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## 1723 **Supplementary Information**

### 1724 **Supplementary Figures**

1725 **Figure S1 *pi5k*- cells display cytokinesis and development defects. (a-b)** Verification of  
1726 CRISPR-mediated disruption of PI5K. **(a)** Genomic PCR sequence analysis confirmed mutations  
1727 on the *PI5K* coding genes of *PI5K* mutant clone. Sequences of WT (Sbjct) and mutation clone  
1728 (Query) are presented. 2 bp deletions are highlighted in the green box, and induced stop Condon  
1729 TAA is highlighted with a red box. **(b)** Representative live-cell time-lapse confocal images (DIC)  
1730 of CRISPR-mediated *pi5k*- fan-shaped (left), and *pi5k*- oscillatory cells (right). **(c)** Images of DIC  
1731 channel and nuclear staining by Hoescht (merged) reveal a big increase in number of nuclei in  
1732 each cell in *pi5k*- cells. **(d)** Histogram quantification of normalized cell number at different nuclei  
1733 numbers for (*pi5k*-) PI5K and *pi5k*- after 65h in suspension. **(e)** WT (top) and *pi5k*<sup>-</sup> cells (bottom)  
1734 were plated on development buffer (DB) medium agar for starvation at 6h, 18h, and 24h. WT cells  
1735 aggregate normally, while *pi5k*- cells fail to aggregate. **(f-h)** Representative live-cell time-lapse  
1736 confocal images of *Dictyostelium pi5k*- oscillatory cells expressing PHcrac-RFP (biosensor for  
1737 PIP3) **(f)** or LimE-mCherry (biosensor for F-actin polymerization) **(g)** or CynA-GFP (biosensor for  
1738 PI(3,4)P2) **(h)**. Scale bars represent 5 mm.

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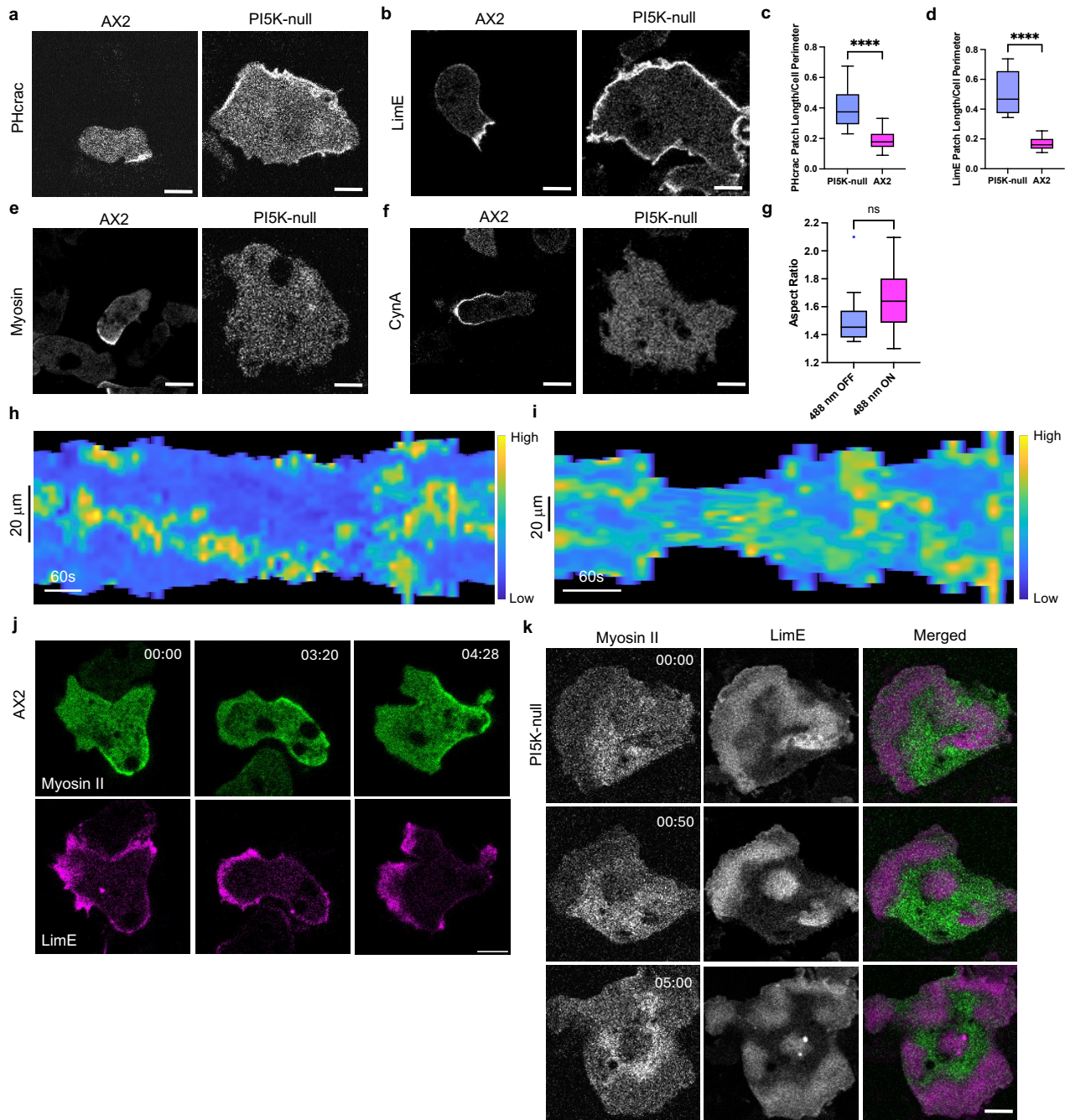
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1754 **Figure S2 *pi5k*- cells display less Myosin and PI(3,4)P2 activities (a-b)** Representative live-  
1755 cell time-lapse confocal images of *Dictyostelium* AX2 (WT) cells (left), and *pi5k*- cells (right)  
1756 expressing PHcrac-RFP (biosensor for PIP3) **(a)** or LimE-mCherry (biosensor for F-actin  
1757 polymerization) **(b)**. Scale bars represent 5  $\mu\text{m}$ . **(c-d)** Box-and-whisker plots of **(c)** PHcrac patch  
1758 length/Cell Perimeter, **(d)** LimE patch length/Cell Perimeter.  $n_c=22$  from at least 3 independent  
1759 experiments; asterisks indicate significant difference, \*\*\*\* $P \leq 0.0001$  (Mann-Whitney test.  
1760 Compare ranks). The median is at the center, and whiskers and outliers are graphed according  
1761 to Tukey's convention (GraphPad Prism 10). **(e-f)** Representative live-cell time-lapse confocal  
1762 images of *Dictyostelium* AX2 (WT) cells (left), and *pi5k*- cells (right) expressing Myosin II-GFP **(e)**  
1763 or CynA-GFP (biosensor for PI(3,4)P2) **(f)**. Scale bars represent 5  $\mu\text{m}$ . **(g)** Box-and-whisker plot  
1764 of aspect ratio corresponds to Figure 1o, 488 nm OFF or 488 nm ON.  $n_c=10$  from at least 3  
1765 independent experiments; 'ns' indicates non-significant difference, ns denotes  $P>0.05$  (Mann-  
1766 Whitney test. Compare ranks). The median is at the center, and whiskers and outliers are graphed  
1767 according to Tukey's convention (GraphPad Prism 10). **(h-i)** Representative membrane  
1768 kymograph of LimE intensity in AX2(WT) cells **(h)** and *pi5k*- cells **(i)** respectively. A linear color  
1769 map shows that blue has the lowest LimE or Myosin II intensity, whereas yellow has the highest.  
1770 **(j-k)** Representative live-cell time-lapse confocal images of *Dictyostelium* AX2 (WT) cells **(j)**, or  
1771 *pi5k*- cells **(k)** expressing Myosin II-GFP and LimE-mCherry (biosensor for F-actin  
1772 polymerization). Cells show ventral wave propagation in the substrate-attached surface of the cell  
1773 in **(k)**. Scale bars represent 5  $\mu\text{m}$ .

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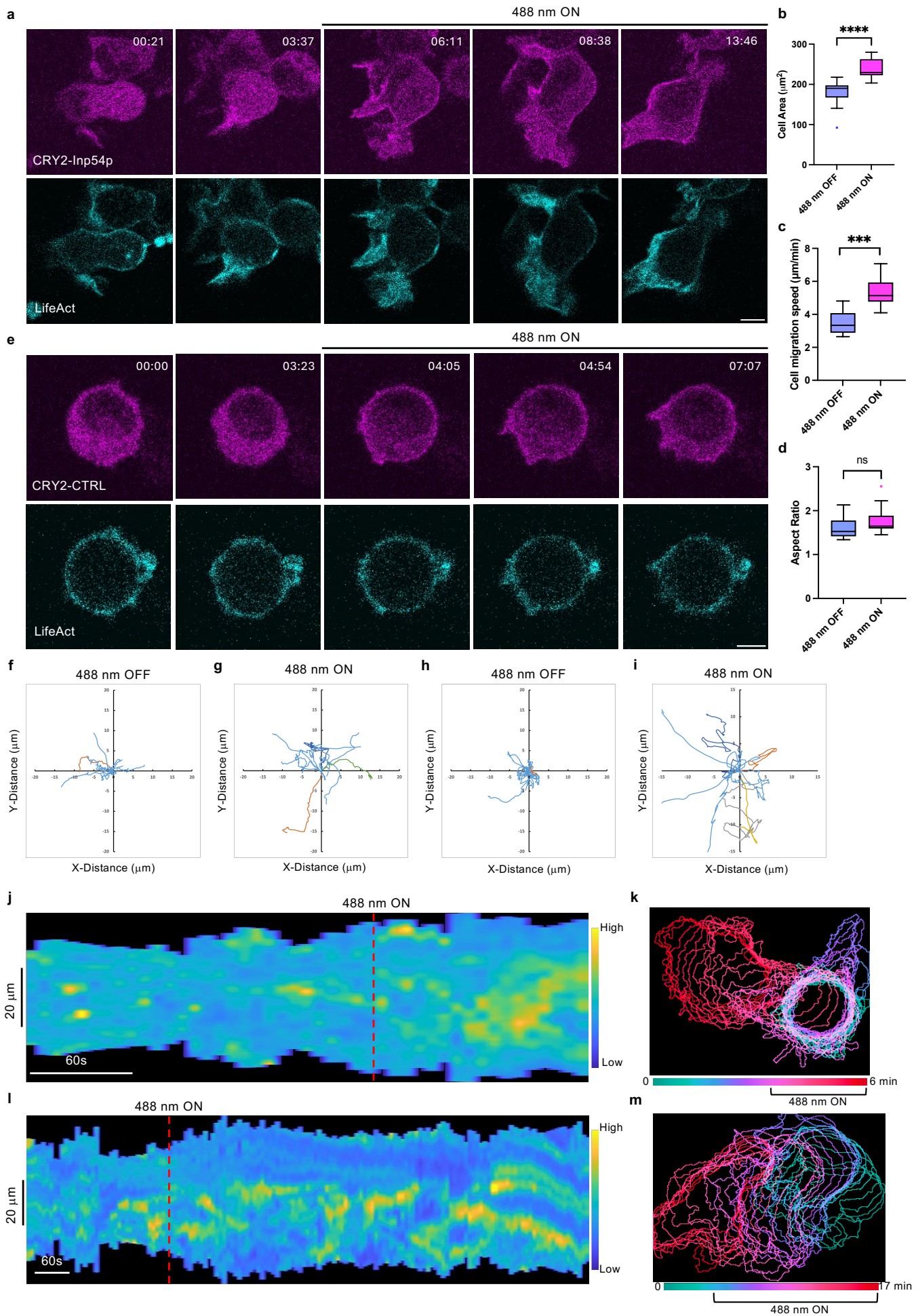
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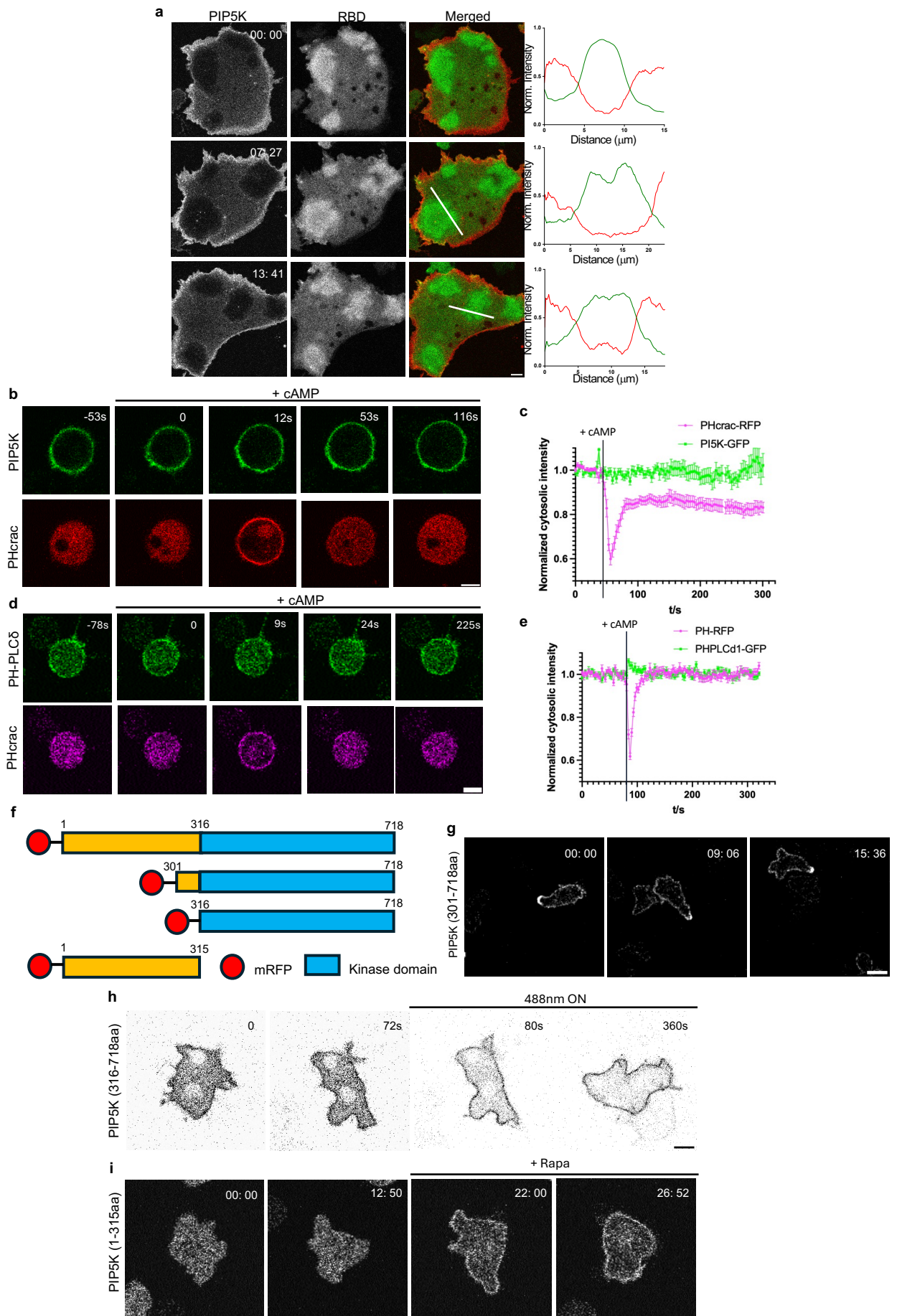




