorporate the noncanonical amino acid and the orthogonal tRNA, and a third plasmid encoding a dominant negative elongation release factor, which increased the efficiency of Tet3-Bu incorporation (Schmied et al., 2014). The cell culture medium was then supplemented with Tet3-Bu. As indicated by the GFP signal and in-gel fluorescence, measured in transfected cells, both TRPV1-T468Tet3-Bu-GFP and InsR-K676Tet3-Bu-GFP expressed at high levels (**Figure 3C,E** and **Figure 3 – figure supplement 1C**). To test whether incorporation of Tet3-Bu interfered with the function of TRPV1, we measured capsaicin-activated currents with whole-cell voltage clamp and found that TRPV1-T468Tet3-Bu-GFP gave robust capsaicin-activated currents (**Figure 3 – figure supplement 1A-B**). To determine whether InsR-K676TAG protein retained its function, we measured insulin-induced endocytosis (Carpentier et al., 1992) and found endocytosis in response to insulin was maintained (**Figure 3 – figure supplement 2**) with approximately the expected kinetics (Figure 3 – supplemental movie).

Developing a membrane-impermeant sTCO dye

Strained trans-cyclooctenes bearing functional groups such as spin labels and fluorophores have been used previously for in-cell studies (Murrey et al., 2015; Ryan et al., 2022; Jana et al., 2023). To determine whether existing sTCO-conjugated dyes would give the required PM-selective labeling, we tested whether sTCO-TAMRA (Jang et al., 2020), or sTCO-JF646 (Jana et al., 2023) were membrane impermeant. We also synthesized and tested sTCO-fluorescein. Unfortunately, these compounds readily equilibrated across the cell PM at the concentrations required for labeling, giving indistinguishable cytosolic fluorescence in untransfected and transfected cells (**Figure 3 – figure supplement 3A-C**). We therefore synthesized a new sTCO-sulfo-Cy5 conjugate, which contains two negative charges at physiological pH to minimize passive diffusion through membrane and therefore selectively label membrane proteins at the cell surface (**Figure 3 – figure supplement 3D**) (Hoffmann et al., 2015; Nikić et al., 2015; Kozma et al., 2016; Lam et al., 2018; Keller et al., 2020).

To determine whether sTCO-sulfo-Cy5 would selectively label a membrane protein with an extracellular Tet3-Bu, we measured Cy5 fluorescence in the presence of 200 nM sTCO-sulfo-Cy5 in the bath, a low concentration that gives very low background fluorescence. The click chemistry reaction between Tet3-Bu incorporated at the extracellular sites and sTCO-sulfo-Cy5 effectively concentrated the dye at the PM, allowing significant membrane labeling to be observed for both membrane proteins examined (TRPV1-T468Tet3-Bu-GFP – **Figure 3C** and InsR-K676Tet3-Bu-GFP – **Figure 3E**). Importantly, sTCO-sulfo-Cy5 did not appear to equilibrate across the cell membrane and did not label untransfected cells (i.e., those without GFP). *In vitro*, the click chemistry reaction between free Tet3-Bu ncAA and sTCO occurs with a rate of 2×10^4 $M^{-1}s^{-1}$; this reaction rate increased by 4 fold when Tet3-Bu was incorporated on the surface of GFP (Jang et al., 2020). The low background fluorescence recorded with 200 nM dye in the bath allowed us to measure the rate of labeling for TRPV1-468Tet3-Bu-GFP and InsR-646Tet3-Bu-GFP with sTCO-sulfo-Cy5. As shown in **Figure 3D** and **Figure 3F**, the reaction rate we measured was approximately 1×10^4 $M^{-1}s^{-1}$ for both proteins, somewhat slower than expected for a protein in solution and on par with the reaction between the free amino acid and sTCO in solution (Meijer et al., 1998; Taylor et al., 2011).

Click chemistry labeling can be used to measure NGF-induced trafficking of TRPV1 to the PM

Based on the extensive contamination of TIRF signals with fluorescence from the ER and other intracellular compartments (Stratiievska et al., 2018), we hypothesized that our previous measurements of NGF-induced trafficking of TRPV1 to the PM using TIRF represent underestimates of the true changes in PM TRPV1. If this were the case, using click chemistry to resolve NGF-induced changes in PM TRPV1 should reveal a greater increase in PM TRPV1. At the beginning of the experiment, we labeled surface TRPV1-468Tet3-Bu-GFP with a pulse of 1 μM sTCO-sulfo-Cy5 and then washed the label from the bath (**Figure 4A**). We used this higher concentration of sTCO-dye (5 times higher than in **Figure 3**) to label the PM channels more rapidly, as the NGF-induced trafficking of TRPV1 to the PM (**Figure 2A**; yellow) occurs with kinetics comparable to labeling with 200 nM sTCO-sulfo-Cy5 (**Figure 3D**; see also **Figure 3 – figure supplement 3D**). We then treated the cells with NGF for ten minutes and again pulse labelled the surface channels with a brief exposure to 1 μM sTCO-sulfo-Cy5. We observed a ~1.4 fold increase in PM Cy5 staining, indicating that NGF induced trafficking of TRPV1 to the surface (**Figure 4B-D**). In contrast, the total number of TRPV1 channels did not change, as evidenced by the total intensity of GFP fluorescence, which was not affected by NGF. This NGF-induced increase in surface expression is indeed greater than the ~1.1-fold increase in NGF-induced surface expression of TRPV1 observed in TIRF experiments (**Figure 2**).

Click chemistry labeling can be used to measure light-induced trafficking to the PM

We next asked whether click chemistry labeling could improve the resolution of PM trafficking in a system in which we have decoupled PI3K activity from other pathways downstream of NGF, i.e., the PhyB/PIF system. Results from these experiments with TRPV1-468Tet3-Bu-GFP and InsR-676Tet3-Bu-GFP and shown in **Figure 5**. After first labeling cells with sTCO-sulfo-Cy5, we exposed cells to an initial 10-minute exposure to 750 nm light and labeled any newly delivered protein at the PM using a second treatment with sTCO-sulfo-Cy5 (**Figure 5A**). The GFP fluorescence remained the same under all conditions indicating that total protein expression did not change over the course of the experiments. For TRPV1-T468Tet3-Bu-GFP, the sTCO-sulfo-Cy5 labeling increased slightly in response to the initial exposure to 750 nm light (**Figure 5B-E**). This small increase in sTCO-sulfo-Cy5 labeling was specific to TRPV1-T468Tet3-Bu, as 750 nm light produced no change in sTCO-sulfo-Cy5 labeling of InsR-K676Tet3-Bu-GFP (**Figure 5F-I**). The TRPV1-specific increase in labeling after 750 nm illumination is likely due to the small, irreversible PM localization of PIF-iSH2-YFP that occurs in TRPV1-expressing cells (**Figure 2 – figure supplement 1**). This small increase in sTCO-sulfo-Cy5 labeling of TRPV1-T468Tet3-Bu was also seen in control experiments using two sequential exposures to 750 nm light (**Figure 5 – figure supplement 1**). Note that the increase in of TRPV1-T468Tet3-Bu labeling, F_2/F_1, in experiments with 650 nm light (**Figure 5**) was significantly greater than F_2/F_1 in experiments with only 750 nm light ($P < 0.001$; **Figure 5 – figure supplement 1**).

After the initial control period with 750 nm light, we exposed the cells to 650 nm light for 10 minutes to activate PI3K and then labeled newly delivered protein at the PM with sTCO-sulfo-Cy5. As shown in **Figure 5B-E** for TRPV1-T468Tet3-Bu and **Figure 5F-I** for InsR-K676Tet3-Bu-GFP, activation of PI3K with 650 nm light increased expression of protein on the PM. For TRPV1, the amplitude of the increase in surface expression, ~1.4-fold, was similar to that observed in click chemistry experiments in response to NGF (**Figure 4**). Surprisingly, the increase in surface expression of InsR-K676-Tet3-Bu was also about 1.4-fold. Like the NGF receptor TrkA, InsR is a receptor tyrosine kinase whose activation stimulates PI3K activity (Saltiel, 2021). Binding of insulin to InsR is a well-studied signal for InsR endocytosis (Carpentier et al., 1992), and PI3K

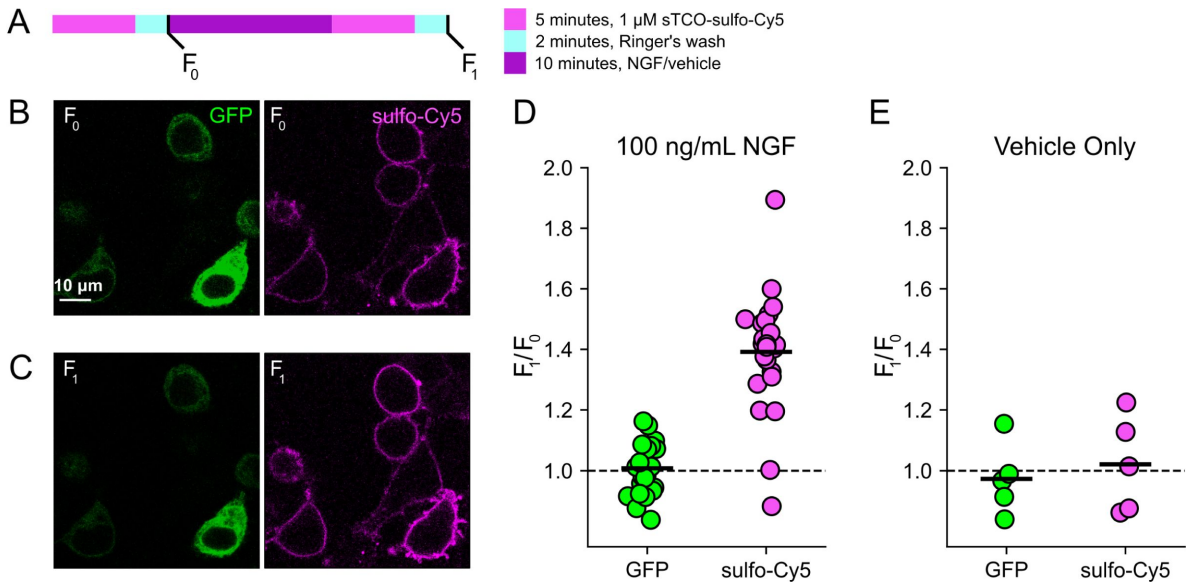

Figure 4.

Click chemistry labeling of TRPV1-468Tet3-Bu-GFP with sTCO-Cy5 to measure NGF-induced trafficking of TRPV1 to the PM.

HEK293T/17 cells expressing TRPV1-Tet3-Bu-GFP and NGF receptor were labeled with extracellular sTCO-sulfo-Cy5 and inspected with confocal microscopy. (A) Experimental protocol. Cells were incubated with 1 µM sTCO-sulfo-Cy5 for 5 min and free dye removed from the bath by washing for 2 minutes with dye-free Ringer's solution ('pulse-chase' labeling. F_0). Then the cells were treated with 100 ng/mL NGF for 10 min before the second sulfo-Cy5 labeling (F_{NGF}). Confocal images after initial sTCO-sulfo-Cy5 labeling (B) and after the 10 minute treatment with NGF and subsequent sTCO-sulfo-Cy5 labeling (C). (D) Summary scatter plot from multiple measurements, with individual experiments shown as dots and the mean of the experiments as black bars. The effect of NGF on GFP and sulfo-Cy5 signals is presented as a ratio of F_{NGF}/F_0 (n = 24). After NGF treatment, the ratio increased for sulfo-Cy5 significantly ($P < 0.001$) but not for GFP ($P = 0.64$). (E) The same experiment without NGF treatment ('Vehicle only', n = 5). Vehicle treatment did not change both GFP ($P = 0.63$) and sulfo-Cy5 ($P = 0.78$).

