

Molecular basis for G $\beta\gamma$ -mediated activation of phosphoinositide 3-kinase γ

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Abstract

The conversion of PIP₂ to PIP₃ by phosphoinositide 3-kinase γ (PI3K γ) is a critical step in neutrophil chemotaxis and is essential for metastasis in many types of cancer. PI3K γ is activated via directed interaction with G $\beta\gamma$ heterodimers released from cell-surface G protein-coupled receptors (GPCRs) responding to extracellular signals. To resolve how G $\beta\gamma$ activates PI3K γ , we determined cryo-EM reconstructions of PI3K γ –G $\beta\gamma$ complexes in the presence of various substrates/analogues, revealing two distinct G $\beta\gamma$ binding sites, one on the p110 γ helical domain and one on the C-terminal domain of the p101 subunit. Comparison of these complexes with structures of PI3K γ alone demonstrates conformational changes in the kinase domain upon G $\beta\gamma$ binding similar to those induced by Ras·GTP. Assays of variants perturbing the two G $\beta\gamma$ binding sites and interdomain contacts that change upon G $\beta\gamma$ binding suggest that G $\beta\gamma$ not only recruits the enzyme to membranes but also allosterically controls activity via both sites. Studies in a zebrafish model examining neutrophil migration are consistent with these results. These findings set the stage for future detailed investigation of G $\beta\gamma$ -mediated activation mechanisms in this enzyme family and will aid in developing drugs selective for PI3K γ .

Phosphoinositide (PI) 3-kinases (PI3Ks) are lipid kinases that phosphorylate PI lipids at the 3'-OH group to produce PI(3)P, PI(3,4)P2, and PI(3,4,5)P3. Although most are coupled to the activation of receptor tyrosine kinases (RTKs)¹, some are also regulated by G protein-coupled receptors (GPCRs). One such isoform is PI3K γ ² whose activity was first noted in activated neutrophils after formyl peptide stimulation (f-Met-Leu-Phe)³. The enzyme is primarily expressed in immune cells, where it converts PI(4,5)P2 (PIP2) to PI(3,4,5)P3 (PIP3) and triggers membrane recruitment of PIP3-dependent effectors such as P-Rex1/2 and AKT. These enzymes initiate downstream signaling pathways involved in actin remodeling, cell growth, proliferation, and cell migration^{4,5}. Like other Class I PI3Ks, PI3K γ consists of a catalytic subunit (p110 γ) and a regulatory subunit (p84 or p101). Lack of p84 expression in neutrophils results in reduced production of reactive oxide species (ROS), whereas lack of p101 significantly impairs chemoattraction and migration of leukocytes⁶. Aberrant expression and activation of PI3K γ has been linked to the progression of rheumatoid arthritis, atherosclerosis, cardiovascular diseases, lupus, and pulmonary fibrosis⁷. Increased expression of PI3K γ has also been observed in pancreatic and prostate cancer^{8,9}.

PI3K γ is stimulated by heterotrimeric G $\beta\gamma$ heterodimers and Ras-GTP, which are released after activation of GPCRs and RTKs¹⁰, respectively. However, beyond membrane recruitment, the molecular mechanisms underlying activation by G $\beta\gamma$ are unclear. The p110 γ catalytic subunit consists of an adaptor binding domain (ABD), a Ras-binding domain (RBD), a C2 domain, a helical domain (HD), and a kinase domain (**Fig. 1A**). At the outset of our study, the structure of p101 was unknown, but was recently shown to consist of three domains which we refer to as the N-terminal domain (NTD), central domain (CD), and C-terminal domain (CTD) (**Fig. 1A**)¹¹. Early studies strongly suggested that G $\beta\gamma$ binds to both p101 and p110 γ subunits^{12,13}, with the p101 domain interaction seeming to be more important for membrane recruitment of PI3K γ ¹³. The two binding sites were further localized using hydrogen-deuterium exchange mass spectrometry (HDX-MS), which pointed to the p110 γ HD and the p101 CTD being the two domains responsible for G $\beta\gamma$ interaction^{11,14}. However, ambiguities remain because changes in HDX-MS signals do not always register direct binding nor can they differentiate between direct binding events or consequent allosteric events. To clarify the regulation of PI3K γ by G $\beta\gamma$, we determined cryo-EM reconstructions of PI3K γ in complex with G $\beta\gamma$, confirming the existence of two discrete G $\beta\gamma$ binding sites. For comparison, we also determined a 3.0 Å structure of heterodimeric PI3K γ in its native conformation. G $\beta\gamma$ binding to the p110 γ subunit drives conformational changes that serve to open the kinase active site. Based on homology, we predict that PI3K β , another isoform regulated by G $\beta\gamma$ ¹⁵, will also engage and be regulated by G $\beta\gamma$ at the analogous site. G $\beta\gamma$ binding to the p101 CTD in our system seems dependent on G $\beta\gamma$ binding to p110 γ and promotes further opening of the kinase active site, consistent with the two binding sites being allosterically coupled. Because these structural changes occur in the absence of membrane, it supports the idea that G $\beta\gamma$ is important for not only membrane recruitment but also

conformational regulation independent of the membrane. Functional studies conducted *in vitro* and in a zebrafish animal model support these conclusions..

G $\beta\gamma$ subunits enage PI3K γ at two distinct binding sites

To capture snapshots of G $\beta\gamma$ activating PI3K γ , we purified functional *Sus scrofa* PI3K γ (p110 γ -p101) (**Extended Data Fig 1**) and incubated the protein with a soluble form of human G $\beta_1\gamma_2$ (G γ^{C68S}) and substrate analogs (ADP·BeF₃ and C₈-PIP₂, although only ADP was ultimately observed). These samples yielded four independent cryo-EM reconstructions at 3.5-3.9 Å resolution (**Extended Data Figs 2&3; Extended Data Table 1**): two distinct conformations (State1 and 2) of PI3K γ ·ADP in complex with one G $\beta\gamma$ heterodimer bound to the p110 γ helical domain (G $\beta\gamma^{HD}$) and another bound to the p101 CTD (G $\beta\gamma^{CTD}$) (**Extended Data Figs 4&5 and Fig 1B-D**), PI3K γ ·ADP in complex with G $\beta\gamma^{HD}$ (**Fig 2A-E**), and PI3K γ ·ADP alone (**Fig 2F**). We did not observe any species corresponding to PI3K γ bound to G $\beta\gamma^{CTD}$ alone, implying that G $\beta\gamma^{HD}$ is the higher affinity site in the absence of a membrane. The G $\beta\gamma^{HD}$ and G $\beta\gamma^{CTD}$ interfaces bury 1600 and 1400 Å², respectively. G $\beta\gamma^{HD}$ and G $\beta\gamma^{CTD}$ bind such that their prenylated G γ C-termini project towards a plane compatible with the membrane binding loops of the C2 and K domains. Thus State1 and 2 could both represent fully activated PI3K γ engaging a lipid bilayer (**Fig 1D**).