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**Figure S3 Globally recruiting Inp54p in neutrophil and macrophage induced fan-shaped phenotype** **(a)** Time-lapse confocal images of differentiated HL-60 macrophage expressing CRY2PHR-mCherry-Inp54p (magenta; upper panel) and LifeAct-miRFP703 (cyan; lower panel), before or after 488 nm laser was switched on globally. Time in min:sec format. Scale bars represent 5  $\mu$ m. **(b-d)** Box-and-whisker plots of **(b)** cell area, **(c)** cell migration speed, and **(d)** aspect ratio correspond to **(a)**, 488 nm OFF or 488 nm ON.  $n_c=10$  from at least 3 independent experiments; \*\*\*\* $P \leq 0.0001$ , 'ns' indicates non-significant difference, ns denotes  $P>0.05$  (Mann-Whitney test. Compare ranks). The median is at the center, and whiskers and outliers are graphed according to Tukey's convention (GraphPad Prism 10). **(e)** Time-lapse confocal images of differentiated HL-60 macrophage expressing CIBN-CAAX, empty vector CRY2PHR-mCherry-CTRL (magenta; upper panel) and LifeAct-miRFP703 (cyan; lower panel), before or after 488 nm laser was switched on globally. Time in min:sec format. Scale bars represent 5  $\mu$ m. **(f-i)** Centroid tracks of differentiated HL-60 neutrophils **(f-g)** or macrophage **(h-i)** ( $n_c=10$ ) showing random motility at 488 nm OFF **(f, h)**, or 488 nm ON **(g, i)**. Each track lasted at least 5 minutes and was reset to the same origin. **(j-k)** Representative kymograph of cortical LifeAct intensity in Inp54p-expressing neutrophil **(j)** or macrophage **(k)** before or after 488 nm laser was turned on. A linear color map shows that blue has the lowest LifeAct intensity, whereas yellow has the highest. Duration of the kymograph is 5 mins. **(l-m)** color-coded temporal overlay profiles of differentiated HL-60 neutrophil **(l)** and macrophage **(m)** expressing CRY2PHR-mCherry-Inp54p. Square brackets indicate the range of recruitment.

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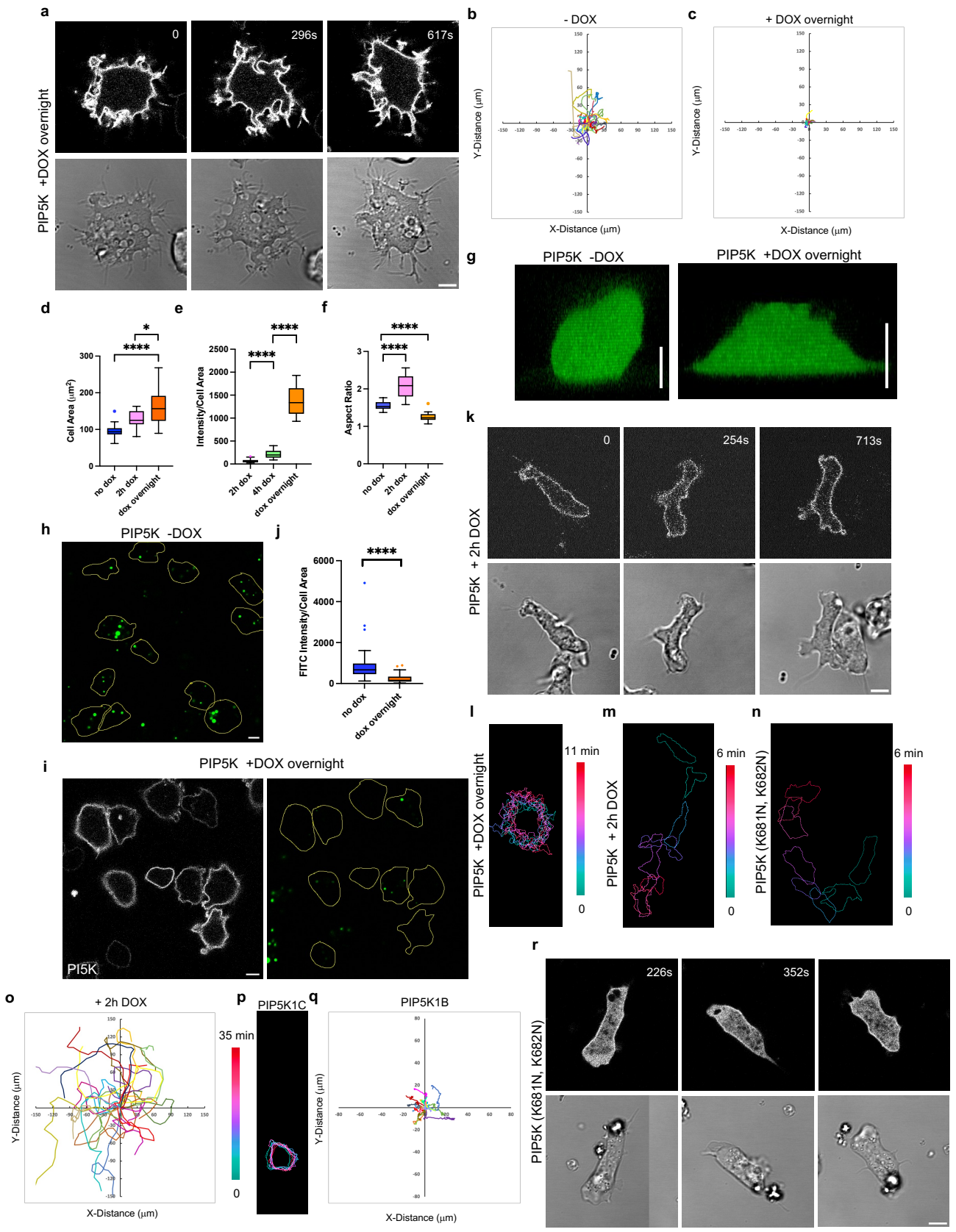


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**Figure S4 PI5K displays dynamic partitioning upon cAMP global stimulation**

**(a)** Representative live-cell time-lapse images of *Dictyostelium* cells coexpressing PI5K-mCherry and RBD-GFP during ventral wave propagation, showing PI5K dynamically localizes to the back-state regions in ventral waves. Line-scan intensity profiles are shown in the bottommost panels. Red line and green line represent PI5K and RBD, respectively. Time in min:sec format. Scale bars represent 5  $\mu\text{m}$ . **(b-e)** Representative live-cell images of *Dictyostelium* cells co-expressing PI5K-GFP and PHcrac-RFP **(b)** or PHPLC $\delta$ -GFP and PHcrac-RFP **(d)** upon global cAMP stimulation, demonstrating that upon transient global activation of cAR1 receptors, PHcrac gets uniformly recruited to membrane whereas PI5K and PHPLC $\delta$  remained steadily membrane-bound throughout the entire time course of the experiment. PHPLC $\delta$  had about 5% response. At time  $t=53\text{s}$  or  $78\text{s}$ , 1  $\mu\text{M}$  (final concentration) cAMP was added. Time series plot of normalized cytosolic intensities of PI5K and PHCrac **(c)** or PHPLC $\delta$  and PHcrac **(e)**, showing the kinetics of the response upon global stimulation with cAMP. In all these figures, vertical dashed lines are used to indicate the time of stimulation. Mean  $\pm$  SEM are shown for  $n_c=18$  cells. **(f)** Schematic representation of PI5K and the derived truncations. **(g)** Representative live-cell time-lapse images of *Dictyostelium* cells expressing PI5K-GFP (301-718aa) during migration showing PI5K (301-718aa) dynamically localizes at the trailing edge in migrating cells. Time in min:sec format. Scale bars represent 5  $\mu\text{m}$ . **(h)** Time-lapse confocal images of *Dictyostelium* cells expressing mRFPmars-SspBR73Q-PI5K(316-718aa), before or after 488 nm laser was switched on globally. Time in sec format. Scale bars represent 5  $\mu\text{m}$ . **(i)** Time-lapse confocal images of *Dictyostelium* cells expressing mCherry-FRB-PI5K(1-315aa), before or after 5  $\mu\text{M}$  Rapamycin was added. Time in min:sec format. Scale bars represent 5  $\mu\text{m}$ .





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**Figure S5 Expressing PI5K induces macropinocytosis defects** (a) Representative live-cell images of *Dictyostelium* cells expressing doxycycline-inducible PI5K with overnight DOX induction (spiky). Time in sec format. Scale bars represent 5  $\mu\text{m}$ . (b-c) Centroid tracks of cells ( $n_c=20$ ) showing random motility in cells expressing doxycycline-inducible PI5K without DOX induction (b) or with overnight DOX induction (c). Each track lasted at least 10 minutes and was reset to the same origin. (d-f) Box-and-whisker plots cell area (d), mean intensity (e), and aspect ratio (f).  $n_c=20$  from at least 3 independent experiments; asterisks indicate significant difference, \*\*\*\* $P \leq 0.0001$  (Mann-Whitney test. Compare ranks). The median is at the center, and whiskers and outliers are graphed according to Tukey's convention (GraphPad Prism 10). (g) Representative z-stack imaging showing the height of *Dictyostelium* cells expressing doxycycline-inducible PI5K without DOX induction (left) or with overnight DOX induction (right). Scale bars represent 5  $\mu\text{m}$ . (h-i) Representative confocal images of *Dictyostelium* cells without DOX induction (h) or with overnight DOX induction (i). Cells were treated with FITC-dextran (green) for 10mins before imaging. The yellow outline corresponds cell area. Scale bars represent 5  $\mu\text{m}$ . (j) Quantification of macropinocytosis uptake.  $n_c=58$  from at least 3 independent experiments; asterisks indicate significant difference, \*\*\*\* $P \leq 0.0001$  (Mann-Whitney test. Compare ranks). The median is at the center, and whiskers and outliers are graphed according to Tukey's convention (GraphPad Prism 10). (k, r) Representative live-cell images of *Dictyostelium* cells expressing doxycycline-inducible PI5K with 2h DOX induction (k) or PI5K (K681N, K682N) (r). Time in sec format. Scale bars represent 5  $\mu\text{m}$ . (l-n) Color-coded temporal overlay profiles of *Dictyostelium* cells expressing doxycycline-inducible PI5K with overnight DOX induction (spiky) (l), or doxycycline-inducible PI5K with 2h DOX induction (m), or PI5K (K681N, K682N) (n). (o) Centroid tracks of cells ( $n_c=20$ ) showing random motility in cells expressing doxycycline-inducible PI5K with 2h DOX induction. Each track lasted at least 10 minutes and was reset to the same origin. (p) Color-coded temporal overlay profiles of differentiated HL-60 neutrophils expressing PIP5K1C (rounded). (q) Centroid tracks of cells ( $n_c=15$ ) showing random motility in differentiated HL-60 neutrophils expressing PIP5K1B. Each track lasted at least 10 minutes and was reset to the same origin.