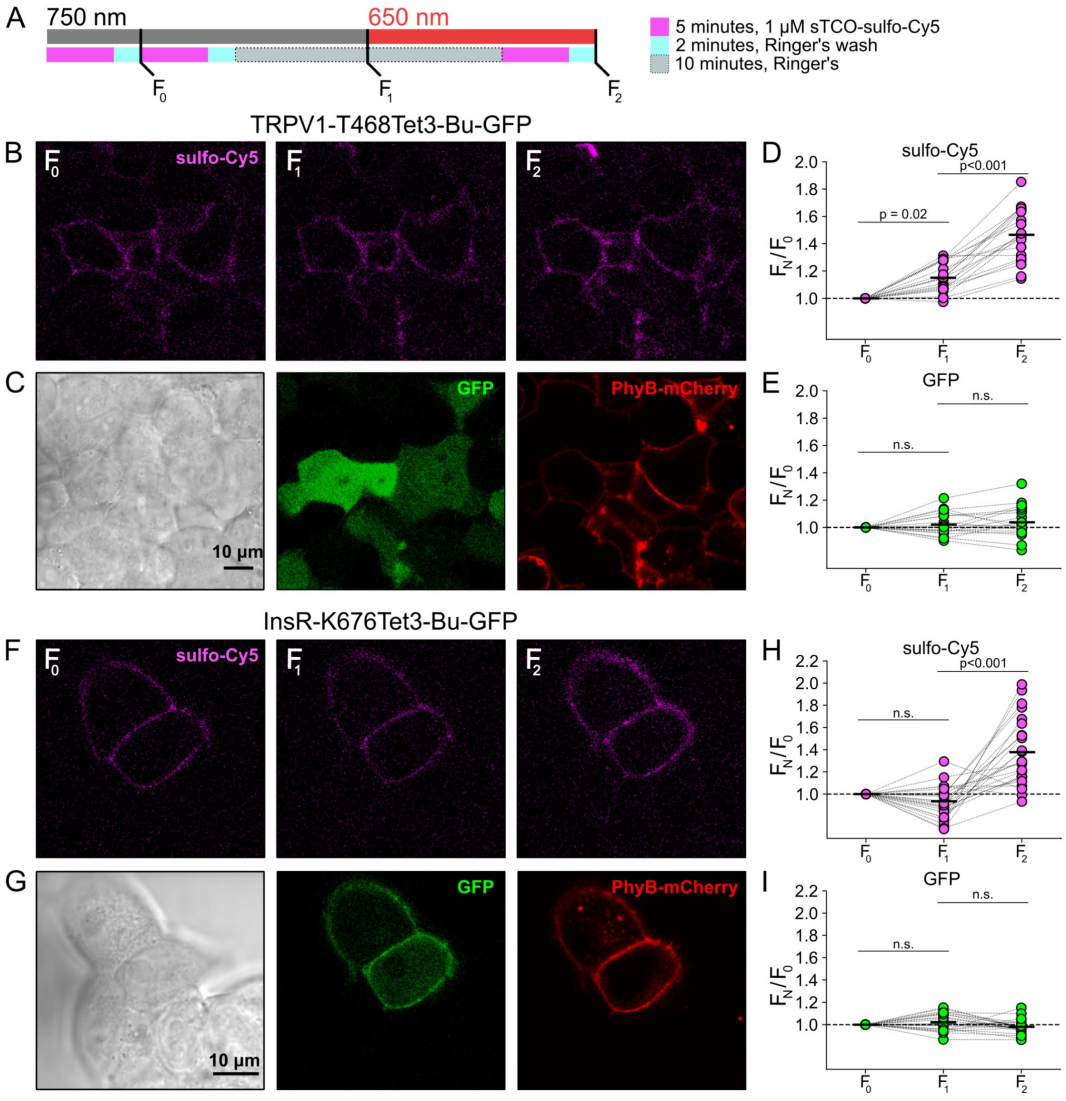

Figure 5.

Measuring light-activated PI3K-induced TRPV1 and InsR trafficking to the PM using click chemistry

(A) Illustration of the experimental protocol. HEK293T/17 cells expressing TRPV1-468Tet3-Bu-GFP and InsR-676Tet3-Bu-GFP. (B) Confocal images sulfo-Cy5 obtained at different stages as depicted in (A). (C) Bright field (left) and confocal (middle and right) images obtained at the end of experiment. Comparison of bright-field (left) and GFP (middle) distinguishes TRPV1-Tet3-Bu-GFP expressing cells from untransfected cells. PhyB-mCherry images (red) indicate that most TRPV1-positive cells expressed significant levels of the PhyB/PIF machinery for activating PI3K. (D and E) Summary scatter plots from multiple experiments, with individual cells shown as dots and the mean shows as black lines (n = 20). The effects of 750 and 650 nm illumination on sulfo-Cy5 (D) and GFP (E) are presented as ratios of fluorescence intensity after illumination with the indicated wavelength of illumination to the initial fluorescence. (F-I) The same experiment for InsR-676Tet3-Bu-GFP trafficking (n = 24).

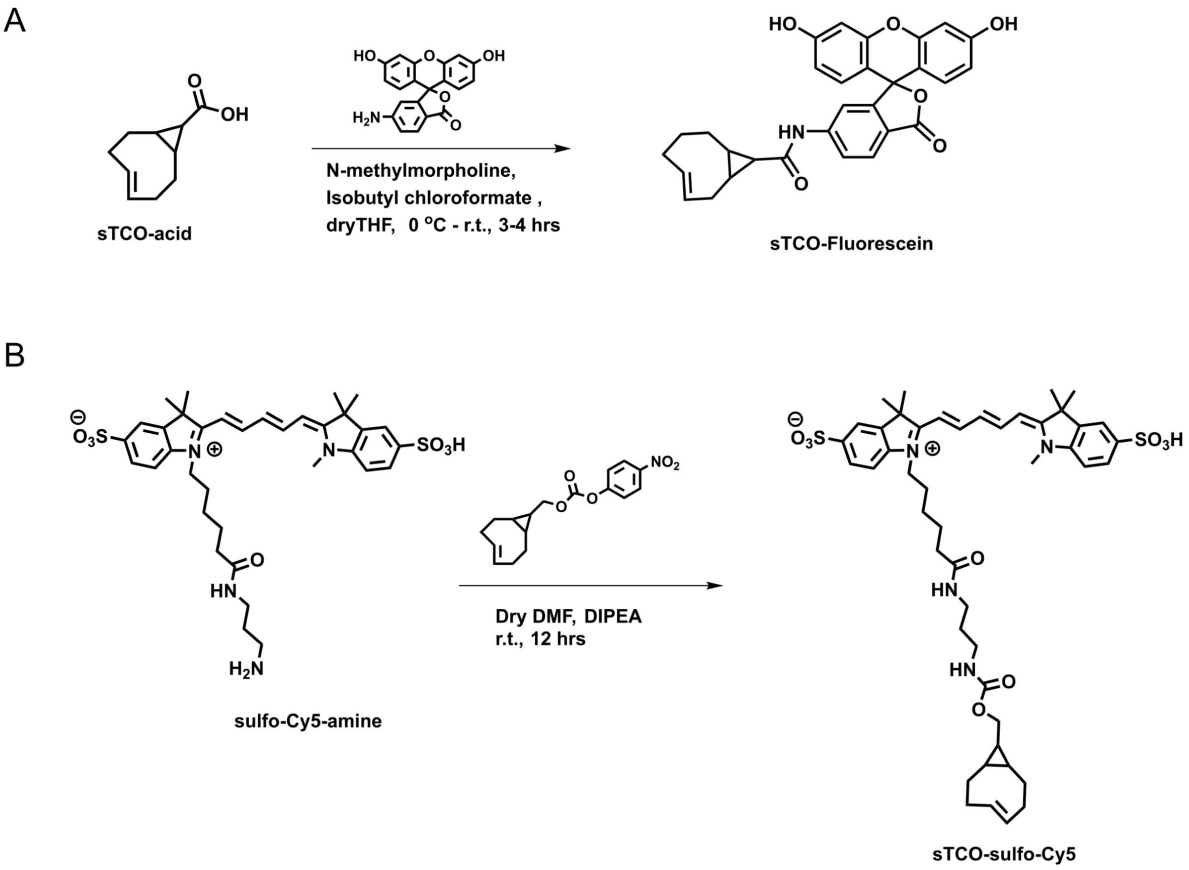

Figure 6.

Synthesis of sTCO-Fluorescein and sTCO-sulfo-Cy5.

activation downstream of InsR is key to delivery of GLUT4 transporters to the PM (Saltiel, 2021). However, to our knowledge, PI3K activity has not been previously known to stimulate InsR delivery to the PM. Although it would be exceedingly interesting if insulin binding to InsR could trigger both InsR endocytosis *and* exocytosis, further studies will be required to determine the physiological significance, if any, of PI3K-stimluated delivery of InsR to the PM.

Discussion

Here, we present proof-of-principle experiments demonstrating the simultaneous use of an optogenetic approach for PI3K activation and genetic code expansion/click chemistry to interrogate regulation of membrane protein trafficking. This combination of approaches is especially useful for membrane proteins with significant expression in the endoplasmic reticulum and/or other intracellular compartments, which makes it very difficult to identify proteins in the PM definitively when using fusions with GFP or similar genetically encoded labels. In addition to distinguishing proteins at the surface, using a small ncAA and click chemistry is likely much less perturbing to protein structure and function than fusion with a ~20-30 kDa protein such as GFP, SNAP-tag or HaloTag. Restricting the ncAA and fluorescent label to an extracellular region/loop is also less likely to interfere with protein-protein interactions on the intracellular side, compared with a large protein fusion to an intracellular N- or C-terminal of a protein of interest.

Successful implementation of this approach required overcoming a number of barriers: efficient incorporation of Tet3-Bu into the extracellular sites; demonstration that the Tet3-Bu-incorporating protein had the expected functional properties; development of a membrane impermeant sTCO-coupled dye; and optimization of the PhyB/PIF and genetic code expansion machinery in the same cells. Incorporation of Tet3-Bu at the extracellular side of TRPV1 was well tolerated in the S1-S2 loop, with two of four sites yielding functional, capsaicin-activated channels. We have found incorporation efficiency to be difficult to predict. Screening through desirable sites typically is the best approach, with approximately one half to one third of selected sites showing reasonable levels of ncAA incorporation.

There is significant room for improvement of these methods, particularly in developing more rapid labeling of the ncAA. As shown in **Figure 3**, complete labeling with a low concentration of sTCO-dye required more than 10 minutes. Monitoring membrane protein trafficking to the surface in real time will require a reaction that is at least five- to 10-fold faster. Increasing the speed of labeling can be accomplished using ≥1 μM sTCO-dye, but at such high concentrations of dye the background signal from dye in solution interferes with imaging. We have recently developed new ncAAs with the tetrazine ring more proximal to the amino acid beta carbon, which we term Tet4, that react with sTCO-based labels with rates as fast as 10^6 $M^{-1}s^{-1}$ (Jana et al., 2023). The aminoacyl tRNA synthetases that incorporate Tet4 amino acids into mammalian cells are not as efficient as those for Tet3 amino acids. Improving the efficiency of incorporating Tet4 amino acids would allow us to express Tet4-incorporating proteins and measure delivery of proteins to the PM in real time.

Another limitation to our approach is the need to co-express five to six plasmids to use PhyB/PIF and Tet/sTCO machinery simultaneously. Developing stable cell lines for ncAA incorporation would reduce the need for multiple plasmid transfection and, perhaps, lead to more uniform expression across cell populations. The PhyB/PIF machinery requires supplementing cell medium with the phycocyanobilin chromophore, making it incompatible with *in vivo* experiments. Introducing four genes, PcyA, H01, Fd, and Fnr, and knocking down/out biliverdin reductase A leads to efficient synthesis of phycocyanobilin in mammalian cells (Uda et al., 2017; Uda et al., 2020). It is therefore possible that genetically modified animals might one day express the

PhyB/PIF machinery and chromophore and allow optogenetic activation of PI3K *in vivo*. However, there is as yet no method for synthesizing the necessary ncAAs within cells, precluding *in vivo* use of the PhyB/PIF with tetrazine-based genetic code expansion.