In the G $\beta\gamma^{HD}$ interface (**Fig 2B-D, Extended Data Fig 4D-F&5D-F**), residues commonly involved in other G $\beta\gamma$ effector interactions, including G β -Ser97, Trp99, and Leu117, engage residues on p110 γ α 13 (residues 568-578), in particular the side chains of Glu573, Leu574, and His577. In addition, G β -Met101, Asp186, Met188, Tyr145, Asp228, Asp246, Arg314, and Trp322 interact with residues at the N-terminus of α 12 (residues 548-560), notably Asn549, Gln550, and Leu551 (**Extended Data Fig 4D-F&5D-F**). The side chains of p110 γ -Arg552 and Lys553, which were previously predicted to contribute to the G $\beta\gamma^{HD}$ interface¹¹, are indirectly involved. The catalytic subunit with p110 γ -⁵⁵²DD⁵⁵³ double mutation, which had significantly reduced sensitivity to G $\beta\gamma$ activation, might have a disrupted interface due to introduction of two helix-destabilizing residues into α 12¹⁴. Based on sequence conservation¹⁶ and the crystal structure of PI3K β (PDB entry 2Y3A¹⁷), we predict that G $\beta\gamma$ could bind the analogous site in the PI3K β HD¹⁷, but not in PI3K α ¹⁸ or PI3K δ ¹⁹ (**Extended Data Fig 6**).

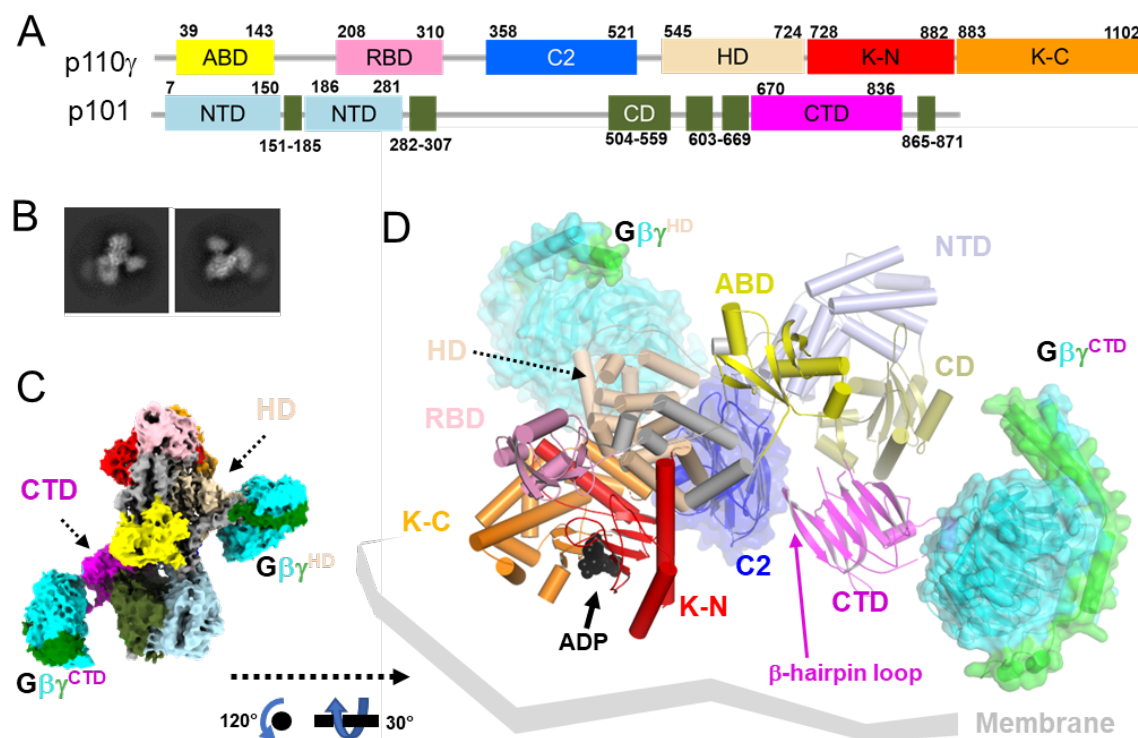


Figure 1. Cryo-EM reconstruction of the PI3K γ -ADP-G $\beta\gamma$ ^{HD}-G $\beta\gamma$ ^{CTD} complex. (A) Domain structure of the PI3K γ heterodimer. The p110 γ subunit is composed of an adaptor binding domain (ABD, yellow), Ras-binding domain (RBD, pink), C2 domain (blue), helical domain (HD, wheat), kinase domain N-lobe (K-N, red), and kinase domain C-lobe (K-C, orange). The p101 subunit is composed of an N-terminal domain (NTD, lightblue), a central domain (CD, deepolive), and C-terminal domain (CTD, magenta). (B) Representative 2D class averages, roughly corresponding to the views in panels C and D. (C) “Top-view” (membrane distal side) of the cryo-EM map of State2 highlighting the distinct G $\beta\gamma$ binding sites in p110 γ and p101 (G $\beta\gamma$ ^{HD} and G $\beta\gamma$ ^{CTD}, respectively). G β and G γ subunits are cyan and green, respectively. (D) The corresponding atomic model can be oriented with the geranylgeranyl sites of the two G $\beta\gamma$ subunits and the basic loops of the p110 γ C2 domain engaging a common membrane surface.