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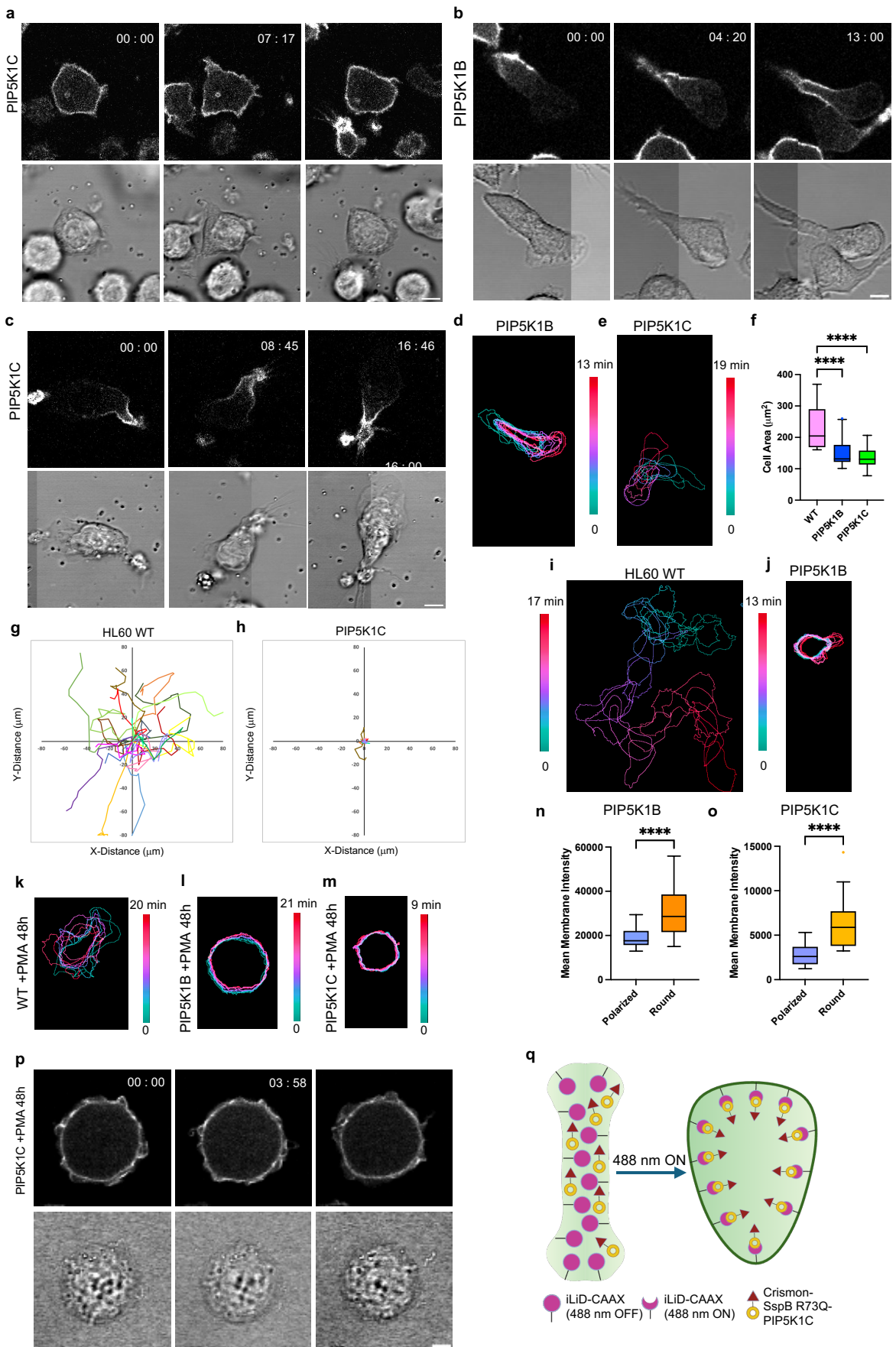
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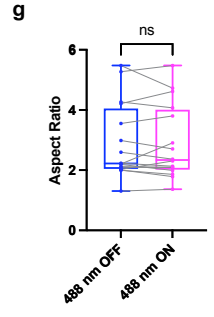
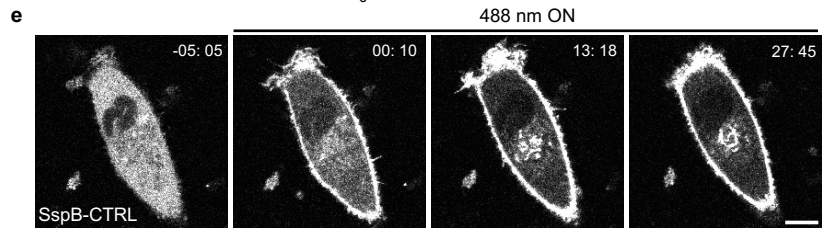
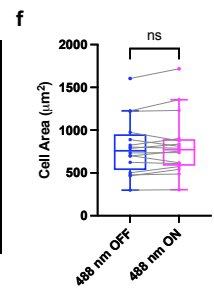
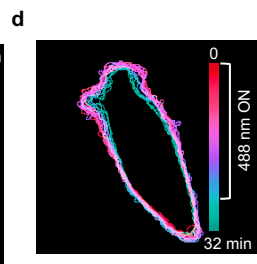
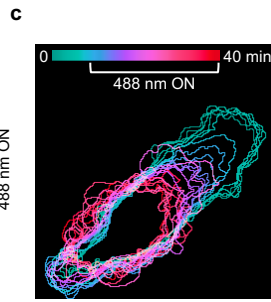
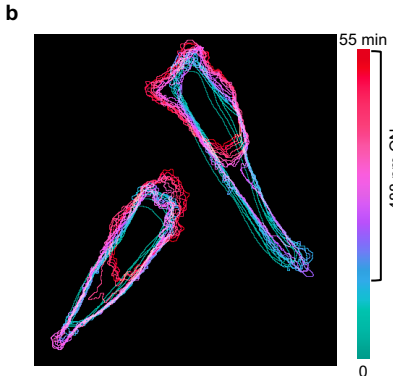
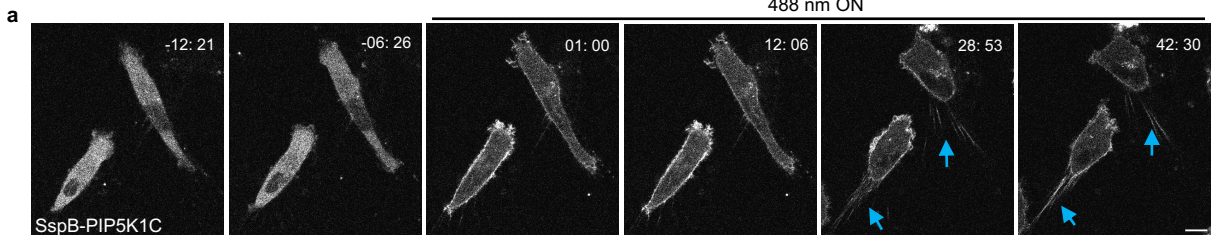
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**Figure S6 Expressing PIP5Ks impairs cell migration and induces cell contraction (a-c)**

Representative live-cell images of differentiated HL-60 neutrophils expressing PIP5K1C (rounded) **(a)**, PIP5K1C (polarized) **(b)**, or PIP5K1C (polarized) **(c)**. Time in min:sec format. Scale bars represent 5  $\mu\text{m}$ . **(d-e)** Color-coded temporal overlay profiles of differentiated HL-60 neutrophils expressing PIP5K1B (polarized) **(d)**, or PIP5K1C (polarized) **(e)**. **(f)** Box-and-whisker plots of cell migration speed.  $n_c=20$  from at least 3 independent experiments; asterisks indicate significant difference, \*\*\*\* $P \leq 0.0001$  (Mann-Whitney test. Compare ranks). The median is at the center, and whiskers and outliers are graphed according to Tukey's convention (GraphPad Prism 10). **(g-h)** Centroid tracks of cells ( $n_c=15$ ) showing random motility in differentiated HL-60 neutrophils WT **(g)**, or expressing PIP5K1C **(h)**. Each track lasted at least 10 minutes and was reset to the same origin. **(i-j)** Color-coded temporal overlay profiles of differentiated HL-60 neutrophils (WT) **(i)**, or expressing PIP5K1B **(j)**. **(k-m)** Color-coded temporal overlay profiles of differentiated HL-60 macrophages (WT) **(k)**, or expressing PIP5K1B **(l)**, or expressing PIP5K1C **(m)**. **(n-o)** Box-and-whisker plots of mean membrane intensity for differentiated HL-60 neutrophils expressing PIP5K1B **(n)** or PIP5K1C **(o)**.  $n_c=20$  from at least 3 independent experiments; asterisks indicate significant difference, \*\*\*\* $P \leq 0.0001$  (Mann-Whitney test. Compare ranks). The median is at the center, and whiskers and outliers are graphed according to Tukey's convention (GraphPad Prism 10). **(p)** Representative live-cell images of differentiated HL-60 macrophages expressing PIP5K1C. Time in min:sec format. Scale bars represent 5  $\mu\text{m}$ . **(q)** Cartoon illustrating mechanism of PIP5K1C global recruitment on MDA-MB-231 cell membrane with the help of iLiD-SspB optogenetic system.