PI3K is a universal signal for trafficking to the PM. In *dictyostelium*, PI3K activation at the leading edge of cells underlies chemotaxis towards nutrients (Nichols et al., 2015). In macrophages, PI3K at the leading edge of cells drives motility towards the object of phagocytosis (Hawkins et al., 2006; Hawkins and Stephens, 2015). In the cases discussed here, membrane proteins constitute the cargo delivered to the PM in response to PI3K signaling. A vast literature has examined the mechanisms by which InsR is recycled from the PM in response to insulin (Knutson, 1991; Ye et al., 2017; Chen et al., 2019), but less is known about ways in which delivery of InsR to the PM is regulated. We show here, for the first time, that activation of PI3K is sufficient to increase InsR in the PM. Future studies will be needed to understand the role for this process in liver and muscle cells. For TRPV1, PI3K was identified as an important signal that enhances surface expression in response to nerve growth factor, but the steps between PI3K activation and fusion of vesicles containing TRPV1 cargo with the PM are a mystery. The tools developed here provide a means by which the cellular mechanisms underlying PI3K-mediated delivery of membrane proteins can be interrogated.

Materials and methods

Molecular Biology and Constructs Used

The cDNAs used in this study were provided by: rat TRPV1 in pcDNA3.1 – David Julius, UCSF, San Francisco, CA (Caterina et al., 1997); the human insulin receptor (accession AAA59452.1) with the K676TAG mutation and C-terminal GFP in a plasmid based on pEGFP– Edward Lemke, European Molecular Biology Laboratory (EMBL), Heidelberg, Germany (Nikić et al., 2015); eukaryotic elongation release factor 1 with E55D mutation in pCDNA5-FRT– Jason Chin, Medical Research Council Laboratory of Molecular Biology, Cambridge, England (Schmied et al., 2014); TrkA (rat) in the pcCMV5 vector and p75NTR (rat) in the pcDNA3 vector from Mark Bothwell, University of Washington, Seattle, WA; PH-Akt-cCerulean in pcDNA3-k vector-Orion Weiner, UCSF, San Francisco, CA (Toettcher et al., 2011) and *Methanosarcina barkeri* R2–84-RS aminoacyl tRNA synthetase/$tRNA_{CUA}$ in the pAcBac1 plasmid – Ryan Mehl, University of Oregon, Corvallis, OR (Jang et al., 2020). Gibson cloning was used to generate the C-terminal GFP fusion for TRPV1 and to introduce 468TAG stop codon. All constructs were verified using Sangar sequencing.

In selecting the extracellular site for ncAA incorporation into TRPV1, we focused on the S1-S2 loop (**Figure 3C**) because the S3-S4 loop is very short (5 amino acids) and we wished to avoid the pore turret between S5 and S6, which has been implicated in gating of the pore (Yang et al., 2015; Bae et al., 2016; Jara-Oseguera et al., 2016; Ma et al., 2016). In our initial screen, we tested incorporation at four positions, R455, V457, K464 and T468 (**Figure 3C**) and used a C-terminal GFP fusion to facilitate screening. We found that TRPV1-K464Tet3-Bu-GFP and TRPV1-T468Tet3-Bu-GFP produced robust, capsaicin-activated currents when interrogated with whole-cell patch-clamp (**Figure 3– figure supplement 1A-B**), whereas TRPV1-R455Tet3-Bu-GFP and TRPV1-V457Tet3-Bu-GFP of incorporation did not (data not shown). We focused on TRPV1-T468Tet3-Bu-GFP, as TRPV1-K464Tat3-Bu-GFP was not efficiently labelled in subsequent experiments (data not shown). We next tested whether expression of full-length TRPV1-T468Tet3-Bu-GFP required incorporation of Tet3-Bu using in-gel GFP fluorescence. As shown in **Figure 3– figure supplement 1C**, detergent solubilized cell lysates from cells expressing TRPV1-T468Tet3-Bu-GFP showed a band at the expected molecular weight when the medium was supplemented with Tet3-Bu, but no GFP fluorescence at this molecular weight was observed when the medium lacked Tet3-Bu. These data indicate that TRPV1-T468Tet3-Bu-GFP incorporated Tet3-Bu without measurable incorporation of natural amino acids at the TAG codon site.

Cell Culture/transfection/solutions

The experiments in **Figure 1** were performed in an NIH/3T3 cell line stably expressing PhyB-Cherry-CAAX, PIF-iSH2-YFP, and Akt-PH-CFP. These cells were a gift from Orion Weiner (UCSF, San Francisco, CA). NIH-3T3 cells were cultured at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen, Grand Island, NY) supplemented with 10% bovine calf serum (Hyclone, Logan, UT), L-Glutamine (Invitrogen), and penicillin/streptomycin (Lonza, Switzerland).

F-11 cells (a gift from M.C. Fishman, Massachusetts General Hospital, Boston, MA; (Francel et al., 1987)), a hybridoma of rat dorsal root ganglion neurons and mouse neuroblastoma cells, were used for experiments in **Figures 1**, **2**, and Figure 3-figure supplement 2, as they are an appropriate model for studying NGF-induced signaling in pain receptor neurons. F-11 cells were incubated in Ham's F-12 Nutrient Mixture Gibco) supplemented with 20% fetal bovine serum, penicillin/streptomycin, and HAT supplement (100 µM sodium hypoxanthine, 400 nM aminopterin, 16 µM thymidine; Invitrogen). Click chemistry experiments in **Figures 3** through 6 were performed in HEK293T/17 cells (Catalog #CRL-11268, ATCC, Gaithersburg, MD) because these cells are optimized for transfection with multiple plasmids. HEK293T/17 cells were incubated according to manufacturer's instructions in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin.

Cells were transfected using Lipofectamine 2000 (Invitrogen) or JetPrime (Polyplus, Illkirch, France) according to the instructions of the manufacturers. Cells were passaged on to 25-mm round glass coverslips (Warner Instruments, Hamden, CT) 12 hours after transfection and cultured until used for experimentation 16–48 hours later. The coverslips were coated with poly-L-lysine to aid cell attachment (Sigma-Aldrich, St. Louis, MO). For the optogenetic system, HEK293T/17 cells were transfected with PhyB-mCherry-CAAX (5 µg/well in 6-well culture plate), PIF-ISH2-YFP (or PIF-YFP) 0.5 µg. The 10:1 ratio helped to express more PhyB membrane target and lesser amount of PIF cargo. When cytoplasmic PIF-ISH2-YFP was highly expressed, its translocation toward the PM upon activating 650 nm light appeared significantly reduced, likely due to the difficulty of separating signal in the cytoplasm from that at the PM. For expression of TAG-encoding cDNAs, HEK293T/17 cells in 6-well plates at 30-50% confluency were transfected target gene cDNAs at 2:1 ratio (proteins with TAG codon: RS 2-84), with a total of 2 µg/well. Dominant negative elongation release factor (DN-eRF-E55D; 0.4 µg) was added to reduce truncation of the protein at the TAG codon (Schmied et al., 2014). For NGF stimulation, F-11 cells were transfected with 1 µg each of TrkA and p75NTR along with 1 µg TRPV1 constructs. For other constructs, 1-3 µg cDNA was used for transfection. During experiments, cells were perfused with Ringer's solution (in mM: 140 NaCl, 4 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES and 5 glucose, pH 7.3). All experiments were performed at room temperature except Figure 3-figure supplement 2.

Optogenetic experiments

Cells were incubated in the dark with 10 µM PCB in the culture medium for at least 1 hour prior to experiments. To avoid photodamage of Opto3K, handling of cells during and subsequent to PCB loading was performed in the dark room using a green safety flashlight (Levskaya et al., 2009). Note that PhyB loaded with PCB is extremely sensitive to light, light of any wavelength between 560 and 650 nm can activate Phytochrome B once PCB is added (Braslavsky et al., 1997; Katrin Anders, 2015). Therefore, experiments were carried out in the presence of 750 nm deactivating light and care was taken to avoid (or minimize) the exposure of cells to 560-650 nm illumination from microscopy or the external environment, including the computer monitor.

TIRF and confocal imaging

The total internal fluorescence (TIRF) imaging set up was as described previously (Stratiievska et al., 2018). We used an inverted microscope (Nikon Ti-E) equipped with a 60x TIRF objective (NA 1.49). For activating/deactivating opto-PI3K with light, we used HQ630/20 X (42490) and HQ760/40 X (226723) excitation filters inserted in the overhead condenser filter slider. The whole experimental chamber was illuminated with activating or deactivating light using the maximum intensity (100 W at full spectrum) of the condenser lamp. Light intensity at the focal plane was 2.4 mW and 3.7 mW for 650 nm and 750 nm illumination, respectively.

Cyan Fluorescent Protein (CFP) fusion proteins were imaged using excitation from a 440 nm laser and a 480/40 nm emission filter. Yellow Fluorescent Protein (YFP) fusion proteins were monitored using the 514 nm line of an argon laser and a 525/50 nm emission filter. mCherry was excited at 561 nm and fluorescence was collected with 605/70 nm emission filter.

Time-lapse images were obtained every 10 or 20 seconds using either a QuantEM or an Evolve EMCCD camera (Photometrics). Movies were then processed using ImageJ software (NIH) (Rasband, 1997; Schneider et al., 2012). Regions of interest (ROI) were drawn around the footprint of individual cells and the average ROI pixel intensity was measured. Background fluorescence from cell-free areas were subtracted. Measurements were analyzed using Excel (Microsoft, Redmond, WA) and Igor Pro (Wavemetrics, Portland, OR) software, Traces were normalized by the average intensity during 1 or 3 min time period prior to activating 650 nm light.

The confocal images were obtained with a Zeiss 710 confocal microscope (Zeiss, Oberkochen, Germany). Illumination of 650-750 nm lights were controlled as described for TIRF microscopy experiments. CFP, YFP, mCherry, and JF646 were excited with 440, 514, 561, 633 nm lasers, respectively. Data were analyzed with ImageJ. The lookup table of images were changed during analysis, but the same range was applied for all images within a time series experiment.

Electrophysiology

Currents were recorded using the standard patch clamp technique (Hamill et al., 1981). Borosilicate glass electrodes had a resistance of 3–6 MΩ when filled with internal solution (in mM; 140 KCl, 5 NaCl, 0.1 EGTA, and 10 HEPES, pH 7.3). Ringer's saline was used in the extracellular bath solution. Current through ion channels was measured in whole-cell configuration and the membrane potential was held at −60 mV. To activate TRPV1, the agonist capsaicin was applied for 10 seconds using a local perfusion system which allowed the solution to exchange within 1 second (Koh and Hille, 1997). Currents were recorded with an EPC-9 amplifier (HEKA Elektronik, Lambrecht (Pfalz), Germany) and analyzed using Igor Pro software.

Endocytosis of insulin receptor

F-11 cells expressing InsR-K676TAG-GFP were treated with 30 μM Tet3.0-Bu in the culture medium for 24 hours, transferred to glass coverslip, and further cultured in serum-deprived medium for 24 hrs. The receptor targeted to the plasma membrane was labeled with membrane-impermeable sTCO-Cy3B dye (**Figure 3 – figure supplement 2C**) for 4 min and free dye molecules were washed with saline solution for 2 min. Confocal images were obtained every 20 s with excitation at 561 nm and emission between 600 and 640 nm. Labeling of the receptor and insulin treatment were performed at around 35 °C to promote the rate of endocytosis using a local perfusion system with heat exchanger (Koh et al., 2011).