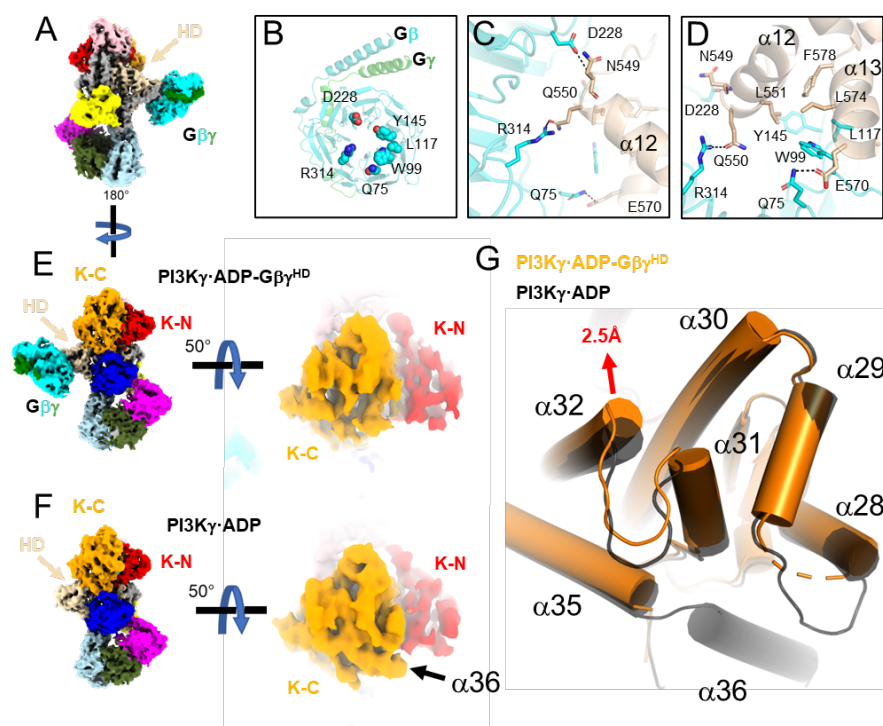


Figure 2. Release of the tryptophan lock and $\alpha 36$ upon $G\beta\gamma$ binding to the p110 γ HD. (A) Cryo-EM map of PI3K γ ·ADP- $G\beta\gamma$ ^{HD} with domains colored as in Fig. 1A. (B) $G\beta\gamma$ residues involved in the p110 γ helical domain (HD) interaction. (C-D) Residues in the $G\beta\gamma$ -HD interface, shown as sticks and colored according to their respective domains. (E-F) Comparison of the maps for PI3K γ ·ADP- $G\beta\gamma$ ^{HD} and PI3K γ ·ADP. Density for $\alpha 36$ is not observed in PI3K γ ·ADP- $G\beta\gamma$ ^{HD}. (G) Overlay of K-C in PI3K γ ·ADP- $G\beta\gamma$ ^{HD} (orange) and PI3K γ ·ADP (black). The movement of the $\alpha 32$ N-terminus is 2.5 Å at the C α of Leu1004.

Due to the lower quality of density corresponding to the p101 CTD (**Extended Data Figs 4G&5G**), AlphaFold2²⁰ was used to guide our modeling of the $G\beta\gamma$ ^{CTD} interface. $G\beta\gamma$ ^{CTD} binds to a flexible loop of the p101 CTD, which is disordered in structures without bound $G\beta\gamma$ ^{CTD}. The loop contains a short helix, modeled as residues 709-713, which binds to the “hotspot” of $G\beta\gamma$ ²¹. A $G\beta\gamma$ sequence previously implicated in $G\beta\gamma$ binding (⁷¹¹KRL⁷¹³) is contained within this element²². In our model, the PI3K γ -Lys711 sidechain electrostatically interacts with $G\beta$ -Asp246, and PI3K γ -Arg712 protrudes its sidechain into the center of the $G\beta$ WD40 domain to form hydrogen bonds with backbone carbonyls of Ser155 and Met188, and PI3K γ -Leu713 forms hydrophobic interactions with $G\beta$ -Trp99 and Met101 (**Extended Data Figs 4J-L&5J-L**). An extended ribbon formed by the $\beta 8$ and $\beta 9$ strands of the CTD may form a secondary contact with a hydrophobic patch on $G\beta\gamma$ ^{CTD} near residues Leu55 and Phe335, but it was not possible to model the density. This unusual loop contains conserved basic and a few conspicuously exposed hydrophobic sidechains (e.g. Phe789) that may form stabilizing interactions with the plasma membrane upon binding of $G\beta\gamma$ ^{CTD}. The basic electrostatic surface created by this loop is continuous with that of the membrane-proximal side of $G\beta\gamma$ ^{CTD}. This may help explain why in cells that the p101 site is more strongly recruited to the plasma membrane than p110 γ ¹³.

States 1 and 2 of the PI3K γ ·ADP–G $\beta\gamma$ ^{HD}–G $\beta\gamma$ ^{CTD} complex primarily differ in the orientation of their p101 CTDs relative to the rest of the protein. Their CTD domains are related by a $\sim 7^\circ$ rotation around the CD and a translation of ~ 3 Å. Consequently, the two G $\beta\gamma$ ^{CTD} domain centers translate ~ 15 Å. Notably, a $\beta 4$ – $\beta 5$ hairpin loop that extends from the central sheet of the p101 CTD domain and contacts the C2 domain (**Fig. 1D, 3C**), although involving a relatively small amount of surface area, is maintained in all the various structures/conformations we determined. This coupling may transmit membrane/G $\beta\gamma$ binding events and conformational changes occurring with the CTD with the membrane binding loops of the adjacent C2 domain and could be a route of overall allosteric control.

Conformational changes induced by G $\beta\gamma$ binding. For comparison with the G $\beta\gamma$ complex data sets, we generated a 3 Å reconstruction of ligand-free PI3K γ (**Extended Data Table 1, Fig 3, Extended Data Fig 7**). We also determined reconstructions of the PI3K γ ·ADP and PI3K γ ·ATP complexes at resolutions of 3.9 and 3.3 Å, respectively (**Extended Data Table 1, Extended Data Figs 2-3 and 8-9**). These structures are nearly identical to that of PI3K γ alone (RMSD of 0.54 Å for 1378 C α atoms comparing PI3K γ ·ADP to PI3K γ ; RMSD of 0.62 Å for 1285 C α atoms comparing PI3K γ ·ATP to PI3K γ ; RMSD of 0.65 Å for 1330 C α atoms comparing PI3K γ ·ADP to PI3K γ ·ATP), suggesting negligible conformational changes occur in PI3K γ upon binding nucleotides in the active site. Density for the K-C C-terminal helix $\alpha 36$ is observed in all these cryo-EM structures (**Fig 2F, Fig 3D, Extended Data Fig 9B**), consistent with ATP or ADP binding not disturbing the structure in the kinase C-lobe and/or releasing the tryptophan lock²³ (**Extended Data Fig 10**).