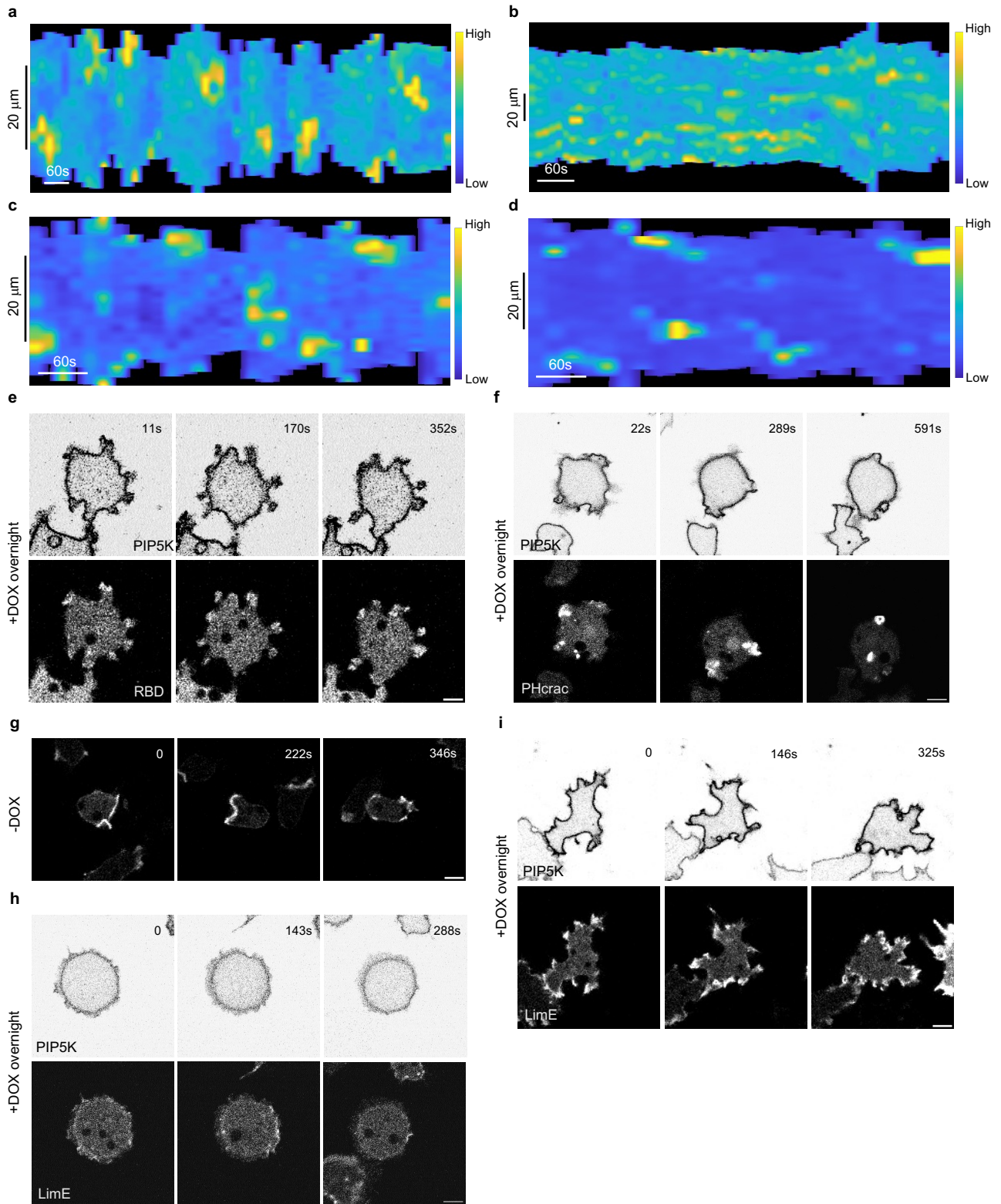


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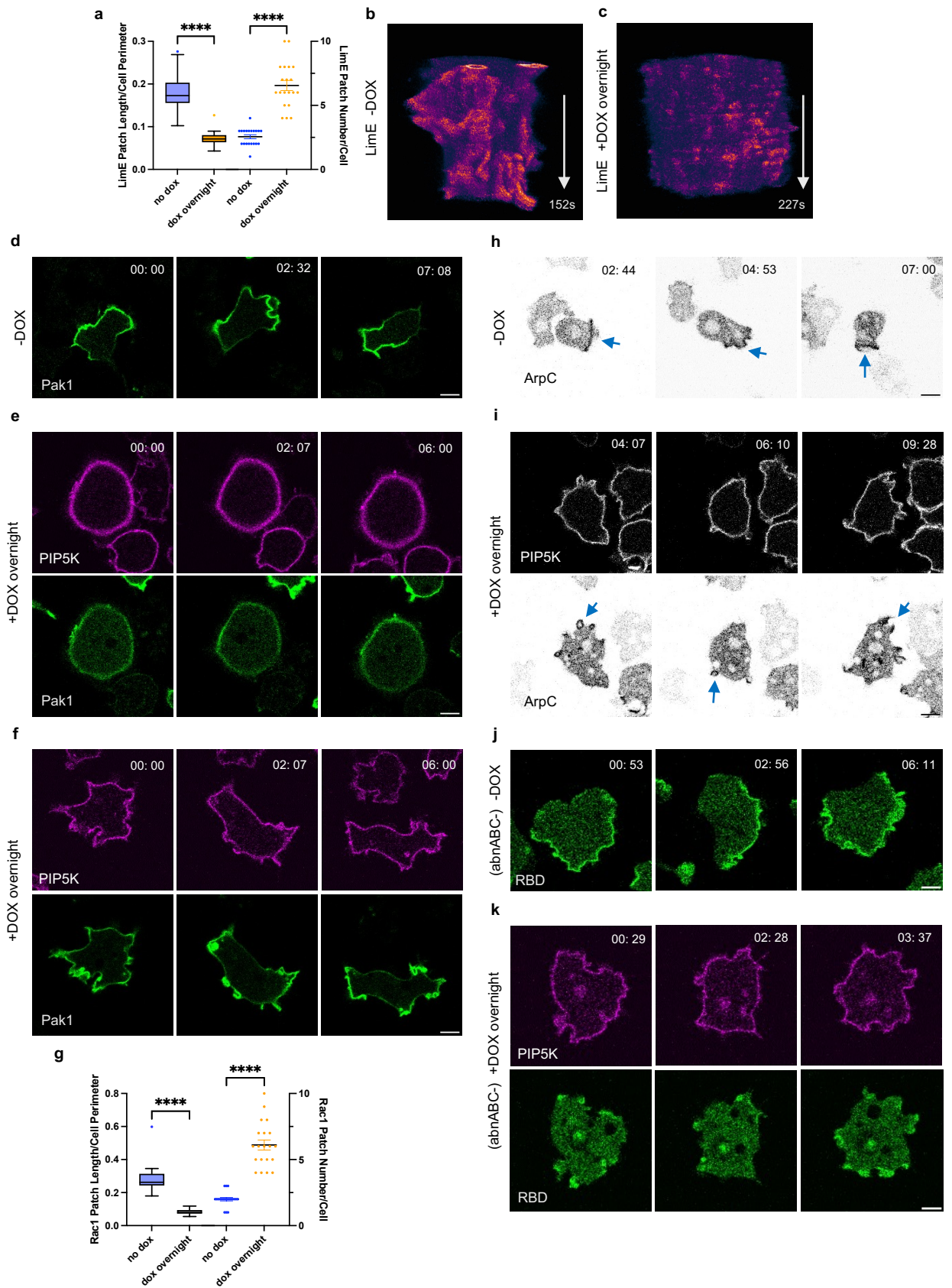
**Figure S7 Globally recruiting empty vector in MDA-MB-231 cells do not induce cell contraction** (a) Time-lapse confocal images of MDA-MB-231 cells expressing crimson-SspB-PIP5K1C-P2A-iLiD-CAAX, before or after 488 nm laser was switched on globally. Time in min:sec format. Scale bars represent 10  $\mu\text{m}$ . Blue arrows indicate where retraction fibers or blebs are formed. Cells are pretreated with 10 ng/ml EGF for 10 mins. (b-d) Color-coded temporal overlay profile corresponds to Figure S7a, 3k, and S7e. (e) Time-lapse confocal images of MDA-MB-231 cells expressing empty vector crimson-SspB-MCS-P2A-iLiD-CAAX, before or after 488 nm laser was switched on globally. Time in min:sec format. Scale bars represent 10  $\mu\text{m}$ . (f-g) Box-and-whisker plots of (f) cell area, (g) aspect ratio correspond to (m-n).  $n_c=10$  from at least 3 independent experiments; asterisks indicate significant difference, 'ns' indicates non-significant difference, ns denotes  $P>0.05$  (Mann-Whitney test. Compare ranks). The median is at the center, and whiskers and outliers are graphed according to Tukey's convention (GraphPad Prism 10).



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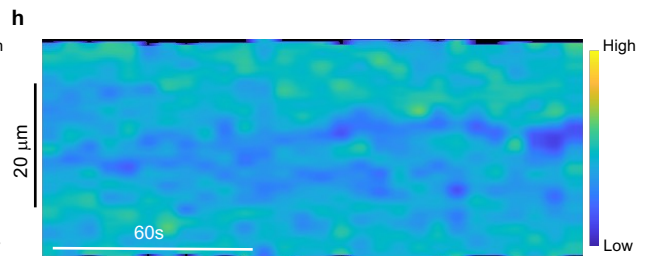
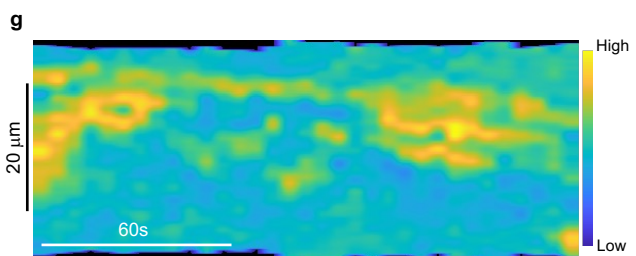
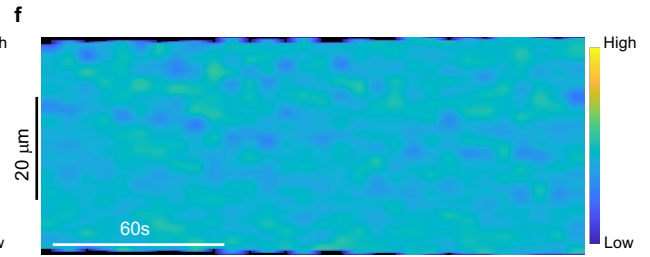
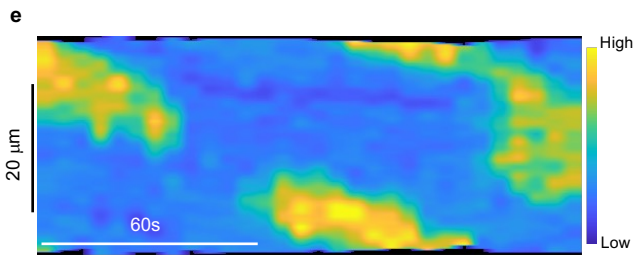
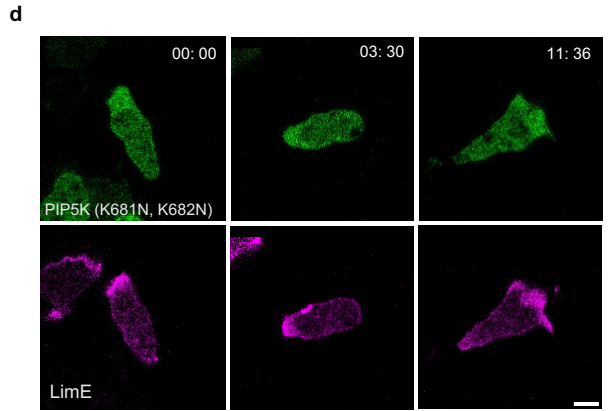
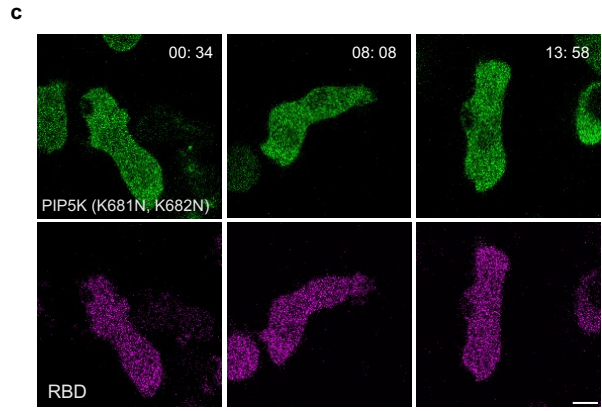
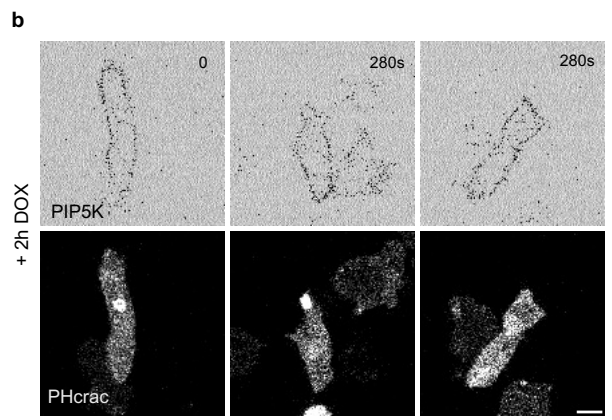
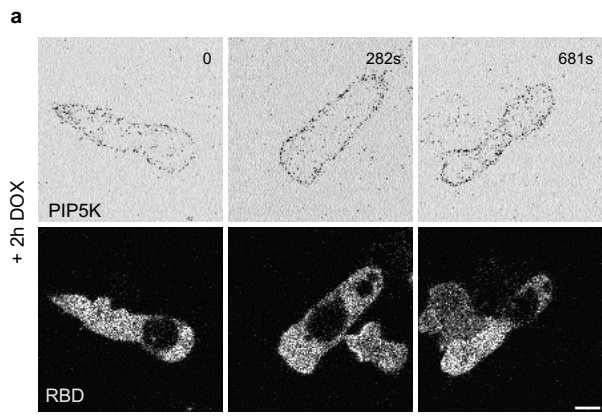
**Figure S8 Expressing PI5K can induce spiky filopods and suppresses signal transduction and cytoskeletal activities (a-b)** Representative membrane kymograph of RBD intensity in *Dictyostelium* AX2 expressing doxycycline-inducible PI5K without DOX induction (**a**) or with overnight DOX induction (spiky) (**b**). A linear color map shows that blue has the lowest RBD intensity, whereas yellow has the highest. (**c-d**) Representative membrane kymograph of PHcrac intensity in *Dictyostelium* AX2 expressing doxycycline-inducible PI5K without DOX induction (**c**) or with overnight DOX induction (spiky) (**d**). A linear color map shows that blue has the lowest RBD intensity, whereas yellow has the highest. (**e-f**) Representative live-cell time-lapse confocal images of *Dictyostelium* AX2 co-expressing doxycycline-inducible PI5K with overnight DOX induction (spiky) and RBD-GFP (biosensor for activated Ras) (**e**) or PHcrac-YFP (biosensor for PIP3) (**f**). Time in sec format. Scale bars represent 5  $\mu\text{m}$ . (**g-i**) Representative live-cell time-lapse confocal images of *Dictyostelium* AX2 co-expressing LimE-mCherry (biosensor for F-actin) and doxycycline-inducible PI5K without DOX induction (**g**) or with overnight DOX induction (rounded) (**h**), or with overnight DOX induction (spiky) (**i**). Time in sec format. Scale bars represent 5  $\mu\text{m}$ .