Chemical synthesis

All purchased chemicals were used without further purification. Thin-layer chromatography (TLC) was performed on silica 60F-254 plates. Flash chromatographic purification was done on silica gel 60 (230-400 mesh size). ^{1}H NMR spectra were recorded at Bruker 400MHz. The chemical shifts were shown in ppm and are referenced to the residual nondeuterated solvent peak CD_3OD (δ =3.31 in ^{1}H NMR), as an internal standard. Splitting patterns of protons are designated as follows: s-singlet, d-doublet, t-triplet, q-quartet, quin-quintet, m-multiplet, bs-broad singlet. sTCO-JF646: Synthesized according to the published procedure (Jana et al., 2023).

sTCO-Fluorescein: In dry tetrahydrofuran (THF) (3 mL), sTCO-CO_2H (10 mg, 0.06 mmol) (O'Brien et al., 2018), and N-methylmorpholine (10 µL, 0.09 mmol) were taken under nitrogen atmosphere and stirrer under ice cold condition. Isobutyl chloroformate (10 µL, 0.07 mmol) was added dropwise to the reaction mixture and stirrer for 5 minutes. After that, 6-aminofluorescein (24 mg, 0.07 mmol) in dry THF (1 mL) was added portion-wise and the reaction mixture was allowed to warm to room temperature and stirring was continued for another 3 hours. The solvent was evaporated, and the residue dissolved in ethyl acetate. The solution was washed with water and saturated sodium bicarbonate solution. The organic layer was dried over sodium sulfate (Na_2SO_4) and the product (16 mg, 0.032 mmol) was purified by silica gel column chromatography (10-15% methanol in dichloromethane). Yield-53%. ^{1}H NMR (400MHz, CD_3OD) δ 7.91 (1H, d), 7.65 (1H, d), 7.06 (1H, d), 6.89 (1H, s), 6.84-6.82 (1H, m), 6.74-6.67 (1H, m), 6.65 (1H, s), 6.59-656 (1H, m), 6.23 (1H, d), 5.92-5.86 (1H, m), 5.27-5.20 (1H, m), 2.48 (1H, d), 2.39-2.25 (3H, m), 2.04-1.94 (2H, d), 1.37-1.32 (1H, m), 1.24 (1H, t), 1.07 (1H, t), 1.01 (1H, d), 0.84-0.75 (1H, m).

sTCO-sulfo-Cy5: In 1 mL of dry dimethylformamide (DMF), 5 mg of the sulfo-Cy5-amine (0.007 mmol) and 3.5 mg (0.01 mmol) of activated 4-nitrophenyl ester of sTCO (Jang et al., 2020) were added under nitrogen atmosphere. Followed by N,N-diisopropylethylamine (DIPEA) (10 µL, 3 equiv.) was added to the reaction mixture and allowed to stirrer at room temperature for 12 hours. After that, the solvent was concentrated onto silica gel under reduced pressure and the product (4 mg, 0.004 mmol) was purified by silica gel column chromatography (20-30% methanol in dichloromethane). At the beginning of column run, added 50 mL of 50% ethyl acetate and hexane to remove the DMF solvent. Yield 57%. ^{1}H NMR (400MHz, CD_3OD) δ 8.31 (2H, t), 7.97 (1H, s), 7.89 (2H, d), 7.87 (1H, dd), 7.33 (2H, d), 6.67 (1H, t), 6.32 (2H, dd), 5.87-5.79 (1H, m), 5.13-5.06 (1H, m), 4.13 (2H, t), 3.88 (2H, d), 3.72 (2H, quin.), 3.64 (3H, s), 3.21 (2H, q), 3.16 (2H, t), 3.09 (2H, t), 2.86 (4H, d), 2.29 (1H, d), 2.23-2.17 (3H, m), 1.89-1.79 (2H, m), 1.60 (2H, t), 1.38 (6H, s), 1.36 (6H, s), 0.91-0.83 (2H, m), 0.59-0.42 (2H, m), 0.45-0.37 (1H, m).

In-gel fluorescence

HEK293T/17 cells were harvested 36-48 hours after transfection and treated with 1x SDS sample buffer (Invitrogen). Equal amounts of proteins were loaded into 3-8% Tris-acetate gradient gels and electrophoresed at 150 V for 1 hr. Fluorescence image of the gel was obtained with an Amersham ImageQuant 800 (Cytiva, Marlborough, MA). GFP fluorescence was collected with excitation at 460 nm and a 525/20 nm emission filter). The gel was subsequently stained with Quick Coomassie solution and destained with distilled water for each 2 hours before image acquisition.

Statistical analysis

Data are presented as mean ± SEM, n is the number of single cells. Student's t test was used to test the significant difference between two groups, with paired test performed as appropriate. $P < 0.05$ was regarded as significant.

Figure Legends

Figure 3– movie supplement 1. Activity of InsR-K676TAG measure as its recycling upon insulin treatment.

The same measurement as **Figure 3– figure supplement 2A**. Each image was obtained every 10 s. The time stamp is indicated on upper right corner and insulin application on upper left corner.

Acknowledgements

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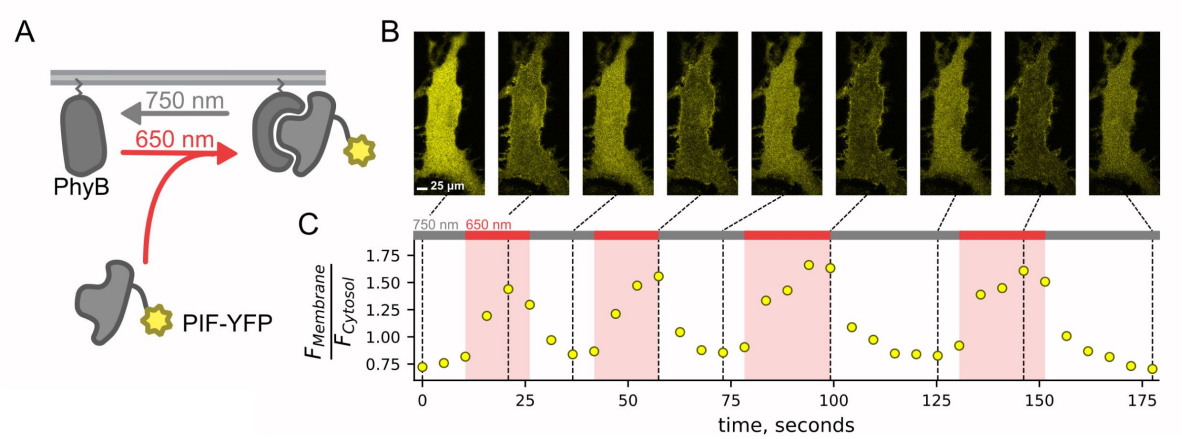

Figure 1 – figure supplement 1.

PIF-YFP translocates to PM in response to 650 nm light and back to the cytosol in response to 750 nm light.

The PhyB –PIF light-inducible interaction is rapid (a few tens of seconds) and fully reversible (within ~30 seconds) and can be repeated multiple times. (A) Schematic diagram for optogenetic PhyB-PIF system. PhyB-mCherry loaded with the chromophore phycocyanobilin localizes to the PM due to a CAAX tag to induce lipidation (squiggly line). Illumination with 650 nm light induces a conformational change in PhyB that increases its affinity for PIF-YFP, effectively recruiting PIF-YFP to the PM. The conformational change in PhyB reverses with illumination with 750 nm light, causing PIF-YFP to dissociate and return to the cytoplasm. (B) A representative confocal experiment with an NIH3T3 cell stably expressing PhyB-mCherry-CAAX and PIF-YFP. Images were obtained at times indicated in C and are all shown on the same lookup table. During illumination with 650 nm light, PIF-YFP translocated to the PM quickly, with a corresponding decrease in cytoplasmic fluorescence. (C) Ratio of measured PIF fluorescence at region of interest (ROI) placed at the PM ($F_{Membrane}$) and cytoplasm ($F_{cytosol}$) from the cell in B. These data recapitulate previously published data (Levskaya et al., 2009; Toettcher et al., 2011).

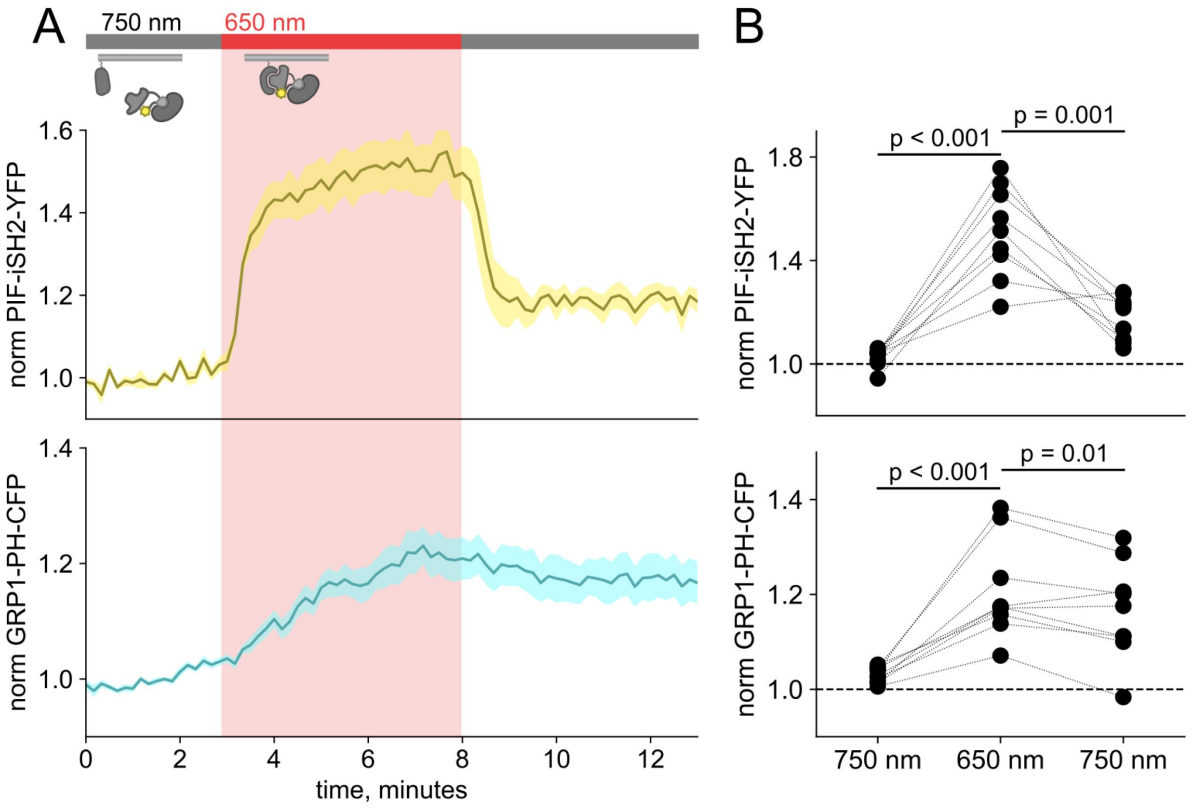

Figure 1 – figure supplement 2.

Detection of PI(3,4,5)P_3 generation by PIF-iSH2-YFP at the PM using GRP1-PH-CFP. (A) TIRF measurements of F-11 cells expressing PhyB-mCherry, PIF-iSH2-YFP, and GRP1-PH-CFP (n = 9). Upon 650 nm illumination, PIF PIF-iSH2-YFP translocated to the PM quickly (upper trace) while GRP1-PH-CFP moved slower (lower trace). Normalized to the initial 3 min control period. (B) Scatter plot of PIF-iSH2-YFP and GRP1-PH-CFP fluorescence for individual cells. Each point represents the 30 s average for 750 nm (2.5-3 min), 650 nm (4.5-5 min), and 750 nm (12.5-13 min). Translocation of both PIF-iSH2-YFP and GRP1-PH-CFP is reversible.