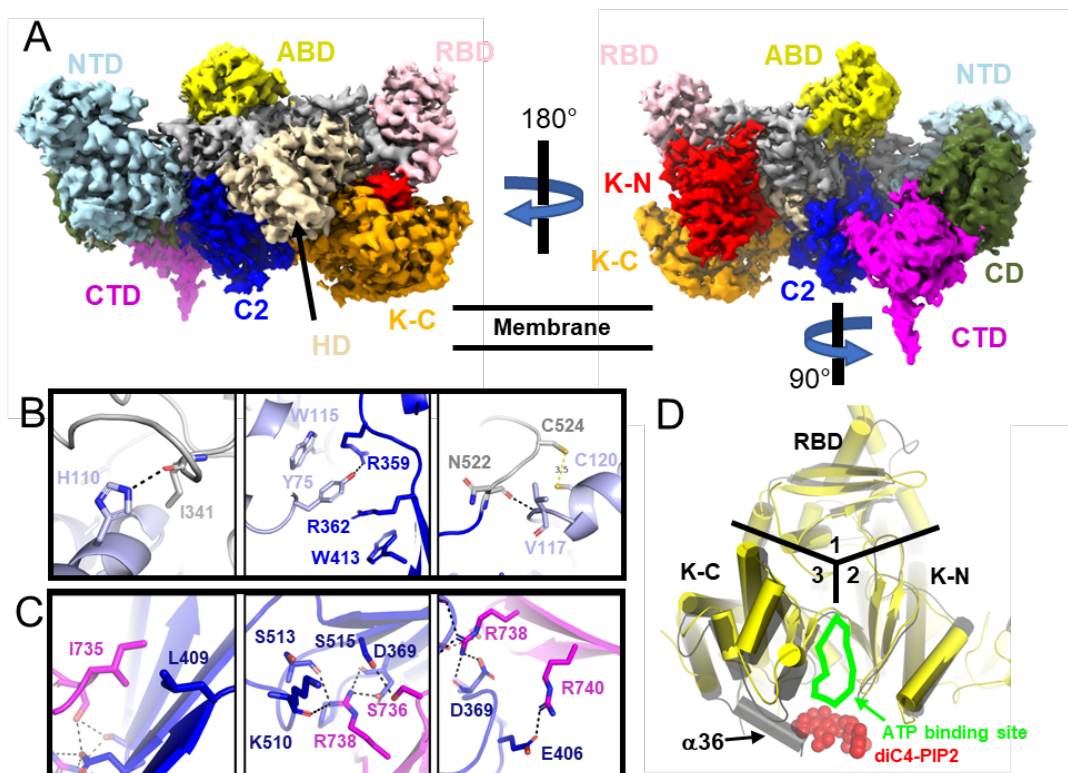


Figure 3. Cryo-EM structure of PI3K γ (A) Composite map (also see the **Extended Data Fig 2H**) colored as described in **Fig 1A**. The NTD (B) and a hairpin loop in CTD (C) of p101 form distinct contact sites with the C2 domain and its preceding linker in p110 γ . The side chains of interacting residues in the p110 γ RBD-C2 linker (grey), p110 γ C2 (blue), p101 NTD (light blue), and p101 CTD (magenta) are shown as sticks. Hydrogen bonds are shown as dashed lines. (D) Superimposition of PI3K γ with the crystal structure of PI3K α :diC4-PIP2. RBD, K-N, and K-C domains are located in areas 1, 2, and 3, respectively. The ATP binding site is indicated, and red spheres show the diC4-PIP2.

In the PI3K γ ·ADP–G $\beta\gamma$ ^{HD} structure, the linker between the C2 and HD (residues 522-545) is better ordered than in the PI3K γ structure. However, the C-terminal helix α 36 (1081-1092) in K-C becomes disordered relative to the PI3K γ ·ADP structure (**Fig 2E-F**). Displacement of α 36 has been proposed to require the release of the tryptophan lock, in which Trp1080 loses its contacts with Pro989 on α 31, Phe902 on the α 28- α 29 loop (residues 894-905), and Asp904 (**Extended Data Fig 10A**). In PI3K γ ·ADP–G $\beta\gamma$ ^{HD}, α 31 and α 32 shift, and the α 28- α 29 loop becomes disordered (**Fig 2G**, **Fig 4A-B**, **Extended Data Fig 10D**). Therefore, binding of G $\beta\gamma$ ^{HD} results in conformational changes in K-C, even in the absence of a lipid membrane or lipid substrates. The packing of a conserved residue, Leu564, in the α 12- α 13 loop of the HD is perturbed upon G $\beta\gamma$ ^{HD} binding (**Fig 4C**) such that it loses buried surface area with helices α 33 and α 34 of the K-C (**Fig 4C**), which connect to the “ α 31, α 32, α 35” bundle (**Extended Data Figs 10E-H**). Reorganization of this contact may be the allosteric conduit by which G $\beta\gamma$ ^{HD} binding opens the kinase active site. Intriguingly, we observed higher movement of helices α 28, α 29, α 30, α 31, α 32, α 35

in K-C upon binding of $G\beta\gamma^{CTD}$ in the State1 and 2 reconstructions (**Fig 4B**). This suggests that $G\beta\gamma^{HD}$ binding may only lead to partial kinase activation (**Fig 4B**).

Upon $G\beta\gamma^{CTD}$ binding, the p101 CTD changes position relative to all the other domains of $PI3K\gamma$, and it is also different between states 1 and 2 of $PI3K\gamma\cdot ADP\text{--}G\beta\gamma^{HD}\text{--}G\beta\gamma^{CTD}$. A conspicuous marker of this conformational variability is how the side chain of Ile689 residue packs against the $\alpha 14$ helix of CD (**Fig 4D**), suggesting that this residue is important for allosteric coupling. Residues 816-830 of p101-CTD form more extensive interactions with the CD in the $G\beta\gamma^{CTD}$ -bound complexes relative to $PI3K\gamma$ alone, consistent with previously observed HDX protection of this region upon $G\beta\gamma$ binding (Vadas, 2013).