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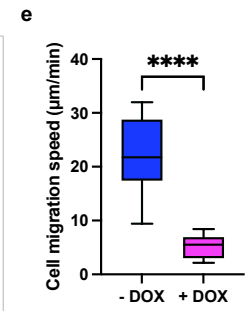
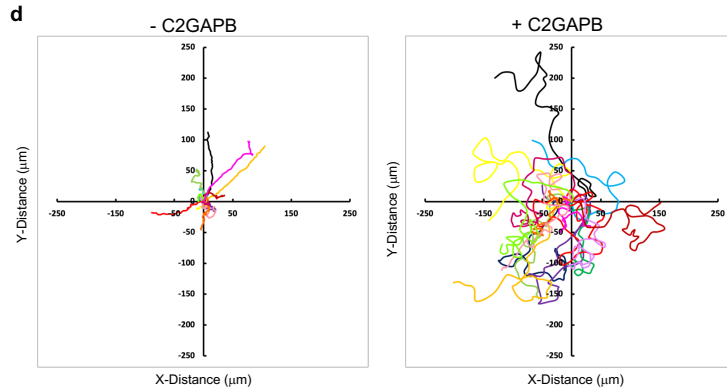
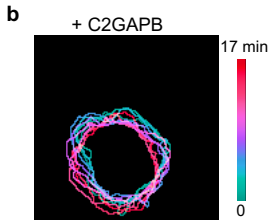
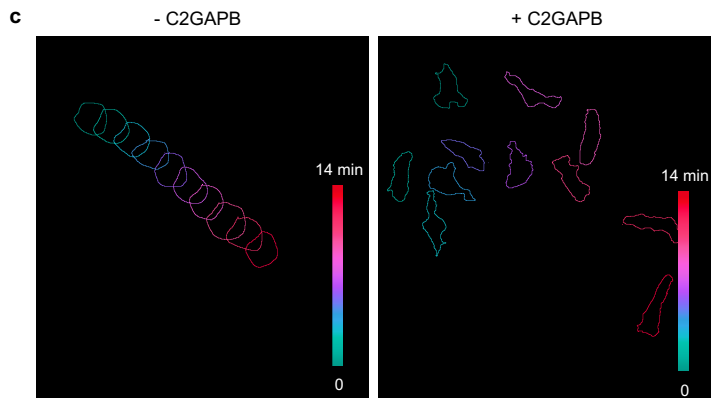
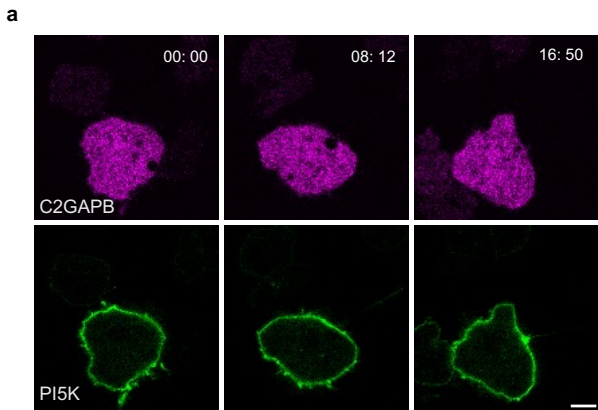
**Figure S9 Expressing PI5K inhibits Rac1/Arp2/3 complex/F-actin axis** **(a)** Box-and-whisker plots of LimE patch size (left axis) and LimE patch number (right axis).  $n_c=20$  from at least 3 independent experiments; asterisks indicate significant difference, \*\*\*\* $P \leq 0.0001$  (Mann-Whitney test. Compare ranks). The median is at the center, and whiskers and outliers are graphed according to Tukey's convention (GraphPad Prism 10). **(b-c)** *t*-stacks from a cell co-expressing LimE-mCherry and doxycycline-inducible PI5K without DOX induction **(b)** or with overnight DOX induction (rounded) **(c)**. The white arrow corresponds to the time duration of the *t*-stack kymograph. **(d-f)** Representative live-cell time-lapse confocal images of *Dictyostelium* AX2 co-expressing Pak1-GFP (biosensor for Rac1) and doxycycline-inducible PI5K without DOX induction **(d)** or with overnight DOX induction (rounded) **(e)**, or with overnight DOX induction (spiky) **(f)**. Time in min:sec format. Scale bars represent 5  $\mu\text{m}$ . **(g)** Box-and-whisker plots of Pak1 patch size (left axis) and Pak1 patch number (right axis).  $n_c=20$  from at least 3 independent experiments; asterisks indicate significant difference, \*\*\*\* $P \leq 0.0001$  (Mann-Whitney test. Compare ranks). The median is at the center, and whiskers and outliers are graphed according to Tukey's convention (GraphPad Prism 10). **(h-i)** Representative live-cell time-lapse confocal images of *Dictyostelium* AX2 co-expressing ArpC-GFP and doxycycline-inducible PI5K without DOX induction **(h)** or with overnight DOX induction **(i)**. Time in min:sec format. Scale bars represent 5  $\mu\text{m}$ . **(j-k)** Representative live-cell time-lapse confocal images of *Dictyostelium* abnABC- cells co-expressing RBD-GFP and doxycycline-inducible PI5K without DOX induction **(j)** or with overnight DOX induction **(k)**. Time in min:sec format. Scale bars represent 5  $\mu\text{m}$ .



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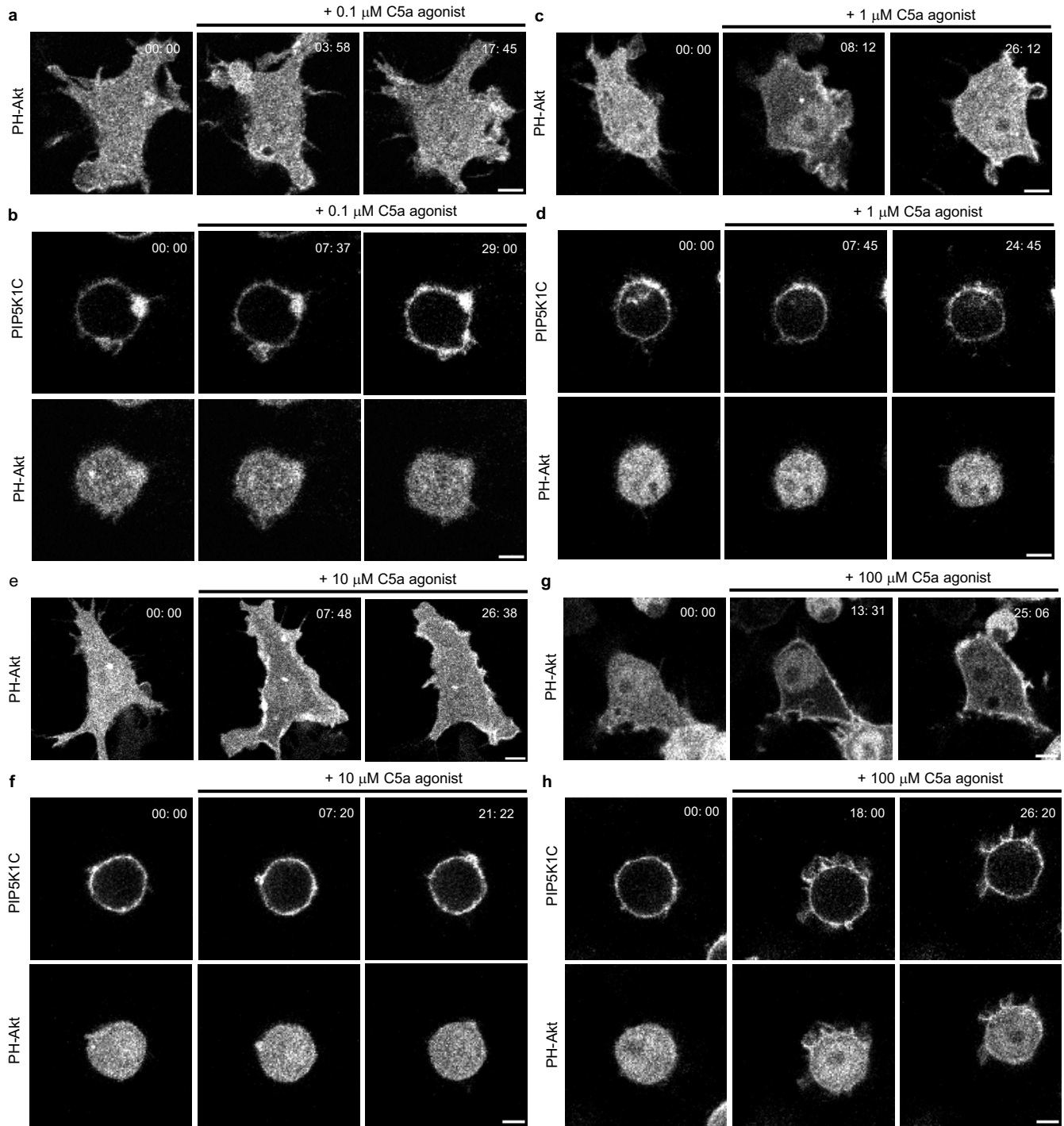
**Figure S10 Expressing less PI5K increases cell polarity but inhibits signal transduction activities (a-b)** Representative live-cell time-lapse confocal images of *Dictyostelium* AX2 co-expressing RBD-GFP **(a)** or PHcrac-YFP **(b)** and doxycycline-inducible PI5K with 2h DOX induction. Time in min:sec format. Scale bars represent 5  $\mu$ m. **(c-d)** Representative live-cell time-lapse confocal images of *Dictyostelium* AX2 co-expressing RBD-RFP **(c)** or LimE-RFP **(d)** and PI5K (K681N, K682N). Time in min:sec format. Scale bars represent 5  $\mu$ m. **(e-f)** Representative membrane kymograph of RBD intensity in *Dictyostelium* AX2 expressing doxycycline-inducible PI5K without DOX induction **(e)** or with overnight DOX induction **(f)**, even in the absence of actin cytoskeleton. Cells were pre-treated with actin polymerization inhibitor Latrunculin A (final concentration 5 $\mu$ M) and caffeine (final concentration 4mM) for 20min. A linear color map shows that blue has the lowest RBD intensity, whereas yellow has the highest. **(g-h)** Representative membrane kymograph of PHcrac intensity in *Dictyostelium* AX2 expressing doxycycline-inducible PI5K without DOX induction **(g)** or with overnight DOX induction **(h)**, even in the absence of actin cytoskeleton. Cells were pre-treated with actin polymerization inhibitor Latrunculin A (final concentration 5 $\mu$ M) and caffeine (final concentration 4mM) for 20min. A linear color map shows that blue has the lowest PHcrac intensity, whereas yellow has the highest.





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**Figure S11 Expressing C2GAPB in *pi5k*- cell increases cell polarity and migration (a)** Representative live-cell time-lapse confocal images of *Dictyostelium* AX2 co-expressing doxycycline-inducible PI5K C2GAPB and doxycycline-inducible PI5K with overnight DOX induction. Time in min:sec format. Scale bars represent 5  $\mu\text{m}$ . **(b)** Color-coded temporal overlay profile of the cell corresponds to **(a)**. **(c)** Color-coded temporal overlay profiles of *Dictyostelium pi5k*- cells expressing doxycycline-inducible C2GAPB without DOX induction (left) or with overnight DOX induction (right). **(d)** Centroid tracks of cells ( $n_c=16$ ) showing random motility in *pi5k*- cells expressing doxycycline-inducible C2GAPB without DOX induction (left) or with overnight DOX induction (right). Each track lasted at least 10 minutes and was reset to the same origin. **(e)** Box-and-whisker plot of cell migration speed corresponds to (c).  $n_c=16$  from at least 3 independent experiments; asterisks indicate significant difference, \*\*\*\* $P \leq 0.0001$  (Mann-Whitney test. Compare ranks). The median is at the center, and whiskers and outliers are graphed according to Tukey's convention (GraphPad Prism 10).