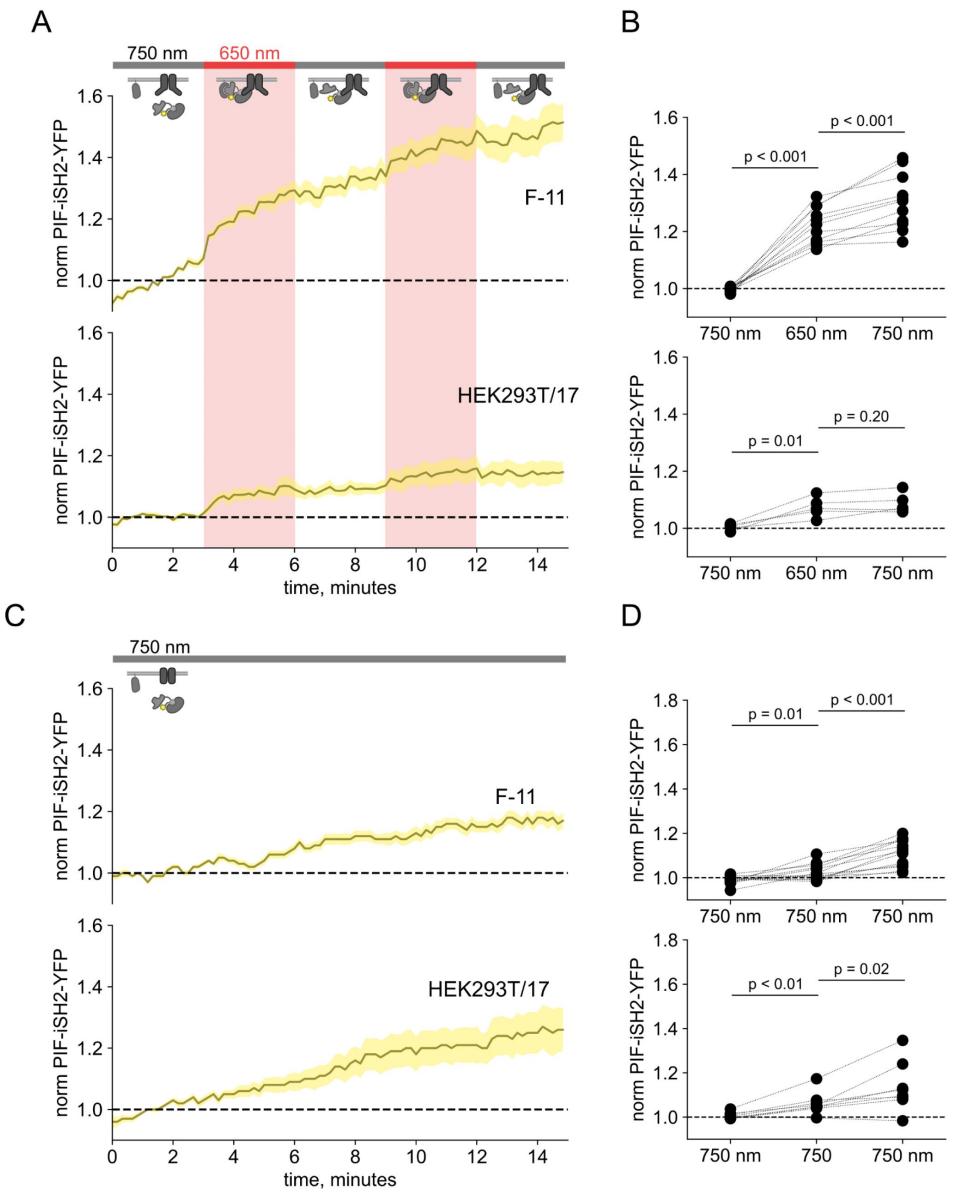

Figure 2 – figure supplement 1.

750 nm light fails to cause iSH2 dissociation from the PM in TRPV1-expressing cells.

(A) TIRF measurements of PIF-iSH2-YFP from F-11 cells expressing TRPV1-CFP in F-11 cells and HEK293T/17 cells, as indicated (n = 5 – 17). The color code and lines/envelopes have the same meaning as in **Figure 2**. Inset cartoons depict the model for retention of iSH2 at the PM via binding to TRPV1, which contains the PI3K-binding ARD domain. (B) Scatter plot of PIF-iSH2-YFP fluorescence for individual cells. Each point represents the 20 s average for 750 nm (1.33 - 1.66 min), 650 nm (4.33 - 4.66 min), and 750 nm (7.33 - 7.66 min). Translocation of PIF-iSH2-YFP is irreversible. (C,D) The same experiments with continuous illumination at 750 nm (n = 5 – 13).

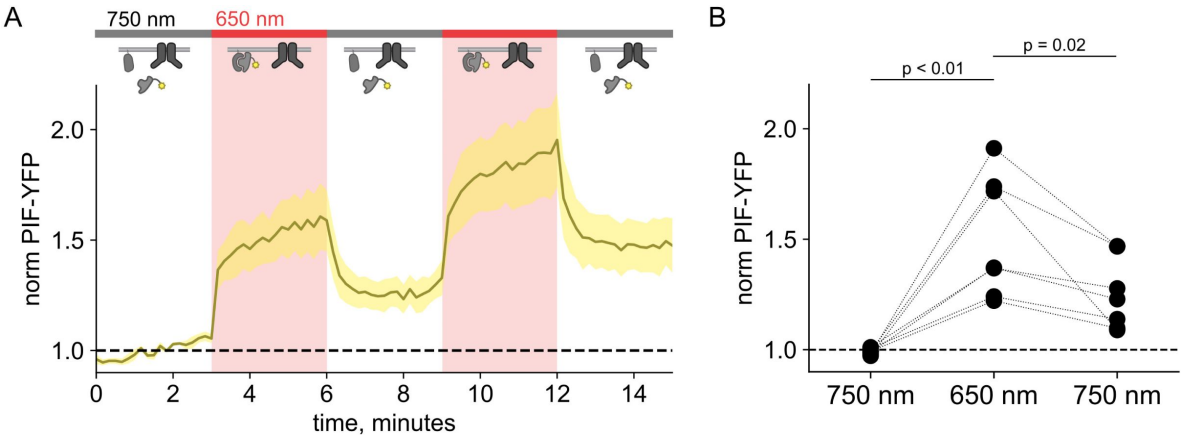

Figure 2 – figure supplement 2.

figure supplement 3. PIF-YFP dissociates from the PM in response to 750 nm light even in TRPV1-expressing cells.

TRPV1 does not retain PIF lacking iSH2 at the PM of F-11 cells. (A) Normalized TIRF fluorescence was recorded in F-11 cells transfected with PhyB-mCherry-CAAX, PIF-YFP (no iSH2 domain), and either TRPV1-CFP or TRPV1 (no fluorescent tag). Reversible translocation of PIF-YFP upon 650 nm illumination demonstrates that PIF lacking iSH2 domain does not interact with TRPV1 channels on the PM (n = 8). (B) Scatter plot of PIF-iSH2-YFP fluorescence for individual cells. Each point represents the 20 s average for 750 nm (1.33 - 1.66 min), 650 nm (4.33 - 4.66 min), and 750 nm (7.33 - 7.66 min).

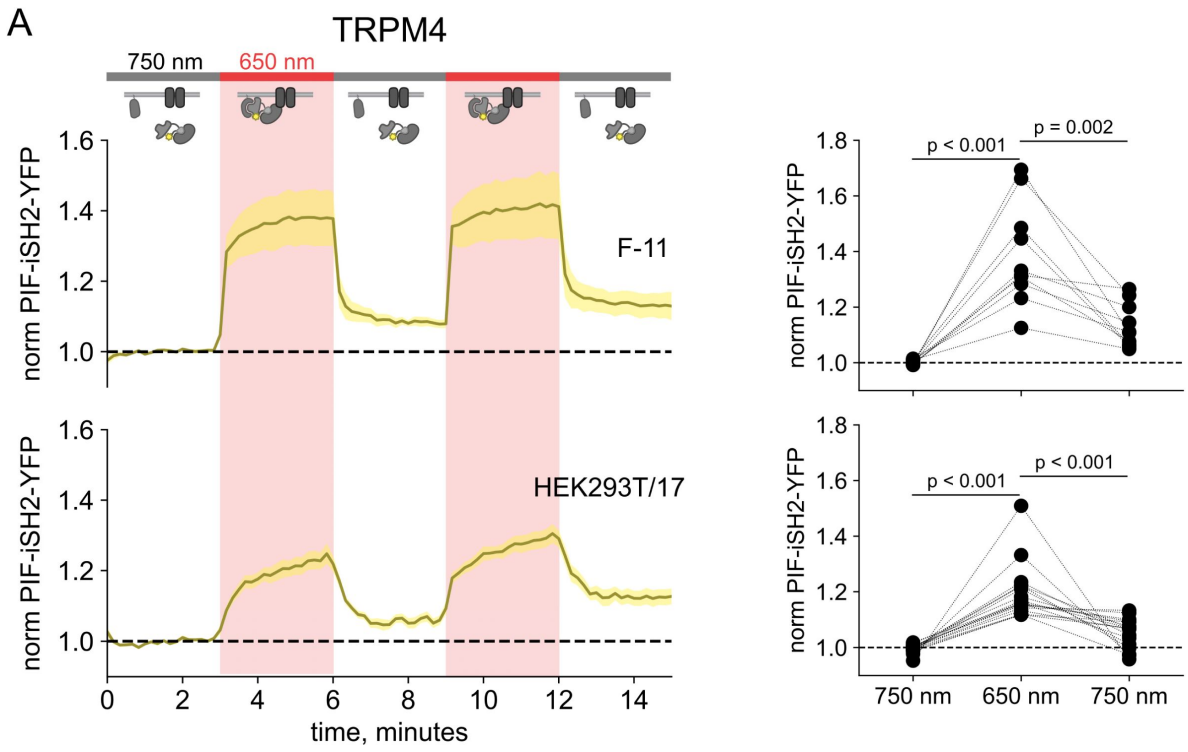

Figure 2 – figure supplement 3.

750 nm light succeeds in causing iSH2 dissociation from the PM in TRPM4-expressing cells.

(A) TIRF measurements of PIF-iSH2-YFP from F-11 and HEK293T/17 cells expressing TRPM4-CFP (n = 7 -17). Inset cartoons depict the model for no retention of iSH2 at the PM via binding to TRPM4, which has no ankyrin repeats. (B) Scatter plot of PIF-iSH2-YFP fluorescence for individual cells. Each point represents the 20 s average for 750 nm (1.33 - 1.66 min), 650 nm (4.33 - 4.66 min), and 750 nm (7.33 - 7.66 min).

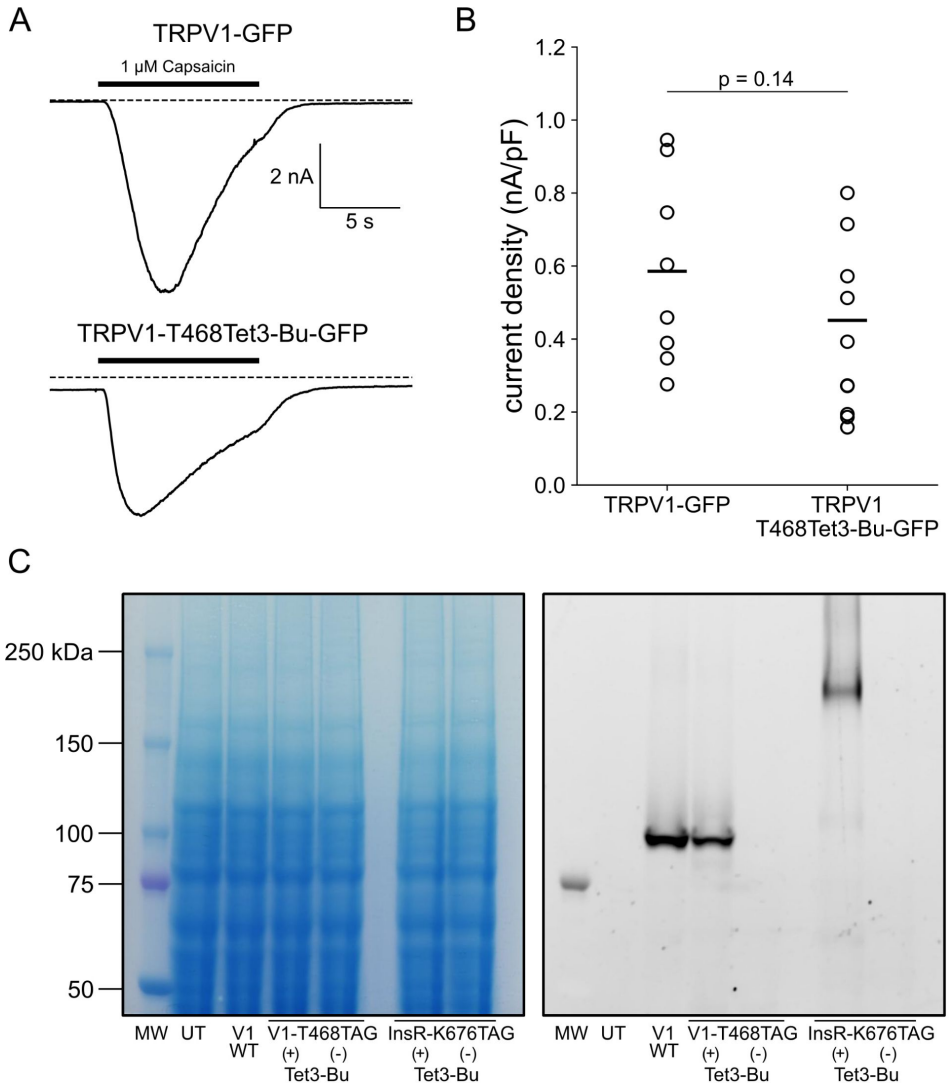

Figure 3– figure supplement 1.

Incorporation of Tet3-Bu into TRPV1 and InsR.