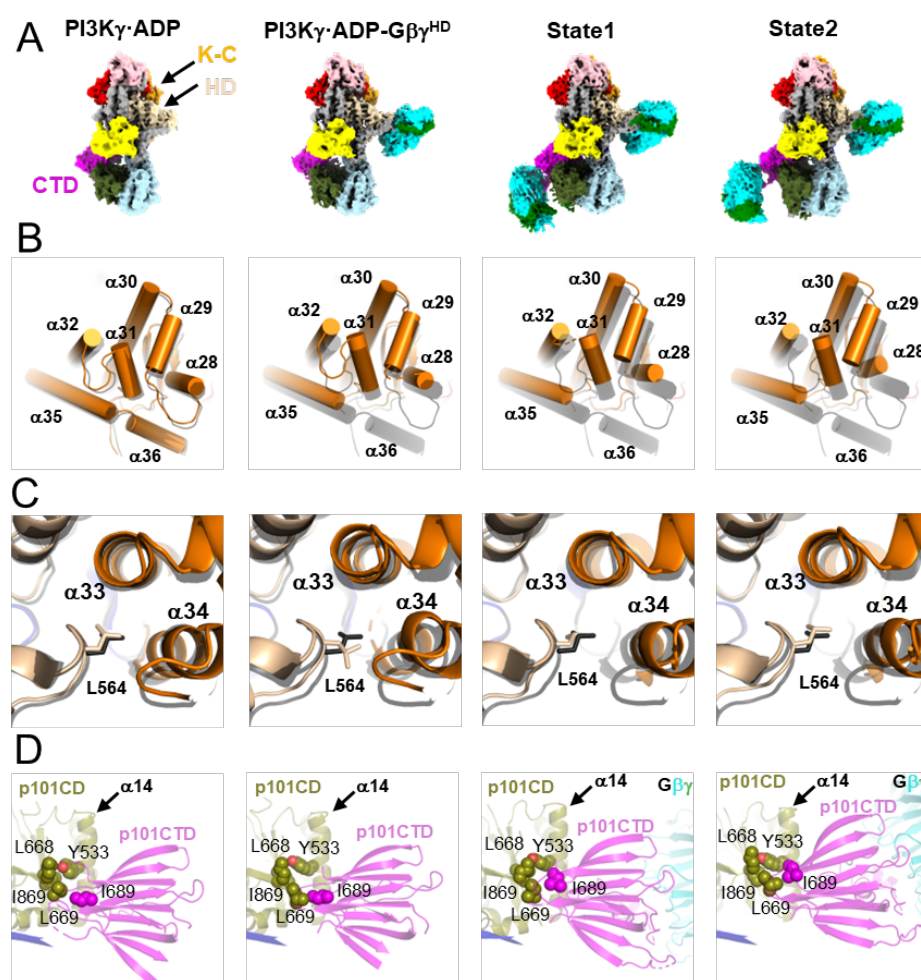
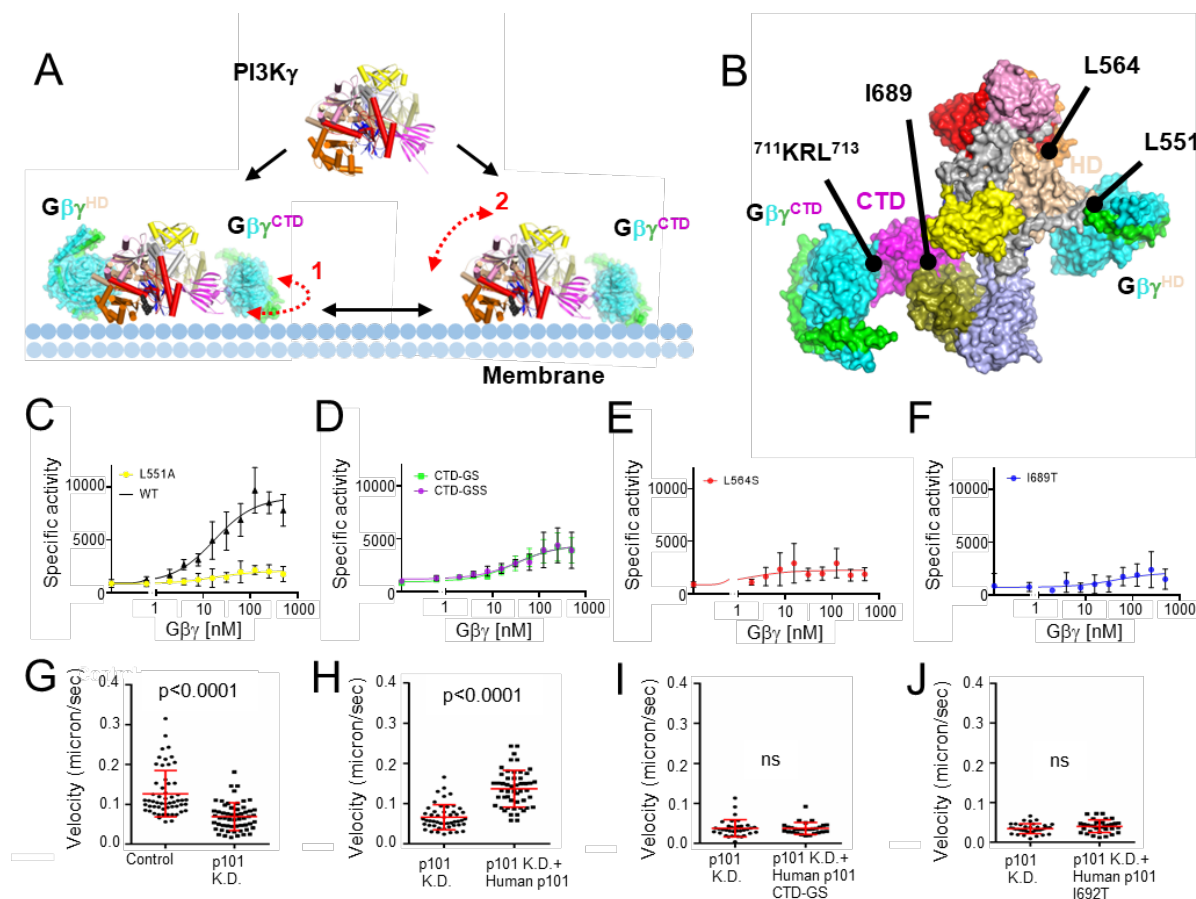


Figure 4. Structural changes in $PI3K\gamma$ as a function of $G\beta\gamma$ binding. (A) Cryo-EM maps of $PI3K\gamma\cdot ADP$, $PI3K\gamma\cdot ADP\text{--}G\beta\gamma^{HD}$, and States 1 and 2 of $PI3K\gamma\cdot ADP\text{--}G\beta\gamma^{HD}\text{--}G\beta\gamma^{CTD}$. (B) Comparison of helices in K-C. (C) Comparison of Leu564 sidechain packing. In (B-C), the structures of $PI3K\gamma\cdot ADP$, $PI3K\gamma\cdot ADP\text{--}G\beta\gamma^{HD}$, State 1 and 2 of $PI3K\gamma\cdot ADP\text{--}G\beta\gamma^{HD}\text{--}G\beta\gamma^{CTD}$ were individually superimposed with that of $PI3K\gamma$ (black). (D) Variability in the position of the p101 CTD. Interacting residues, Tyr533, Leu668, Leu669, and Ile869 in the p101 CD and Ile689 in the p101 CTD are shown as spheres.

Functional consequences of Gβγ binding.

Based on the structural results we hypothesized that the binding of Gβγ^{HD} or Gβγ^{CTD} allosterically triggers conformational changes in the K-C that facilitate catalysis (**Fig 4, Fig 5A**). To validate each observed binding site, we mutated key residues in each site: p110γ-L551A, p101-⁷¹¹Lys-Arg⁷¹² to ⁷¹¹Gly-Ser⁷¹² (CTD-GS), and p101-⁷¹¹Lys-Arg-Leu⁷¹³ to ⁷¹¹Gly-Ser-Ser⁷¹³ (CTD-GSS) (**Fig 5B**). We also generated L564S and I689T variants (**Fig 5B**) to probe the importance of allosteric transitions we observed to be mediated by the binding of Gβγ^{HD} and Gβγ^{CTD}, respectively. We then compared the specific enzyme activity of each variant to that of wild-type (WT) PI3Kγ at varying concentrations of Gβγ using liposome-based kinase assays (**Fig. 5C-F, Extended Data Table 3**). Disruption of either Gβγ binding site significantly impaired enzyme activity (**Fig. 5C-D**). The L564S mutation resulted in a total loss of Gβγ-mediated activation (**Fig. 5E**), consistent with it playing critical role in propagating signals from both Gβγ binding sites. This position is conserved as Leu547 in PI3Kβ (**Extended Data Fig 6B**). Significantly decreased activity in the dose-response curve for the p101^{I689T} mutant (**Fig 5F**) likely reflects less efficient coupling between the p101-CTD and CD (**Figs 1D&4D**).