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2108 **Figure S12 Expressing PIP5Ks in RAW 264.7 cells increases the threshold for PI3K**  
2109 **activation (a-h)** Representative live-cell time-lapse confocal images of responses of PH-Akt-  
2110 mCherry to global simulation C5aR agonist at 0.1  $\mu\text{M}$  in RAW 264.7 WT cells **(a)** or cells  
2111 overexpressing PIP5K1C **(b)**; or at 1  $\mu\text{M}$  in RAW 264.7 WT cells **(c)** or cells overexpressing  
2112 PIP5K1C **(d)**; or at 10  $\mu\text{M}$  in RAW 264.7 WT cells **(e)** or cells overexpressing PIP5K1C **(f)**; or at  
2113 100  $\mu\text{M}$  in RAW 264.7 WT cells **(g)** or cells overexpressing PIP5K1C **(h)**. Time in min:sec format.  
2114 Scale bars represent 5  $\mu\text{m}$ .

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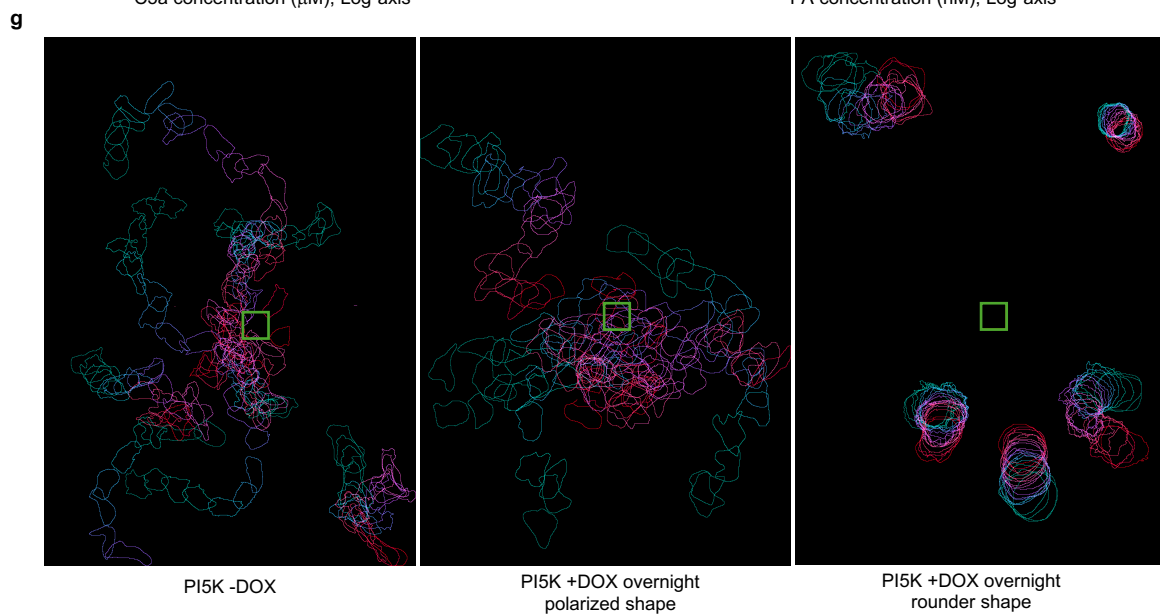
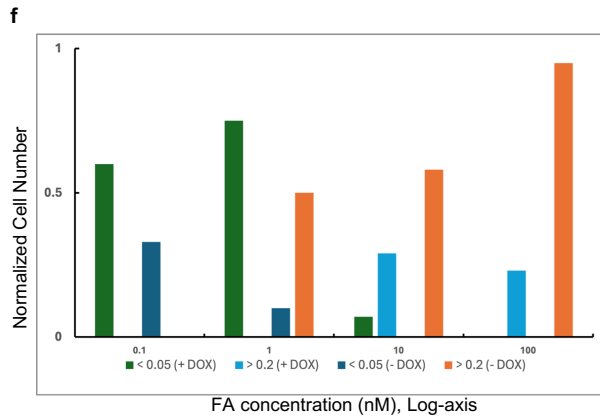
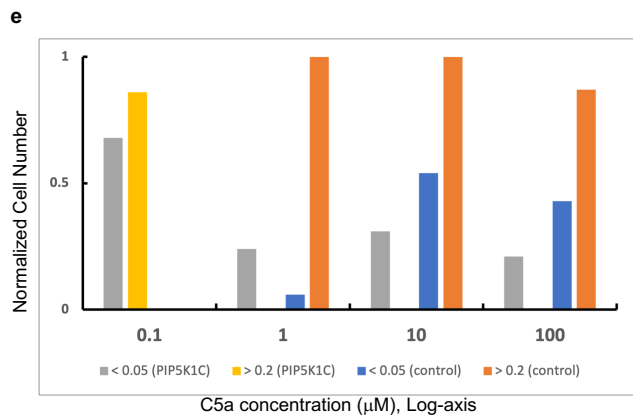
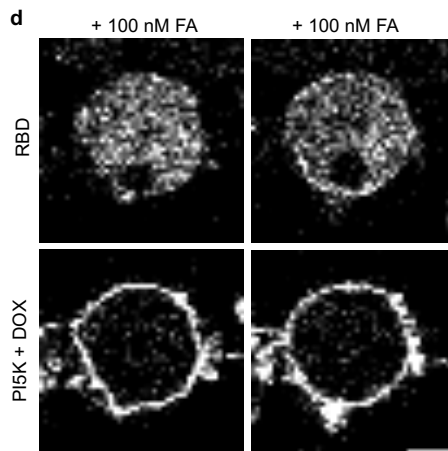
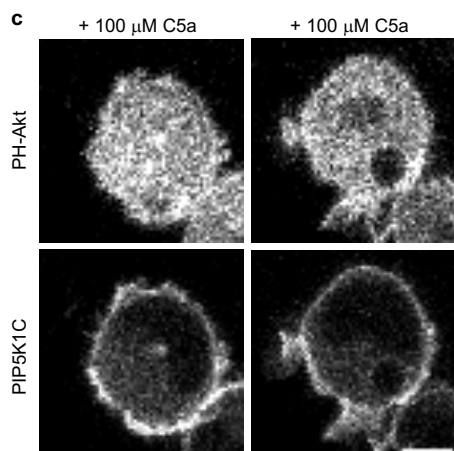
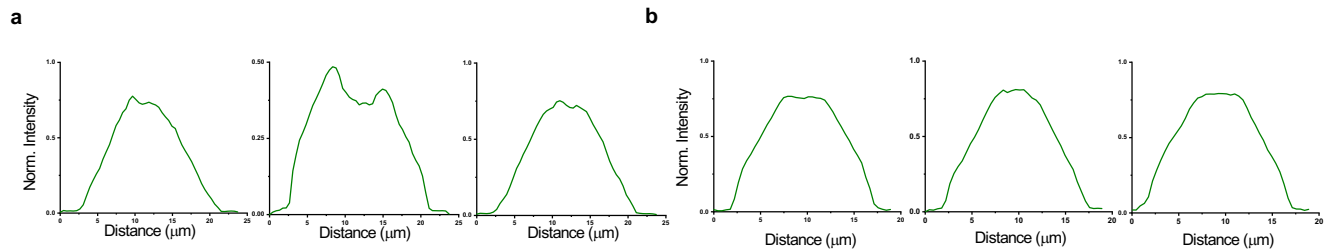
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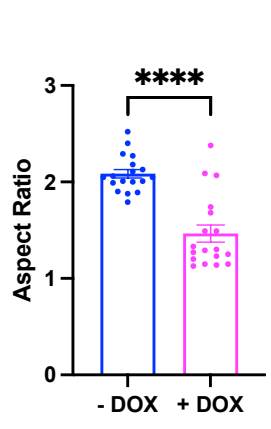
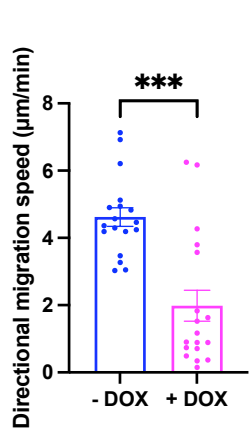
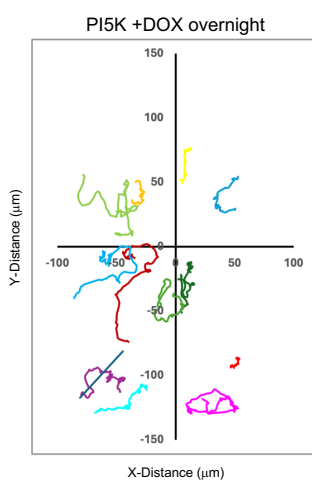
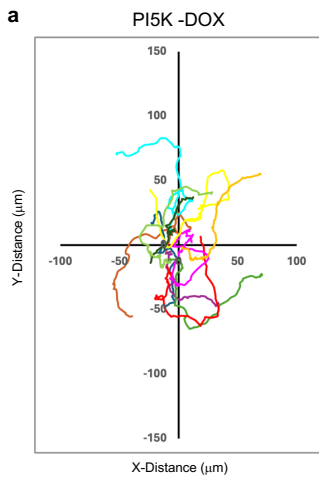
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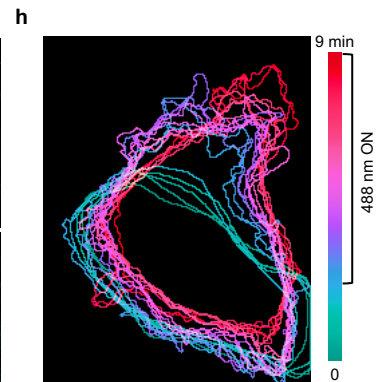
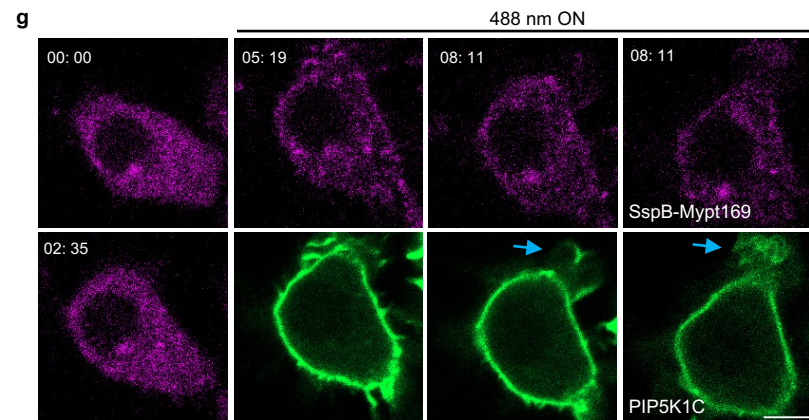
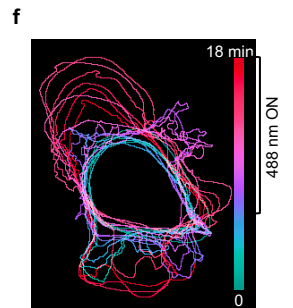
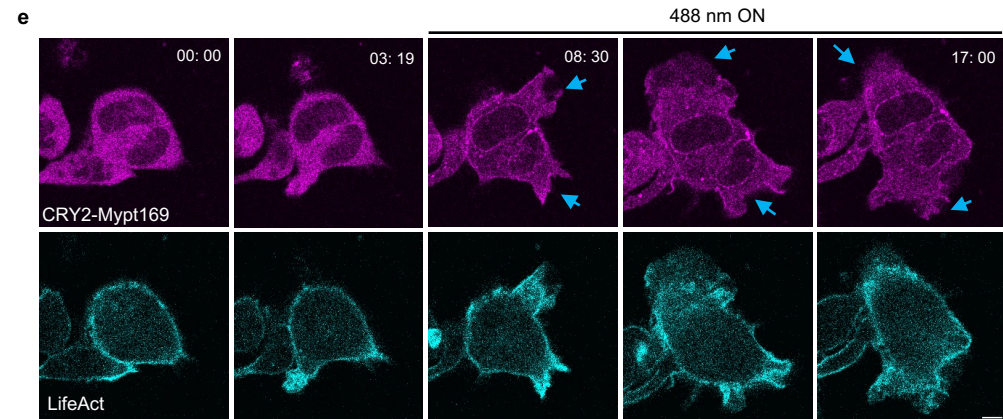
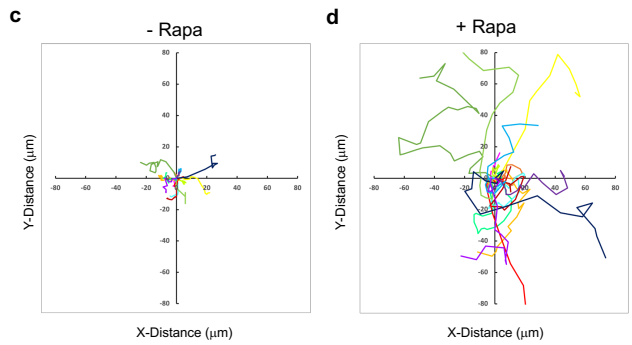
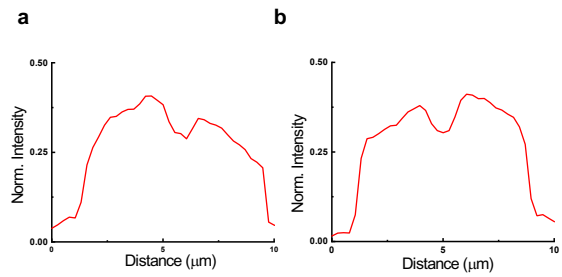
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**Figure S13 Expressing PI5K induces chemotaxis defects (a-b)** Line-scan intensity profiles correspond to Figure 5d-e. **(c)** Representative live-cell time-lapse confocal images of 5% (left) or 20% (right) responses of PH-Akt-mCherry to global simulation C5a agonist at 100  $\mu$ M in RAW 264.7 cells overexpressing PIP5K1C. **(d)** Representative live-cell time-lapse confocal images of 5% (left) or 20% (right) responses of RBD-GFP to global simulation folic acid (FA) at 100 nM in *Dictyostelium* AX2 expressing doxycycline-inducible PI5K with overnight DOX induction. **(e)** Histogram quantification of normalized cell number at different doses of C5a agonist in RAW 264.7 WT cells or cells overexpressing PIP5K1C. Gray columns represent PIP5K1C cells that have < 5% responses. Yellow columns represent PIP5K1C cells that have > 20% responses. Blue columns represent WT cells that have < 5% responses. Orange columns represent WT cells that have > 20% responses. **(f)** Histogram quantification of normalized cell number at different doses of folic acid (FA) in *Dictyostelium* AX2 expressing doxycycline-inducible PI5K without DOX induction (- DOX) or with overnight DOX induction (+ DOX). Green columns represent + DOX cells that have < 5% responses. Cyan columns represent + DOX cells that have > 20% responses. Dark blue columns represent - DOX cells that have < 5% responses. Orange columns represent - DOX cells that have > 20% responses. **(g)** Color-coded temporal overlay profiles of vegetative *Dictyostelium* AX2 expressing doxycycline-inducible PI5K without DOX (left), or polarized cells with overnight DOX induction (middle), or rounded cells with overnight DOX induction (right), chemotaxing to 10  $\mu$ M folic acid. The green box is where the center of the chemoattractant source.