(A) Whole-cell patch clamp recording demonstrates that TRPV1 incorporating Tet3-Bu remains functional. Application of 1 μM capsaicin to HEK-293T/17 cells transfected with either TRPV1-GFP (top) or TRPV1-468Tet3-Bu (bottom) induced inward currents at a holding potential of -60 mV. (B) Collected whole-cell electrophysiology data from all cells with peak capsaicin-activated currents normalized to the area of cell membrane (n = 8 and 9 for TRPV1-GFP and TRPV1-468Tet3-Bu, respectively). (C) In-gel fluorescence screening demonstrates that expression of full-length TRPV1-468Tet3-Bu or InsR-676Tet3-Bu requires the presence of Tet3-Bu ('Tet3 (+)'). Wild type TRPV1-GFP is shown for reference. Detergent-extracted cell lysates were run on SDS/PAGE. GFP fluorescence in the gels was measured and then cells were stained with Coomassie blue dye.

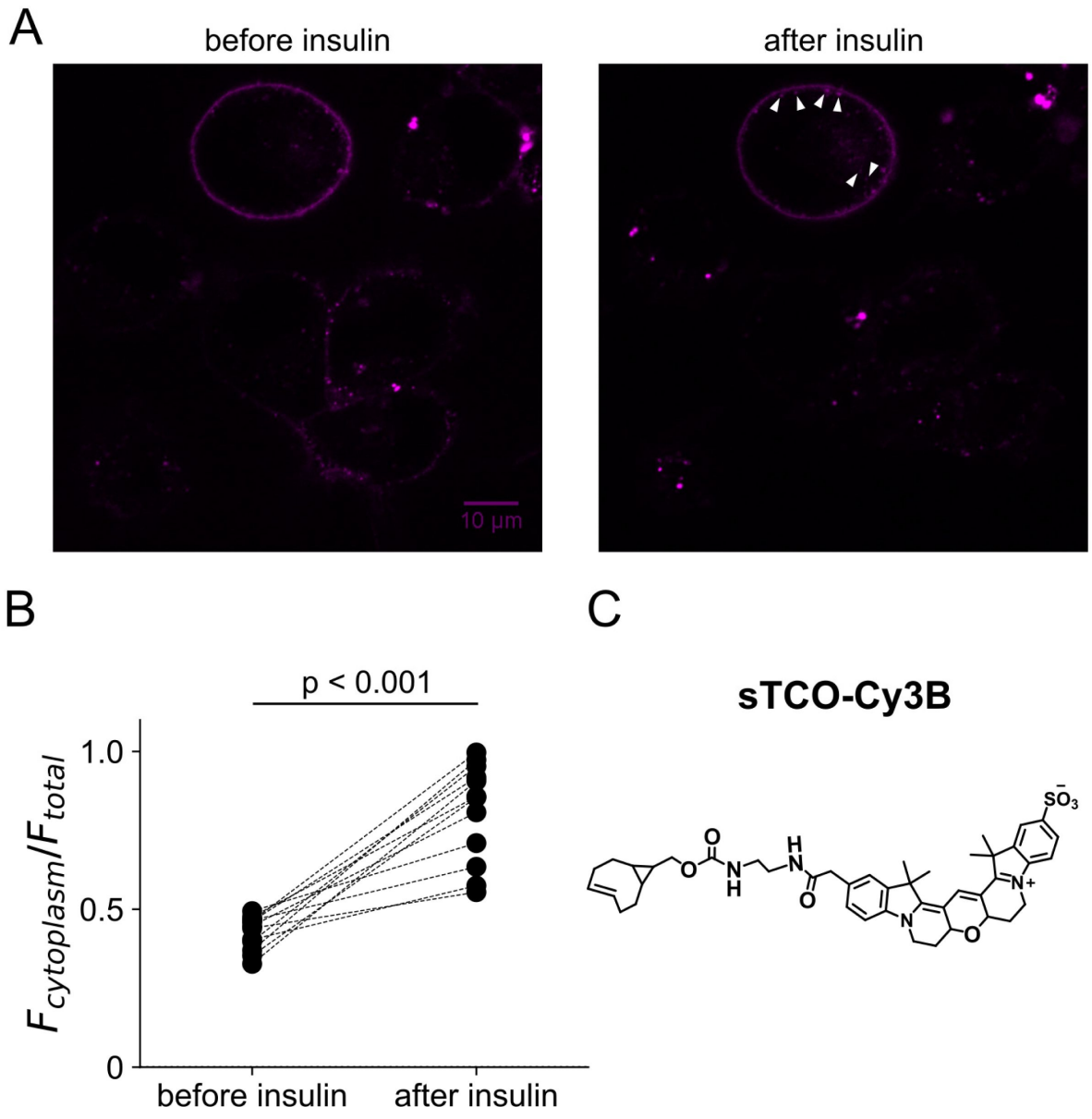

Figure 3– figure supplement 2.

Activity of InsR-K676TAG measure as its recycling upon insulin treatment.

F-11 cells were transfected with the genetically modified receptor and inspected with a confocal microscope. (A) After labeling with membrane-impermeable sTCO-Cy3B dyes, the receptor was visible in the PM. Application of 100 nM insulin triggered the generation of endocytic granules (arrow heads). In some cells the receptor recycling was so vigorous that PM receptors nearly disappeared. (B) Scatter plot of the ratio of fluorescence between the cytoplasm and total cell area in individual cells (n = 12). (C) Structure of sTCO-Cy3B.

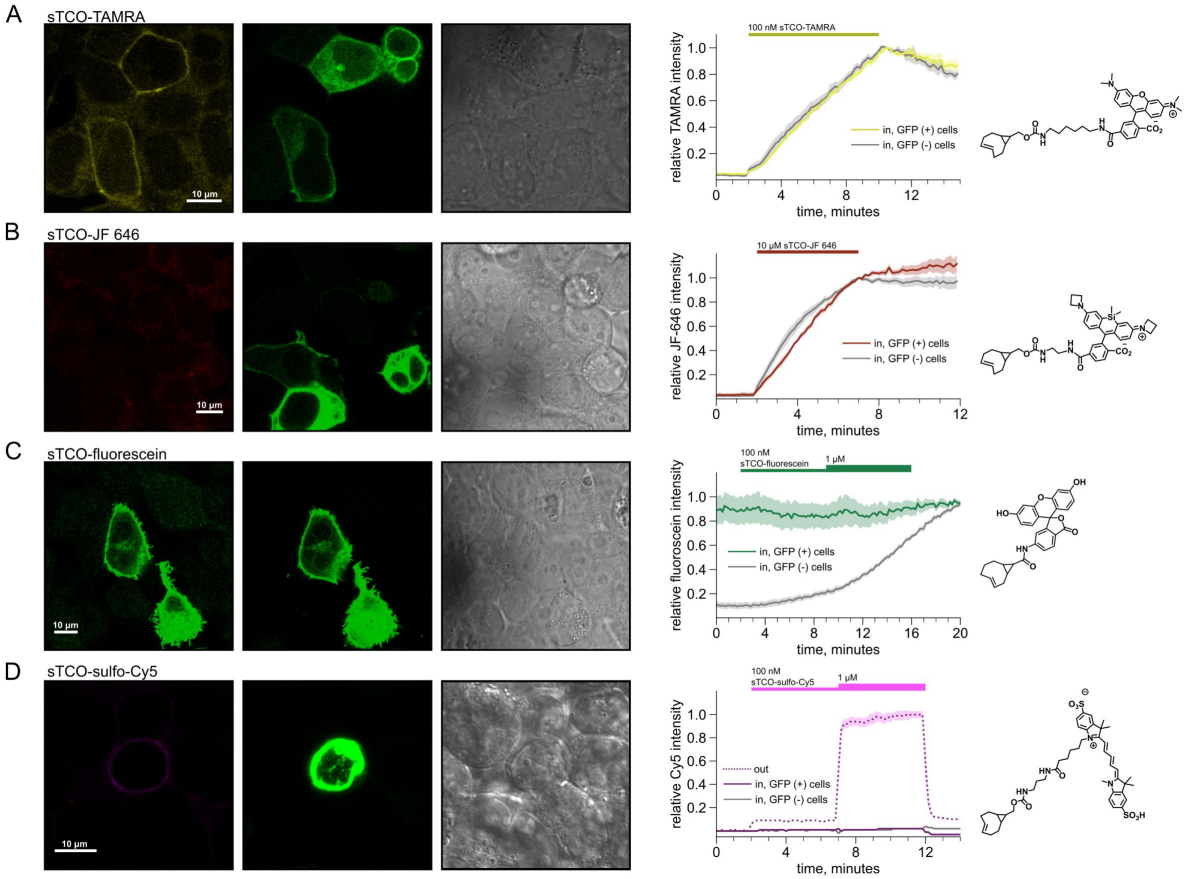

Figure 3 – figure supplement 3.

Labelling of cells with sTCO conjugates of TAMRA, JF 646, fluorescein, and sulfo-Cy5.

HEK293T/17 cells expressing InsR-Tet3-Bu-GFP were labeled with sTCO dyes. For all dyes, shown are (left) confocal images of a representative field of HEK293T/17 cells incubated with the indicated concentration of sTCO-conjugated dye, (center) plots of the kinetics of cell labeling, and (right) a diagram of the fluorescent dye. The confocal images were obtained ~30 s after washing off of extracellular dyes. GFP and bright field images are presented to demonstrate GFP-expressing (GFP(+)) and non-expressing (GFP(-)) cells. (A) 100 nM sTCO-TAMRA; (B) 10 µM sTCO-JF 646; (C) 100 nM and 1 µM sTCO-fluorescein; and (D) 100 nM and 1 µM sTCO-sulfo-Cy5. For the plots, the intracellular fluorescence was normalized to the peak value at the end of the dye applications (A-C) or to the fluorescence from 1 µM extracellular sTCO-sulfo-Cy5 (D). The thick lines represent the mean of 6 - 9 cells for each dye, and the envelopes represent the standard errors of the mean.

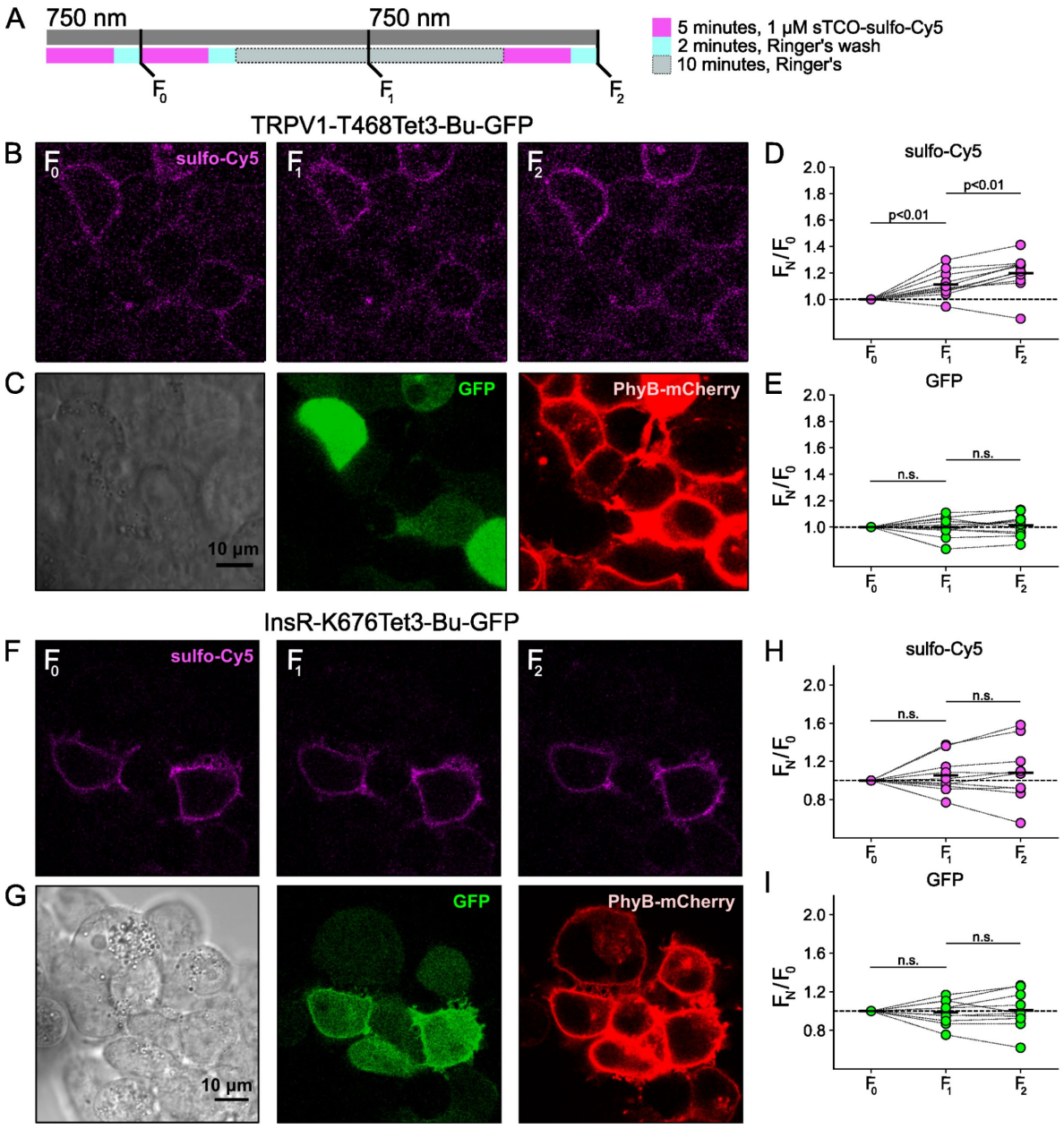

Figure 5 – figure supplement 1.