To further examine regulatory functions of the Gβγ^{CTD} site of p101 on PI3Kγ activation, we created morpholino p101 knock-down zebrafish animals (**Fig 5G-J, Extended Data Fig 11, and Movies 1, 2**) and measured neutrophil motility either without (**Fig 5G**) or with the expression of WT (**Fig 5H**) or mutant human p101 proteins: human p101 CTD-GS (**Fig 5I**) and human p101 I692T (equivalent to I689T in *Sus scrofa*) (**Fig 5J**). WT human p101 (**Fig 5H**) rescued neutrophil motility and their resulting behavior was comparable to normal zebrafish neutrophils (**Fig 5G**). However, with the mutant human p101 variants neutrophil motility remained impaired (**Figs. 5H-J**). These results suggest that the p101 regulatory subunit not only functions to recruit PI3Kγ to the plasma membrane but also activates PI3Kγ allosterically because the I692T mutant should not be impaired in binding membrane-associated Gβγ (**Fig 4D**), yet still fails to rescue. We could not rescue p110γ knock-out zebrafish model animals with human WT p110γ, which prevented us from testing mutations at the Gβγ^{HD} site.



Discussion

HDX-MS and other biochemical studies have previously provided insights into possible $G\beta\gamma$ binding interfaces on $PI3K\gamma$ and regions that potentially undergo conformational changes, such as residues 579-607 in the p110 γ helical domain, helices $\alpha 31$ (989-995), $\alpha 35$ (1060-1078), and $\alpha 36$ (1085-1090) in the p110 γ K-C, and the p101 CTD. Because these same regions also showed perturbed HDX signals when $PI3K\gamma$ attaches to the liposome vesicles, a model of membrane-induced conformational change was proposed to explain activation by $G\beta\gamma$ ^{11,14}. Based on our data, we propose that in addition to any effects the lipid bilayer might have, soluble (unprenylated) $G\beta\gamma$ can itself trigger an allosteric transition involving p110 γ -Leu564, which disseminates the $G\beta\gamma$ -mediated activation signal through helices $\alpha 32$ and $\alpha 31$ to the $\alpha 28$ - $\alpha 29$ loop, leading to an opening of the tryptophan lock and release of $\alpha 36$ blocking the active site of the kinase domain (**Figs 2G, 4B, Extended Data Fig 10**). Comparing our cryo-EM structure of $PI3K\gamma$ and $PI3K\gamma$ -ATP with the crystal structure of $PI3K\alpha$ bound to diC4-PIP2 (PDB entry 4OVV), the PIP2 headgroup would clash with $\alpha 36$ in both $PI3K\gamma$ (**Fig 3D**) and $PI3K\gamma$ -ATP (**Extended Data Fig 9B**). Consistent with this idea, $\alpha 36$ was not ordered in the $PI3K\alpha$ crystal structure. The binding of $G\beta\gamma^{CTD}$ alters the interface between the p101-CTD and CD domains with the assistance of Ile689 and also likely pulls on the C2 domain via the $\beta 4$ - $\beta 5$ hairpin loop (**Fig 1D&4D**). Although the long-range allosteric pathway to the kinase domain is less clear, $G\beta\gamma^{CTD}$ binding leads to an even greater displacement of helices in the K-C in the $PI3K\gamma$ -ADP- $G\beta\gamma^{HD}$ - $G\beta\gamma^{CTD}$ complexes (**Fig 4D**). We have yet been unable to isolate $PI3K\gamma$ in complex with $G\beta\gamma^{CTD}$ alone, which suggests that either it is dependent on the binding of $G\beta\gamma^{HD}$ in our system, or, trivially, that it is too disordered for the processing programs to reconstruct in the absence of $G\beta\gamma^{HD}$. Because both the p110 γ -L564S and p101-I689T mutations individually nearly eradicate activation by $G\beta\gamma$, it implies these regions are involved in a shared allosteric network necessary for full activation. The residual activity of these variants in our assays could then reflect activation via membrane recruitment. Finally, because perturbation of the $G\beta\gamma^{CTD}$ site does not reduce activation to the level of p110 γ -L551A, it implies that the HD is the dominant site involved in allosteric regulation, a conclusion that has been suggested previously in cell based data¹³. In the crystal structure of p110 γ -H-Ras-GDPPNP (PDB entry 1HE8)²⁴, similar helical movements in K-C were observed (**Extended Data Fig 12A-D**), suggesting that both Ras and $G\beta\gamma$ binding can release the tryptophan lock and promote PIP2 binding by allosterically tuning the surrounding helices (**Fig 4B**).

In summary, we have described cryo-EM structures of $PI3K\gamma$ in various ligand/ $G\beta\gamma$ protein-bound states that provide new insights into mechanisms underlying $G\beta\gamma$ -mediated activation of $PI3K\gamma$ (**Fig 4 & Fig 6**), and which parallel those of the Ras binding to the RBD (**Fig 6 and Extended Data 12A-D**). There likely exist other paths for $PI3K\gamma$ activation as evidenced by cancer-related somatic mutations, such as E347K in the RBD-C2 linker (residues 311-357) found in pancreatic ductal carcinoma and large intestine

adenocarcinoma²⁵, and R472C in the C2 domain found in prostate cancer²⁵, which show enhanced G $\beta\gamma$ -mediated activation in *in vitro* assays¹¹.

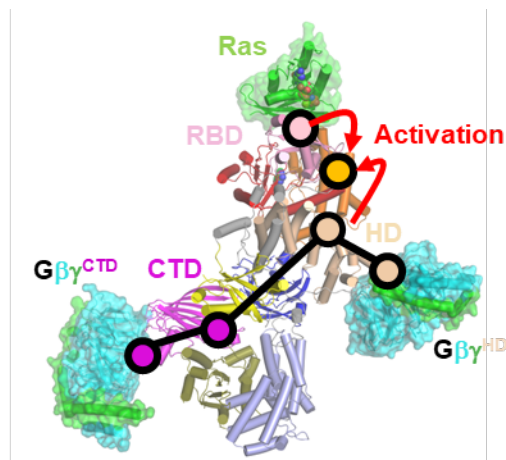


Figure 6. Proposed allosteric pathways in PI3K γ . Purple circles indicate domains proposed to be involved in allosteric activation by G $\beta\gamma$ ^{CTD}. Orange circles indicate key regions involved in allosteric activation by G $\beta\gamma$ ^{HD}. Based on our data, the two networks would seem to converge on the interface between the HD and K-C domain. The pink circle indicates the Ras binding interface with RBD, demarking the route for allosteric activation by Ras. The orange circle demarks the kinase active site. Note that the NTD and CD of p101 seem to form a rigid scaffold with the C2, HD, and K-N domains of p110 γ , providing a framework within which the p101 CTD and K-C domains exhibit dynamics essential for allosteric control of PIP3 production.