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**Figure S14 Quantifications of directed migration speed with or without PI5K expression (a)**  
Centroid tracks of cells ( $n_c=13$ ) showing chemotaxis motility in cells expressing doxycycline-inducible PI5K without DOX induction (left) or with overnight DOX induction (right). Each track lasted 30 minutes, and the center of the chemoattractant source was reset to origin. **(b-c)** Scatter dot plots of directed cell migration speed (b) and aspect ratio (c) corresponds to Figure S12e.  $n_c=18$  from at least 2 independent experiments; asterisks indicate significant difference, \*\*\*\* $P \leq 0.0001$ , \*\*\* $P \leq 0.001$  (Mann-Whitney test. Compare ranks). The median is at the center, and whiskers and outliers are graphed according to Tukey's convention (GraphPad Prism 10).





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2205 **Figure S15 Globally recruiting Mypt169 in neutrophil and macrophage induces cell polarity**  
2206 **and protrusive activities (a-b)** Line-scan intensity profiles correspond to Figure 6b time point  
2207 30: 40 (a) or 35: 47 (b). (c-d) Centroid tracks of cells correspond to Figure 6b ( $n_c=15$ ), showing  
2208 random motility before (c) and after (d) 5  $\mu$ M rapamycin treatment. Each track lasted at least 15  
2209 minutes and was reset to the same origin. (e) Representative live-cell time-lapse confocal images  
2210 of differentiated HL-60 macrophage expressing CIBN-CAAX, CRY2PHR-mCherry-Mypt169  
2211 (magenta) and LifeAct-miRFP703 (Cyan), before or after 488 nm laser was switched on globally.  
2212 Blue arrows indicate where protrusions are formed. Time in min:sec format. Scale bars represent  
2213 5  $\mu$ m. (f) Color-coded temporal overlay profile corresponds to (j). (g) Representative live-cell time-  
2214 lapse confocal images of RAW 264.7 cells expressing CIBN-CAAX, CRY2PHR-mCherry-Mypt169  
2215 (magenta) and PIP5K1C-GFP (green), before or after 488 nm laser was switched on globally.  
2216 Blue arrows indicate where protrusions are formed. Time in min:sec format. Scale bars represent  
2217 5  $\mu$ m. Cells are pretreated with 10  $\mu$ M C5a agonist for 10 mins. (h) Color-coded temporal overlay  
2218 profile corresponds to (l).

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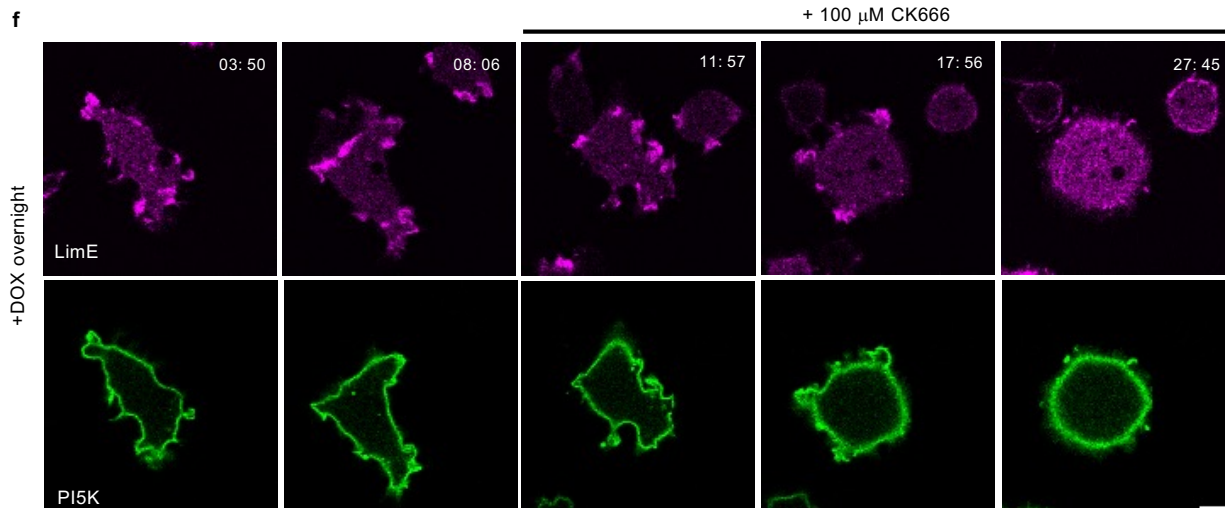
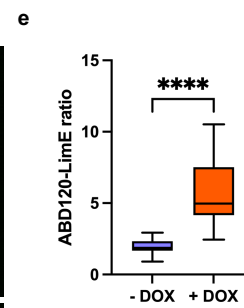
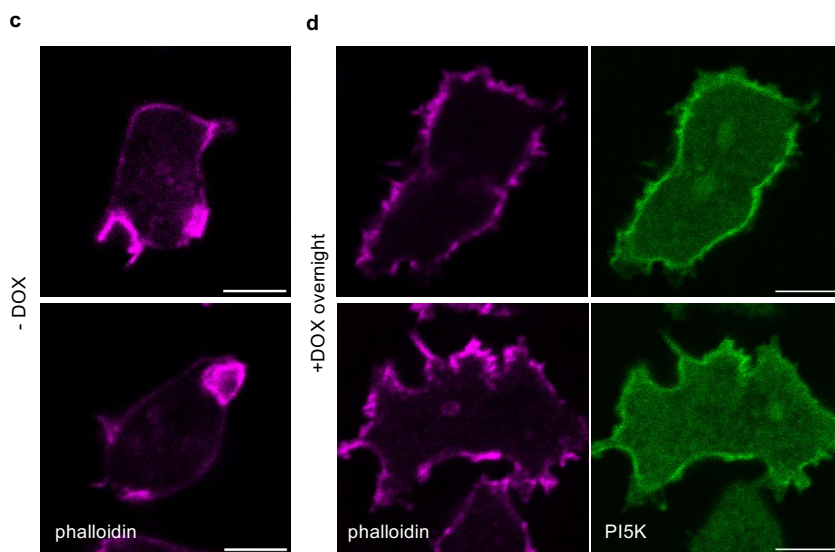
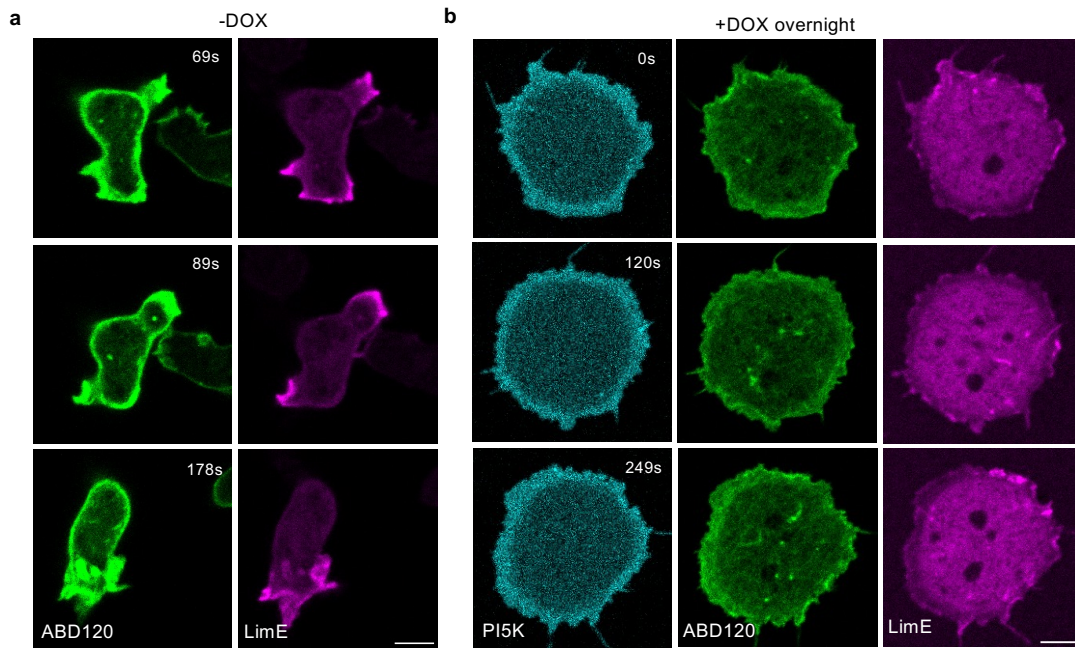
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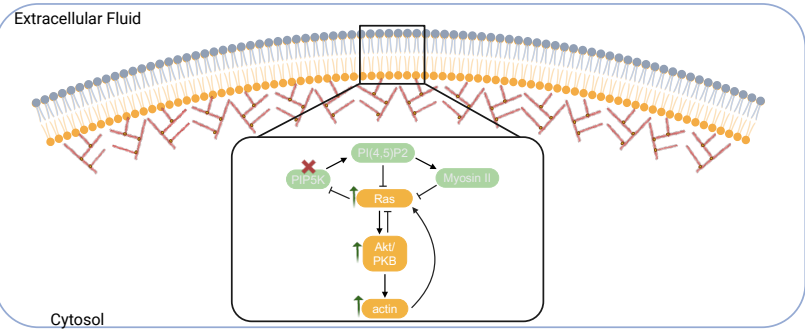
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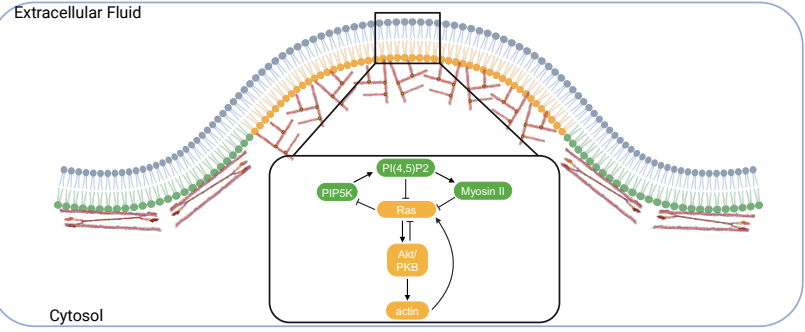
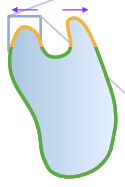
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**Figure S16 Expressing PI5K induces a shift to cortical actin at the cell rear (a-b)**  
Representative live-cell time-lapse confocal images of *Dictyostelium* AX2 co-expressing ABD120-GFP (green), LimE-Halo (magenta), and doxycycline-inducible PI5K without DOX induction (**l**) or with overnight DOX induction (**m**). Time in sec format. Scale bars represent 5  $\mu\text{m}$ . (**c-d**) Phalloidin stain of *Dictyostelium* AX2 doxycycline-inducible PI5K without DOX induction (**c**) or with overnight DOX induction (**d**). (**e**) Box-and-whisker plot of ABD120-LimE ratio corresponds to (a-b).  $n_c=17$  from at least 3 independent experiments; asterisks indicate significant difference, \*\*\*\* $P \leq 0.0001$  (Mann-Whitney test. Compare ranks). The median is at the center, and whiskers and outliers are graphed according to Tukey's convention (GraphPad Prism 10). (**f**) Representative live-cell time-lapse images of *Dictyostelium* cells coexpressing LimE-mCherry and doxycycline-inducible PI5K with overnight DOX induction, before and after 100  $\mu\text{M}$  CK666 treatment. Time in min:sec format. Scale bars represent 5  $\mu\text{m}$ .