Control experiment for light-activated PI3K-induced TRPV1 and InsR trafficking to the PM.

The same experiment as **Figure 5** without 650 nm illumination. The cells were exposed to 750 nm throughout the recording (n = 11 and 10 for TRPV1-Tet3-Bu-GFP and InsR-676Tet3-Bu-GFP, respectively)

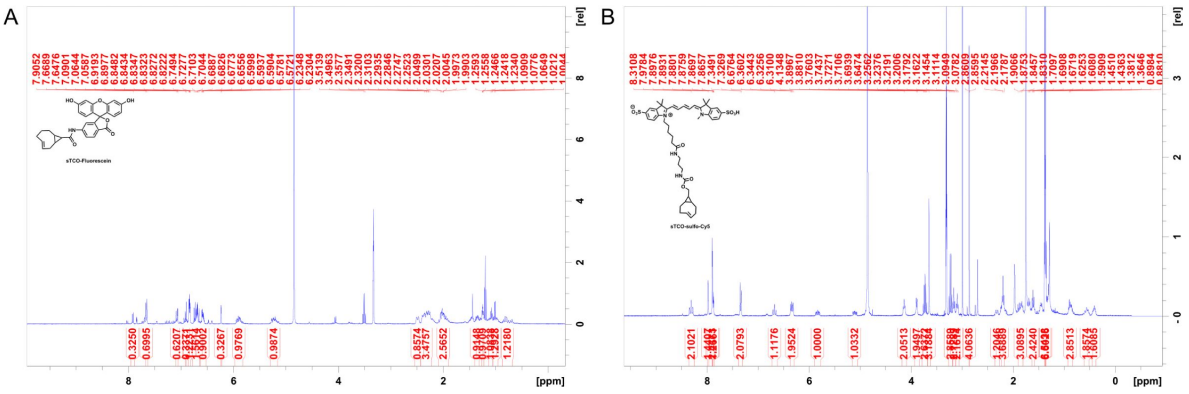

Figure 6 – figure supplement 1.

^{1}H NMR spectra of synthesized compounds (A) sTCO-fluorescein and (B) sTCO-sulfo-Cy5.

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Editors

Reviewing Editor

Leon Islas

Universidad Nacional Autónoma de México, México City, Mexico

Senior Editor
Volker Dötsch
Goethe University, Frankfurt am Main, Germany

Reviewer #1 (Public Review):

Summary:

This work seeks to isolate the specific effects of phosphoinositide 3-kinase (PI3K) on the trafficking of the ion channel TRPV1, distinct from other receptor tyrosine kinase-activated effectors. It builds on earlier studies by the same group (Stein et al. 2006; Stratiievska et al. 2018), which described the regulatory relationship between PI3K, nerve growth factor (NGF), and TRPV1 trafficking. A central theme of this study is the development of methods that precisely measure the influence of PI3K on TRPV1 trafficking and vice versa. The authors employ a range of innovative methodologies to explore the dynamics between TRPV1 and PI3K trafficking.

Strengths:

A major strength of this study is the application of innovative methods to understand the interaction between PI3K and TRPV1 trafficking. The key techniques presented include:

(1) The optogenetic trafficking system based on phytochrome B, introduced in this research. Its interaction mechanism, dependent on reversible light activation, is comprehensively explained in Figures 1 and 2, with the system's efficacy demonstrated in Figure 3.

(2) An extracellular labeling method using click chemistry, which although not exclusive to this study, introduces specific reagents engineered for membrane impermeability.

The central biological insight presented here is the sufficiency of PI3K activation to guide TRPV1 trafficking to the plasma membrane. An additional notable discovery is the potential regulation of insulin receptors via this mechanism.

The paper's strengths are anchored in its innovative methodologies and the valuable collaboration between groups specializing in distinct areas of research.

Weaknesses:

The paper might benefit from a more streamlined structure and a clearer emphasis on its findings. A possible way to enhance its impact might be to focus more on its methodological aspects. The methodological facets stand out as both innovative and impactful. These experiments are well-executed and align with biological expectations. It's evident how these techniques could be tailored for many protein trafficking studies, a sentiment echoed in the manuscript (lines 287-288). When seen through a purely biological lens, some findings, like those concerning the PI3K-TRPV1 interaction, are very similar to previous work (Stratiievska et al. 2018). A biological focus demands further characterization of this interaction through mutagenesis. Also, the incorporation of insights on the insulin receptor feels somewhat tangential. A cohesive approach could be to reshape the manuscript with a primary focus on methodology, using TRPV1 and InsR as illustrative examples.

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Reviewer #2 (Public Review):

Summary

The authors developed new tools for isolating PI3K activity and for labeling newly made membrane proteins for monitoring membrane trafficking. They found that PI3K activity alone was able to explain the increased presence of TRPV1 on the membrane independent of other cascades induced by NGF signaling. They also showed an interesting feedback between PI3K and the insulin receptor trafficking to the membrane.

Strengths:

A major strength of the paper is the innovative combination of techniques. The first technique used the optogenetic PhyB/PIF system. They anchored PhyB to the membrane and fused PIF with the interSH2 domain from PI3K. This allowed them to use 650nm light to induce an interaction between the PhyB and PIF resulting in a recruitment of the endogenous PI3K to the membrane through the iSH2 domain without actual activation of an RTK. This allowed them to dissect out one function, just PI3K recruitment/activation from the vast number of RTK downstream cascades.

The second technique was the development of a new non-canonical amino acid that is cell-impermeant. The authors synthesized the sTSO-sulfa-Cy5 compound that will react with the Tet3 ncAA through click chemistry. They showed that the sulfa-Cy5 did not cross the membrane and would be used to track protein production over time, though the reaction rates were slow as noted by the authors. The comparison of the sulfa-Cy5 data with the standard GFP with TIRF showed a clear difference indicating the useful information that is gained with the ncAA.

Another strength comes from the discovery that an isolated PI3K is responsible for increasing TRPV1 and InR trafficking to the plasma membrane.

Weakness:

The discussion does not go into much detail regarding the importance of their discovery of TRPV1 and InR increases trafficking due to PI3K activation. It also jumps to the limitations of in vivo implementation prematurely. These weaknesses are minor however.

The authors achieved their goal of creating the tools needed to separate out one of the many RTK signals and give a strong proof of concept implementation of their tools. Their results support their conclusions and will help understand how TRPV1 is regulated by signals other than the traditional channel activators. The tools developed in the article will be of use to the broader cell biology and biophysics community, not just the channel community. The opto control of the PhyB/PIF system makes it more convenient than other systems since it does not take the typical wavelengths needed for fluorescence. The cell-impermeant ncAA will also be a great tool for those studying membrane proteins, protein trafficking and protein dynamics.

https://doi.org/10.7554/eLife.91012.2.sa1

Reviewer #3 (Public Review):

Summary:

In this manuscript, Koh, Stratiievska, and their colleagues investigate the mechanism by which TRPV1 channels are delivered to the plasma membrane following the activation of receptor tyrosine kinases, specifically focusing on the NGF receptor. They demonstrate that the activation of the NGF receptor's PI3K pathway alone is sufficient to increase the levels of TRPV1 at the plasma membrane.

Strengths:

The authors employ cutting-edge optogenetic, imaging, and chemical-biology techniques to achieve their research goals. They ingeniously use optogenetics to selectively activate the PI3K pathway without affecting other NGF pathways. Additionally, they develop a novel, membrane-impermeable fluorescent probe for labeling cell-surface proteins through click-chemistry.

Comment on revised version:

We commend the authors on the significant improvements made to the manuscript. They have adequately addressed our comments. Notably, the new control experiments shown in Figure 4E and Figure 5 Fig. Supp 1 convincingly demonstrate the specificity of the NGF and 650 nm light stimuli, respectively. The addition of quantitative analyses strengthens the findings significantly. Furthermore, the manuscript is now presented in a much linear manner, enhancing its clarity and impact.

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Author response:

The following is the authors' response to the original reviews.

We thank the reviewers for their careful reading of our manuscript and their constructive comments. We have significantly improved the writing, consolidated figures, and include new experiments (see below). We now center the manuscript on the methods used and have updated the title to reflect this new emphasis. We have also added quantification with statistics, as described below. A detailed description of our improvements is provided below.

New data figures:

• Fig 3 – fig supp 2 – new experiment with insulin-triggered endocytosis of InsR

• Fig 3 – fig supp 3 – new experiments, all using the same protein construct

• Fig 3 – movie– new experiment with insulin-triggered endocytosis of InsR

• Fig 4 – added new vehicle-only negative control experiments

• Fig 5 – fig supp 1 – new negative control experiments with sequential exposures to 750 nm light

Added figure panels with quantification/statistics for: Fig. 1F; , Figure 1- figure supp 2B, Figure 2B, D, Fig. 2 – fig supp 1B, D; Fig 2 – fig supp 2B; Fig 2 – fig supp 3B;

> ***Reviewer #1:***
>
> *(1) The paper might benefit from a more streamlined structure and a clearer emphasis on its findings. A possible way to enhance its impact might be to focus more on its methodological aspects. The methodological facets stand out as both innovative and impactful.*

We thank the reviewer for this suggestion and have rewritten the manuscript to center the methods, with our applications to TRPV1 and the InsR serving as examples.

> *(2) Line 243: Please provide a reference for Tet3-Bu or clarify its origin in this study. A concise description would be helpful.*

The Jang et al., 2020 and Jana et al., 2023 studies are cited and give the structure of Tet3-Bu in Figure 3A.

> *(3) Consider merging Figures 1 and 2 for clarity.*

Because the cell types and constructs expressed differ for the figures, we did not merge them. However, we moved Figure 1 to the supplement because it repeats previously published data.

> *(4) Lines 281 and 293 should refer to Figure 5C, not 5B.*

This is now corrected.

> *(5) Should the paper pivot towards methodology, combining Figures 6 and 7 might be more coherent.*

The experiments in Figures 6 and 7 are different, making it difficult to merge them. However, Figures 7 and 8 describe the same experimental approach applied to two different membrane proteins. To align with our new focus on the methods and deemphasis of the biological system, we have merged Figures 7 and 8.

> *(6) A brief discussion comparing the cell surface labeling techniques and the merits of the presented system would offer valuable context.*

We agree that additional discussion here would be helpful but were also trying to satisfy Reviewer #3's request to reduce review-like content that disrupts the flow of the primary results. We therefore did not add a discussion of cell-surface labeling techniques.