Methods

Expression constructs and purification of PI3K γ variants. Full-length p110 γ and p101 from *Sus scrofa* were inserted into the pFastBacTM Dual vector (Thermo Fisher) for use in the Bac-to-Bac Baculovirus Expression System (used without authentication or testing for mycoplasma). A His₆-tag was added to the N terminus of p110 γ , and a FLAG-tag to the C terminus of p101. Mutations in p110 γ and p101 were created using the overlap PCR technique (**Extended Data Table 2**), followed by restriction enzyme digestion and ligation (*EcoRI* and *XbaI* for p110 γ , and *NheI* and *KpnI* for p101). Sf9 insect cells were infected by baculovirus for each PI3K γ variant for two days and harvested (6,000xg for 15 minutes). For purification, freshly prepared or thawed cell pellets were resuspended in cold lysis buffer containing buffer A (20 mM HEPES pH 8.0, 100 mM NaCl) and 20 mM EDTA, 0.1 mM each of PMSF, leupeptin (10 μ M) and lima bean trypsin protease inhibitor (0.1 mg/ml), and the SIGMAFASTTM protease inhibitor cocktail (1 tablet in 100 ml buffer). The cells were resuspended and then lysed using an Avestin C3 emulsifier. The lysed cells were centrifuged in a Beckman JA-25.5 rotor at 30,000xg for 20 min. A 40 ml supernatant was then rocked with ~80 μ l anti-FLAG magnetic beads (pre-equilibrated with lysis buffer) for at least 2 hours. The anti-FLAG magnetic beads bound with proteins were washed with 10 ml lysis buffer for 5 times, followed by elution with 50 μ l buffer A with Flag peptide at a final concentration of 1 mM. We assessed the purity and the

quantity of PI3K γ proteins via SDS-PAGE. Freshly purified protein samples were used immediately for cryo-EM and biochemical studies.

Expression and Purification of WT ($G\beta\gamma^{WT}$) and soluble $G\beta\gamma$ ($sG\beta\gamma^{C68S}$). The WT human geranylgeranylated $G\beta_1\gamma_2$ protein ($G\beta\gamma^{WT}$) with an N-terminal His₆ tag on $G\beta$ was expressed using the Sf9 expression system and purified by Ni-NTA affinity chromatography as described previously⁴. Briefly, the cells were infected by baculovirus for two days and harvested (6,000xg for 15 minutes). The cell pellet was resuspended with Buffer A (20 mM HEPES pH 8, 100 mM NaCl, and 1 mM MgCl₂) and lysed by a high-pressure homogenizer (Emulsiflex C3). The cell debris was pelleted using centrifugation (30,000xg for 15 minutes). The membrane fraction in the supernatant was pelleted by ultracentrifugation (186,000xg for 40 mins), followed by solubilization with Buffer A supplemented with 1 % sodium cholate. After another run of ultracentrifugation, the supernatant containing $G\beta\gamma^{WT}$ was loaded onto Ni²⁺ resin (HisPurTM Ni-NTA resin, ThermoFisher) equilibrated with Buffer A. Impurities were removed from the resin during the wash step with 20 column volumes of Buffer B (20 mM HEPES pH 8, 100 mM NaCl, 1 mM MgCl₂, 10 mM CHAPS, and 10 mM imidazole pH 8), and the $G\beta\gamma$ protein was eluted with 5 column volumes of Buffer C (20 mM HEPES pH 8, 100 mM NaCl, 1 mM MgCl₂, 10 mM CHAPS, and 150 mM imidazole pH 8). The eluted protein was dialyzed against Buffer D (20 mM HEPES pH 8, 0.5 mM EDTA, 2 mM MgCl₂, 10 mM CHAPS, and 1 mM DTT) and loaded onto an anion exchange column (Q column). The column was first washed with Buffer D until the UV280 signal was stable. $G\beta\gamma^{WT}$ was eluted with a linear gradient to 30 % Buffer E (20 mM HEPES pH 8, 1 M NaCl, 0.5 mM EDTA, 2 mM MgCl₂, 10 mM CHAPS, and 1 mM DTT). The eluted fractions were analyzed by SDS-PAGE and further purified by size exclusion chromatography (SEC) using Buffer F (20 mM HEPES pH 8, 100 mM NaCl, 0.5 mM EDTA, 2 mM MgCl₂, 10 mM CHAPS, and 1 mM DTT). Peak fractions from SEC were pooled, fresh-frozen in liquid nitrogen, and stored at -80°C. The expression and purification steps for the soluble form of $G\beta\gamma$ ($sG\beta\gamma^{C68S}$) are similar to that for $G\beta\gamma^{WT}$, albeit the steps for collecting membrane fraction by ultracentrifugation were excluded, and no detergents (cholate, CHAPS) were added to the buffer solutions²⁶.

PI3K Assay. The ADP-Glo lipid kinase assay (Promega) was used to monitor ADP production, which corresponds to PIP3 production, following the manufacturer's protocol. Liposomes containing phosphatidylserine and PIP2 (3:1 ratio mol/mol) were purchased (Promega) and prepared to a final concentration of PIP2 at 25 μ M. A varied concentrations of $G\beta\gamma^{WT}$ (500, 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9, 1.95, 0.978 and 0 nM) were added to the liposomes, followed by addition of the WT or mutant PI3K γ proteins (1-10 nM) with ATP (final concentration of 100 μ M). Reactions were performed at room temperature for 40 minutes, followed by the addition of the ADP-Glo reagent for 40 minutes and the addition of the fluorescent substrate. The specific activity of each variant is summarized in **Extended Data Table 3** [N=8~14, 3 parameter curve fitting using GraphPad Prism 9].

Cryo-EM sample preparation and data collection and processing. Cryo-EM samples consisted of PI3K γ (0.05-0.2 mg/ml), PI3K γ supplemented with 2 mM of either ATP or ADP·BeF₃²⁷, 2 mM C₈-PIP₂, and sG β γ ^{C68S} (0.2 mg/ml)²⁸ were prepared for data collection. The UltrAuFoil gold grids (R1.2/1.3, 300 mesh) were glow-discharged (25 mA, 60 seconds) and soaked into 100 mM MgCl₂, followed by applying 3.5 μ l of protein samples onto the grids before plunge-freezing using the Vitrobot Mark IV system (Thermo Fisher Scientific)(blotting force=2, blotting time=3.5 seconds, wait time= 5 minutes). All datasets were collected using a Titan Krios cryo-electron microscope, and micrographs were recorded as movies on the Gatan K3 detector (nominal magnification: 81,000x, magnified pixel size: 0.54 Å in super-resolution mode). The total electron dose for data collection was ~ 56 e-/Å². Motion correction to align the movie frames for each of the data sets was performed using motioncor2 software²⁹ implemented within CryoSPARC. The CTF of each aligned micrograph was estimated using the CTFFind4 module of CryoSPARC. Particle picking, 2D classification, initial model generation, 3D classification, and 3D refinement were also performed in CryoSPARC. Each dataset was split into two independent sub-datasets and then subjected to individual data processing for map reconstructions and resolution estimation. The nominal resolution was determined based on a Fourier shell correlation cutoff of 0.143³⁰. Statistics for all datasets are summarized in **Extended Data Table 1**. For processing the data of PI3K γ supplemented with ADP·BeF₃ and sG β γ ^{C68S}, we used RELION 3.1³¹ for additional 3D classification during the process (**Extended Data Fig 2**). In the case of PI3K γ (**Extended Data Fig 7**), there was a well-resolved p110 γ density but relatively low-resolution (3.5~4.5Å) p101 density (**Extended Data Fig 7E**). The density in the N-terminal and C-terminal halves of p101 was improved by local refinement with different fulcrum points and varying sizes of mask applied (**Extended Data Fig 7F-G**). The locally refined maps were combined to generate a composite map (**Fig 3A, Extended Data Fig 7H-I**) (phenix_combine.focus.maps)³² for model building.

Structure modeling and refinement. Model building was performed using Coot³³, and alternated with real-space structure refinement using phenix.real_space_refine in Phenix³² with refinement strategies of global minimization (minimization_global) and B-factor refinement (adp). Because the p101 structure was unknown at the time the first maps were generated, the NTD and CD of the protein were built *ab initio* into locally refined maps. The advent of AlphaFold2 contributed to predicting the structure of p101, helped verify our modeling, and allowed us to build the p101 CTD density. During this time, the structure of PI3K γ (human p110 γ , pig p101) in complex with a nanobody (PI3K γ -Nb) that binds the p101 CTD became available and was also assessed for consistency (RMSD of 1.34 Å for 972 C α atoms in p110 γ ; and RMSD of 2.9 Å 501 C α atoms in p101). As indicated by the high RMSDs, the structures have significant differences, most notably in the registration of several secondary structure elements in the p101 NTD and CD, and in regions of the ABD of p110 γ , as well as in the topological modeling of the p101 CTD (**Extended Data Fig 13A-B**). The bound nanobody also seems to alter the orientation of the CTD¹¹. Thus, we used our base atomic model of PI3K γ as the seed for all the other

reconstructions. The initial models of the $G\beta\gamma^{HD}$ and $G\beta\gamma^{CTD}$ complexes were generated by AlphaFold multimer²⁰, and the resulting $G\beta\gamma$ -bound p110 γ helical domain and the p101 CTD were then merged with the rest of the PI3K γ model to set up MDFF procedures³⁴⁻³⁶. The grid scaling (gscale) value was set to 0.5. Suggested restraints regarding secondary structures, peptide bonds, and chirality also were applied during MDFF fitting^{35,36}. For State1 and 2 of PI3K γ -ADP- $G\beta\gamma^{HD}$ - $G\beta\gamma^{CTD}$, the MDFF simulation was performed for 300 picoseconds until convergence of the model RMSD³⁶ (**Extended Data Fig 14A&C**), followed by 3000 steps (3 picoseconds) of energy minimization. The quality of the MDFF fittings was estimated using the CCC generated by VMD³⁶ (**Extended Data Fig 14B&D**). All models were subjected to rounds of cryo-EM real-space refinement in Phenix³² alternating with manual adjustments using Coot. DAQ-score³⁷ was used to validate final models against the 3D reconstruction data, with an example shown in **Extended Data Fig 15**. Results are summarized in **Extended Data Table 1**.

Morpholino oligonucleotides (MO) microinjection. Splice morpholino for pik3r5-202 e2i2 (5'-TACAAACATTCTCACCTCTCTGGCT-3') was purchased from GeneTools, LLC, and resuspended in distilled water. Stocks are stored at RT with a concentration of 1 mM. For the knock-down experiments, one nanoliter of MO was injected into the yolk of 1-cell stage zebrafish embryos at 0.2 mM concentration. The human PIK3R5 sequence was cloned from Plasmid #70463, R777-E179 Hs.PIK3R5 (Addgene) into the *tol2* transposon system with *lyzC* promoter for neutrophil-specific expression³⁸. GFP with 2A peptide was inserted to generate the Tol2-*lyzC*-GFP-2A-PIK3R5 (p101) plasmid. For the following rescue experiments, microinjections of zebrafish embryos were performed by injecting 1 nL of a mixture containing 25 ng/ μ L plasmid and 35 ng/ μ L Tol2 transposase mRNA in an isotonic solution into the cytoplasm of embryos at the 1-cell stage. Mutations were generated using primers that bind to human PIK3R5 sequences: 5'-GATCTTCACCCACTCCTTGGAGCTGGGTCAC-3' and 5'-GAGTGGGTGAAGATCTCTGTCGTCTTCTCCCC-3' for the I692T mutation; 5'-GCAGCGGGTCGCTGGGCATCGATGGCGACC-3' and 5'-CCAGCGACCCGCTGCCAGGACCTGACGC-3' for the p101 CTD-GS mutation.

Live imaging. Larvae at 3 days post fertilization (dpf) were mounted with 1% low melting agarose and 0.02% tricaine on a glass-bottom dish, and imaging was performed at 28°C. Time-lapse fluorescence images in the head mesenchyme were acquired with 10x magnification on BioTek Lionheart FX Automated Microscope (Agilent). The green and red channels were acquired sequentially with 488-nm laser 561-nm laser, respectively. Videos of neutrophil motility were taken with stack slices less than 10 μ m. The fluorescent stacks were flattened using the maximum intensity projection and overlaid with each other for representation. MtrackJ in ImageJ was used for tracking neutrophil motility.

Data Availability. All data needed to evaluate the conclusions in the paper are presented in the paper and/or the Supplementary Materials. Additional data related to this paper are available upon reasonable request from the authors. The structures of PI3K γ , PI3K γ -ATP, PI3K γ -ADP, PI3K γ -ADP- $G\beta\gamma^{HD}$, State1 and 2 of PI3K γ -ADP- $G\beta\gamma^{HD}$ - $G\beta\gamma^{CTD}$, as well as

their associated cryo-EM reconstructions, have been deposited into the Protein Data Bank under accession codes 8SO9, 8SOA, 8SOB, 8SOC, 8SOD and 8SOE, and the Electron Microscopy Data Bank under accession codes EMD-40650, EMD-40651, EMD-40652, EMD-40653, EMD-40654, and EMD-40655, respectively.

Statistics. *In vitro* and *in vivo* assays were conducted with at least three technical replicates (n=3). The dose response curves were fitted to the *in vitro* lipid kinase assays with a three-parameter logistic nonlinear regression model implemented in GraphPad Prism 9. For the Zebrafish K.D. and rescue experiments, three independent experiments with n>20 are compiled. The p values represent overall effects using the Gehan–Breslow–Wilcoxon test.

Author Contributions

C.-L.C. and J.J.G.T. conceptualized the study. S.K.R. created the WT PI3K γ construct and C.-L.C. and Y.-C.Y. created the mutant PI3K γ constructs. C.-L.C. produced and purified WT and mutant PI3K γ proteins for conducting lipid kinase assays. Y.-C.Y. produced the WT G $\beta\gamma$ proteins. C.-L.C. and T.K. collected cryo-EM data, and C.-L.C. performed cryo-EM data analysis. C.-L.C. performed molecular dynamics flexible fitting. C.-L.C. and J.J.G.T. performed atomic modeling for all the cryo-EM reconstructions of PI3K γ without or bound with ligand, and G $\beta\gamma$. R.S and Q.D. conducted the morpholino knock-down and rescue experiments using the zebrafish model system. C.-L.C. and J.J.G.T. wrote the original draft, and all authors further edited the manuscript.

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