> ***Reviewer #2:***
>
> *(1) To monitor the phosphatidylinositol-3,4,5-trisphosphates, the pleckstrin homology (PH) domain from Akt was used. This PH domain is not specific for just PI(3,4,5)P3 as stated by the authors. The Akt PH domain also binds PI(3,4)P2. The observed PI3K localization increase will also increase PI(3,4)P2 concentrations so the observed responses may not be solely because of PI(3,4,5)P3…*
>
> *…Repeating the PH domain experiments with a PH domain that is specific for just PI(3,4,5)P3, like GRP1 or Btk, would be useful to separate out any contributions from PI(3,4)P2.*

We have repeated key experiments demonstrating optogenetic activation of PI3K with the Grp1-PH domain and included these data in Figure 1-figure supplement 2.

> *(2) The data in Figure 4 supplement was confusing to interpret since it is unclear whether a membrane protein with the Tet3 is being expressed at the same time as the ncAA for labeling or if the observed labeling is endogenous. If the observed labeling in Figure 4 supplement D is endogenous, then significant concerns come up regarding the background labeling of the sTCO-sulfo-Cy5 used in the rest of the experiments.*

We have updated the data in this figure using the same protein (InsR-Tet3-Bu-GFP) for every sTCO-conjugated dye tested. The protein is also labelled with GFP, making it clear which cells in the field were transfected and which were not. The new panels showing the bright field images for each field further aid readers in identifying untransfected cells. We believe the new presentation addresses the reviewer's concerns about distinguishing sTCO labeling of Tet3-Bu-incorporating protein from labeling of endogenous proteins.

> *(3) I recommend reorganizing the article to be more linear. For example, Figure 4 is not fully explained until after Figure 4 supplement and Figure 5. This non-linear organization required a lot of back and forth reading to fully understand the logic of the experiments as well as the conclusions.*

We have improved the presentation along the lines suggested by the reviewer.

> *(4) The InsR data is interesting as a proof of concept however the writing around the InsR looks like an afterthought. The explanation for why InsR is chosen, what is known and unknown about its trafficking is given secondary importance in the writing but not in the figures. This difference weakens the article.*

We have improved the presentation along the lines suggested by the reviewer.

> *(5) Line 244 should read Figure 4A.*

This is now corrected.

> *(6) Line 281 should read Figure 5C.*

This is now corrected.

> *(7) Line 645. Fig 4, says C and E were shown as inverted b&w images when they aren't.*

This is now corrected.

> *(8) Fig 8. Line 702. States that these are TRPV1 positive cells but the figure is about InsR.*

This is now corrected.

> ***Reviewer #3:***
>
> *(1) The Results section is lengthy and disorganized. Consider revising it for better clarity and conciseness. For instance, moving lines 157 and 166-170 to the Discussion or Methods section can streamline the Results section.*

We have improved the presentation along the lines suggested by the reviewer.

> *(2) Provide more specificity in reporting: In lines 139-170, clarify why you chose to use PhyB and this particular technique. Eliminate extraneous details and maintain a more concise narrative.*

We have improved the presentation along the lines suggested by the reviewer.

> *(3) Avoid excessive review-like content, and keep the Results section focused on presenting novel findings. Simplify lines 4 173-185 to provide a straightforward presentation of results rather than extensive references to previous work.*

We have improved the presentation along the lines suggested by the reviewer.

> *(4) Reevaluate lines 196-204 to determine if they are best suited for the Results section or if they could be moved to the Discussion or Methods for improved focus.*

We have improved the presentation along the lines suggested by the reviewer.

> *(5) 231-238, revise the content to be more concise and directly to the point.*

We have improved the presentation along the lines suggested by the reviewer.

> *(6) Limit the number of figures to a maximum of five and restructure them to enhance readability. Consider consolidating panels from Figures 1 (which replicates previouslypublished work), 2, and 3 into a single figure to improve organization and information flow.*

See response to Reviewer #1, Comment #3. Although we did not merge Figures 2 and 3, we have consolidated the writing to improve the flow of the writing.

> *(7) Move Fig 5, which depicts control experiments, to supplementary information to improve the overall flow of the paper. Also, Figure 5 comes in the text before Figure 4 C-F and before Figure 4- supp1, so placing it in supplementary information would fix this issue.*

We have moved this figure to the supplement as Figure 3 – figure supplement 1.

> *(8) Merge Figures 6, 7, and 8 (or at least 7 and 8) to facilitate the comparison of data obtained with different proteins or conditions.*

We have merged Figures 7 and 8.

> *(9) Line 303: when referring to the chemical structure of sTCO-sulfo-Cy5, refer to Figure 4 Supp 1 and not Figure 9. Alternatively, consider moving Fig 9 to supplementary information or placing it earlier in the figure list.*

We now refer to the earlier supplemental figure when describing the structure of sTCO-sulfo-Cy5.

> *(10) Ensure proper referencing of Figure 4E in the text, particularly since it's vital to understanding the selection of mutation sites for the Insulin receptor, as discussed in lines 392-400.*

We have made this correction.

> *(11) Maintain citation consistency by verifying that all references cited in the text, including those in the Introduction, Results, and Discussion sections, are included in the References list at the end of the paper.*

We have reviewed all our citations for consistency.

> *The reviewer is also concerned by the lack of any statistical analyses, and of appropriate control experiments:*
>
> *(1) The trapping of PI3K at the plasma membrane, shown in Figure 3 supplementary 1, is not very convincing. It is unclear whether PI3K is trapped at the membrane, as claimed by the authors, or whether PI3K slowly accumulates at the membrane independently of the light stimulation. Indeed, the baseline fluorescence isn't flat to start with (especially in F-11 cells), and the change in fluorescence under 650 nm light is very modest, much weaker, in fact, than in control experiments without TRPV1 (Figure 2C). Do the authors*

> *observe a similar drift in fluorescence in absence of photostimulation at 650 nm? Such control experiment needs to be performed and discussed. More importantly, authors need to provide quantitative (and not just qualitative) measures of the changes in fluorescence observed in the different conditions, and run adequate statistical analyses to compare the different conditions (for all the figures of the manuscript where this applies).*

We can see that the language of "trapped at the membrane" is more of an interpretation than a description. We now describe this result as a lack of dissociation of PIF-iSH2 from the membrane in response to 750 nm light. We more clearly explain our interpretation and label it as speculative.

> *(2) Consider moving Figure 3 Supplementary 1 from supplementary information to the main document due to its importance. It seems like an important finding to me, and I believe also to the authors, who wrote a whole paragraph on PI3K trapping in the discussion section (lines 361-380).*

We agree that the results from this figure are important. To better align with the request of all reviewers to shorten the manuscript and reduce the number of figures in the main text, however, we have left the figure in the supplement.

> *(3) Figure 3: why is the increase in IP3 levels not reversible as in Figure 2? Is this because IP3 is detected only at the membrane level (TIRF experiment) and not the entire cell? Authors should comment on this aspect.*

As described in response to Comment#2, we now better explain our interpretation. Briefly, we speculate that the PIF-iSH2 that encounters TRPV1 in the plasma membrane binds to the ankyrin repeat domain of TRPV1 and, therefore, does not readily dissociate from membrane in response to 750 nm light.

> *(4) Figure 4E: Verify the functionality of the Insulin receptor mutants, as was done for TRPV1.*

We have added new experiments to demonstrate that the insulin receptor incorporating Tet3-Bu is functional. Because the insulin receptor is not electrogenic, we could not use electrophysiology to validate its function. Instead, we measured the insulin-dependent endocytosis of the receptor. These data are now presented in Figure 3 – figure supplement 2 and Figure 3 – supplemental movie.

> *(5) Figures 6 to 8: The authors quantify the change in plasma membrane expression of TRPV1 and insulin receptors after NGF treatment (or photoactivation), but an important control experiment is missing. They first label cells with sulfo-Cy5, then treat them with NGF (or photoactivate them with 650 nm light), and then label them again with sulfo-Cy5, supposedly to label only the TRPV1 receptors that newly arrived at the membrane. However, we have no evidence that the first sulfo-Cy5 labeling (1 uM, 5 min) was complete. In fact, labeling with sulfo-Cy5 (200 nM) in Figure 4 never reaches saturation, not even after 20 min. The authors need to control for this, by comparing the change in fluorescence with and without NGF treatment. The GFP control is simply not sufficient. Also, include Figure 8 in the text, as it is missing from the results section, and discuss the results in more detail. Indeed, the current data is appealing as it suggests that what was observed with TRPV1 is also true for the Insulin receptor, but without a proper control this could just be an artefact.*

We have performed several new control experiments to address the reviewer's concerns. (1) For NGF-induced increase in TRPV1 at the plasma membrane, we repeated the experiment using a vehicle instead of NGF. These data, added to Figure 4E, demonstrate that the increase in plasma membrane TRPV1 depends on NGF. (2) For the light-activated increase in plasma membrane TRPV1, we repeated the experiment using a second exposure to the deactivating 750 nm light instead of the activating 650 nm light and added the data as Figure 5, figure supplement 1A-E. These new data demonstrate that the increase in plasma membrane TRPV1 occurred only in response to the activating wavelength of light. (3) To address the same as the previous comment, but for the insulin receptor, we repeated the insulin receptor experiments also using a second exposure to the deactivating wavelength of light. These data are now shown in Figure 5, figure supplement 1F-I and demonstrate that the increase in the insulin receptor levels in the plasma membrane required the activating wavelength of light.

> *(6) Line 313: "Importantly, sTCO-sulfo-Cy5 did not appear to equilibrate across the cell membrane and did not label untransfected cells (i.e., those without GFP; Figure 4 - figure supplement 1)". I don't see where the absence of labeling of untransfected cells is shown. The authors should show fluorescence changes on the surface of both transfected and untransfected cells and, as discussed above, quantify the data and provide statistical analyses.*

See response to Reviewer #2, Comment #2.

> *Minor Comments:*
>
> *(1) Define « PM » and « RTK » in abstract We have made the requested changes.*
>
> *(2) Consider presenting the signaling pathways defined in the introduction in a scheme to improve readability.*

We have added the signaling pathways defined in the introduction to Figure 1A.

> *(3) In Figure 1A, include the CAAX lipidation signal in the schematic representation.*

We had already shown the lipidation itself, but we have added the lipidation signal as a magenta star, with its meaning explained in the figure legend. We hope the reviewer finds this useful.

> *(4) Terminology clarification: Given the broad readership of Elife, provide clearer explanations for terms and techniques used, such as the function of PIF (line 144).*

We define the acronym PIF in the text, but do not further elaborate on the biological function of PIF to align with other reviewers' requests that we reduce the review-type material in the manuscript.

> *(5) Correct "m-1s-1" to "M-1s-1" in line 119.*

This is now corrected.

> *(6) Replace "activate" with "activation" in line 122.*

This is now corrected.

> *(7) Indicate 650 nm and 750 nm next to the arrows in Figure 2B for reader clarity.*

We have added the requested arrow labels.

> *(8) Correct Figure 5A to Figure 4A in line 244.*

This is now corrected.

> *(9) Correct Figure 5B to Figure 5C in line 293.*

This is now corrected.

> *(10) In lines 274, 293, 312 and 329, clearly specify which panels of the referenced figures are being discussed to avoid confusion.*

We have now clearly specified which panels are being referenced.

> *(11) Figure 1B: it is unclear how long after 650 nm light switching the image is taken. The red bar indicating 650 nm light makes it look like the image is taken right after light switching, which would suggest that PIF-YFP trafficking to the membrane takes milliseconds in response to 650 nm light. However, the legend says that photoactivation kinetics are in the range of 10 seconds. Please accurately position the red bar in Figure 1B to reflect the time between light switching and imaging, and specify the time between light switching and imaging in the figure legend.*

We have more accurately shown the timing of image acquisition in what is now Figure 1, figure supplement 1.

> *(12) Please add a merged image for all the immune data figure.*

We are uncertain about which figures the reviewer is referring to. We do not have any immunohistochemistry in the manuscript.

> *(13) Line 205: "we found that expression of TRPV1 trapped PIF-iSH2 at the PM upon stimulation with 650 nm light, so that it no longer translocated to the cytoplasm in response to 750 nm light (Figure 3B and Figure 3 - figure supplement 1A)." This is shown in the supplementary figure but not in Figure 3B. Same issue with the following sentence.*

We have corrected the figure references in the text.

> *(14) For Figures 7 and 8, the authors state ""We next asked whether click chemistry labeling could be executed in cells in which we also used the PhyB/PIF machinery for activating PI3K." Is this really the main motivation for conducting these experiments?*

Good point. We have improved the writing around this issue.

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