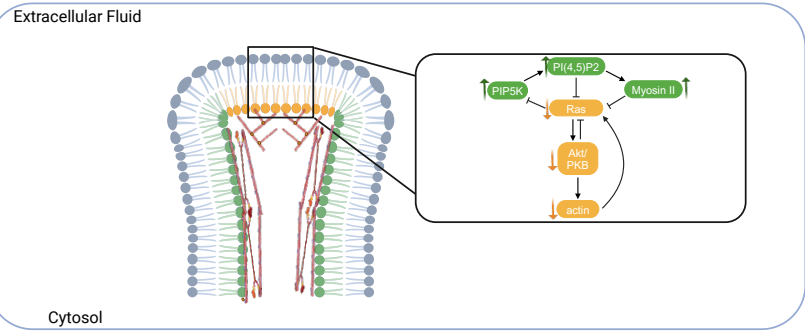
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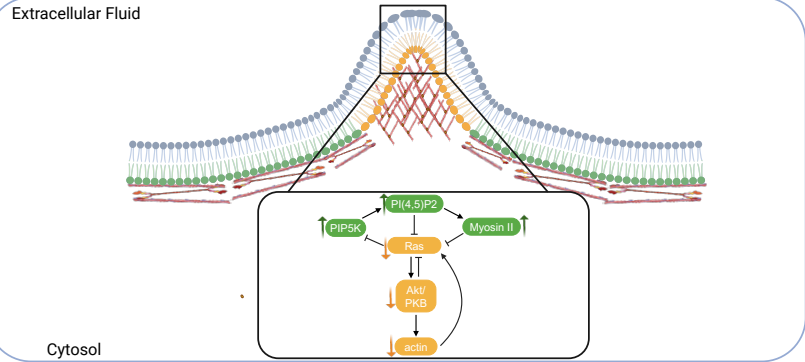
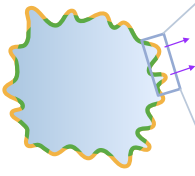
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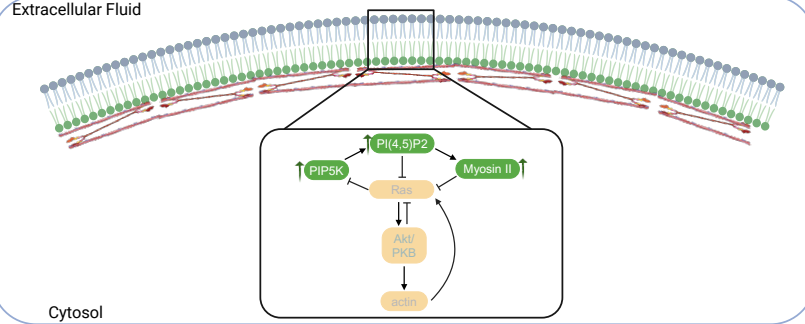
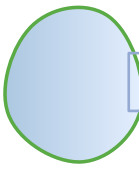
low PIP5K expression



High PIP5K expression



High PIP5K expression



Increased Signal Transduction Network Activity



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2272 **Figure S17 Schematic illustration showing the effect of PIP5Ks on cell morphology, signal**  
2273 **transduction, and cytoskeletal dynamics.** Cells display different morphology at different  
2274 PIP5Ks expression levels as shown on the left of this figure. The front region of the cell at each  
2275 cell morphology is shown in yellow, while the back region of the cell is shown in green. The blue  
2276 box on each cell represents the zoom-in region on the right side of the figure. The yellow or green-  
2277 shaded lipid head groups at each zoomed-in box represent the inner leaflet membrane. The  
2278 headgroups of the inner leaflet lipid molecules that are enriched in front-state are shown in yellow,  
2279 while the headgroups that are enriched in back-state are shown in green. Actin or actomyosin  
2280 structures are shown at the front-state or bac-state of the cell, respectively. The black box on each  
2281 lipid bilayer represents the zoom-in region of the lipid bilayer and the signaling pathways within  
2282 this region. In each condition, the lighter color icons represent the depleted signaling components.  
2283 Green arrow represents the increased change of this signaling molecular, while orange arrow  
2284 represents the decreased change.

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## 2305 **Supplementary Video Legends**

2306 Video S1

2307 Representative live-cell time-lapse confocal images of *Dictyostelium* AX2 (WT) cells and *pi5k*-  
2308 cells expressing PHPLC $\delta$ -YFP (biosensor for PI(4,5)P<sub>2</sub>), CynA-GFP (biosensor for PI(3,4)P<sub>2</sub>),  
2309 and mhcA-GFP. Top left corner shows time in min:sec format. Scale bar represents 10  $\mu$ m.

2310 Video S2

2311 Representative live-cell time-lapse confocal images of *Dictyostelium* AX2 (WT) cells and *pi5k*-  
2312 cells expressing RBD-GFP (biosensor for activated Ras), PHcrac-RFP (biosensor for PIP<sub>3</sub>), and  
2313 LimE-mCherry (biosensor for actin polymerization). Top left corner shows time in min:sec format.  
2314 Scale bar represents 10  $\mu$ m.

2315 Video S3

2316 Representative live-cell time-lapse confocal images of *Dictyostelium* AX2 (WT) cells and *pi5k*-  
2317 cells expressing mhcA-GFP (green) and LimE-mCherry (magenta). In *pi5k*- cells, ventral wave  
2318 activities of mhcA and LimE can be observed. Top left corner shows time in min:sec format. Scale  
2319 bar represents 10  $\mu$ m in *pi5k*- cells and 5  $\mu$ m in AX2 cells, respectively.

2320 Video S4

2321 Time-lapse confocal images of differentiated HL-60 neutrophil and macrophage expressing CIBN-  
2322 CAAX, CRY2PHR-mCherry-Inp54p (magenta) and LifeAct-miRFP703 (cyan), or differentiated  
2323 HL60 macrophage expressing untagged CIBN-CAAX, CRY2PHR-mCherry-empty vector  
2324 (magenta) and LifeAct-miRFP703 (cyan), before or after 488 nm laser was switched on globally.  
2325 Top left corner shows time in min:sec format. To start recruitment (magenta), the laser was  
2326 switched on at '03:09', or '03:51', or '03:30', once '488 nm ON' appears at the top of the video.  
2327 Cell was not exposed to chemoattractant during the experiment. Scale bar represents 5  $\mu$ m.

2328 Video S5

2329 Representative live-cell time-lapse images of *Dictyostelium* cells coexpressing PI5K-GFP and  
2330 PHcrac-RFP (biosensor for PIP<sub>3</sub>), differentiated HL-60 neutrophil expressing PIP5K1B, or  
2331 PIP5K1C and LifeAct (Cyna) during migration showing PI5K dynamically moves away from  
2332 protrusions in migrating cells. Top left corner shows time in min:sec format. Scale bars represent  
2333 5  $\mu$ m.

2334 Video S6

2335 Representative live-cell time-lapse images of *Dictyostelium* cells coexpressing PI5K-GFP and  
2336 PHcrac-RFP (biosensor for PIP<sub>3</sub>), PI5K-mCherry and RBD-GFP (biosensor for activated Ras),  
2337 PI5K-mCherry and PHPLC $\delta$ -GFP (biosensor for PI(4,5)P<sub>2</sub>), differentiated HL-60 macrophage  
2338 expressing PIP5K1B and LifeAct, and *Dictyostelium* cells coexpressing PI5K-mRFP and RBD-  
2339 GFP (biosensor for activated Ras) upon Latrunculin A treatment, during ventral wave propagation,  
2340 showing PI5K dynamically localizes to the back-state regions in ventral waves. Top left corner  
2341 shows time in min:sec format. Scale bars represent 5  $\mu$ m.

2342 Video S7

- 2343 Representative live-cell images of *Dictyostelium* cells co-expressing PI5K-GFP and PHcrac-RFP,  
2344 or PHPLC $\delta$ -GFP and PHcrac-RFP upon global cAMP stimulation. Top left corner shows time in  
2345 min:sec format. To start global stimulation, cAMP was added at '00:53', or '01:17', once '+ cAMP'  
2346 appears at the top of the video. Scale bar represents 5  $\mu$ m.
- 2347 Video S8
- 2348 Representative live-cell images of *Dictyostelium* cells expressing doxycycline inducible KikGR-  
2349 PI5K with overnight DOX incubation. Top left corner shows time in min:sec format. Photo  
2350 conversion happened at '01:50', '05:30', or '11:34'. Scale bar represents 5  $\mu$ m.
- 2351 Video S9
- 2352 Representative live-cell time-lapse images of *Dictyostelium* cells expressing PI5K-GFP (301-  
2353 718aa) during migration showing PI5K (301-718aa) dynamically localizes at the trailing edge in  
2354 migrating cells, or expressing mRFPmars-SspBR73Q-PI5K(316-718aa), before or after 488 nm  
2355 laser was switched on globally, or expressing mCherry-FRB-PI5K(1-315aa), before or after 5  $\mu$ M  
2356 Rapamycin was added. Top left corner shows time in min:sec format. To start recruitment, the  
2357 laser was switched on at '01:20', once '488 nm ON' appears at the top of the video, or Rapamycin  
2358 was added at '12:51', once '+ Rapamycin' appears at the top of the video. Scale bars represent  
2359 5  $\mu$ m.
- 2360 Video S10
- 2361 Representative live-cell images of *Dictyostelium* cells expressing doxycycline-inducible PI5K  
2362 without DOX induction, or with 2h DOX incubation, or with overnight DOX induction (rounded), or  
2363 with overnight DOX induction (spiky), or expressing PI5K(K681N, K682N). Top left corner shows  
2364 time in min:sec format. Scale bars represent 5  $\mu$ m for first 3 videos and 10  $\mu$ m for last video.
- 2365 Video S11
- 2366 Representative live-cell images of differentiated HL-60 neutrophils (WT) expressing LifeAct as the  
2367 biosensor, or expressing PIP5K1B (rounded), or expressing PIP5K1B (polarized), or expressing  
2368 PIP5K1C (rounded), or expressing PIP5K1C (polarized). Top left corner shows time in min:sec  
2369 format. Scale bars represent 5  $\mu$ m.
- 2370 Video S12
- 2371 Representative live-cell images of differentiated HL-60 macrophage (WT) expressing PH-Akt as  
2372 the biosensor, or expressing PIP5K1B (rounded), or expressing PIP5K1C (rounded). Top left  
2373 corner shows time in min:sec format. Scale bars represent 5  $\mu$ m.
- 2374 Video S13
- 2375 Time-lapse confocal images of MDA-MB-231 cells expressing crimson-SspB-PIP5K1C-P2A-iLiD-  
2376 CAAX or expressing crimson-SspB-empty vector-P2A-iLiD-CAAX, before or after 488 nm laser  
2377 was switched on globally. Top left corner shows time in hour:min:sec or min:sec format. To start  
2378 recruitment, the laser was switched on at '00:12:21', or '09:44', or '04:55', once '488 nm ON'  
2379 appears at the top of the video. Scale bar represents 10  $\mu$ m.
- 2380 Video S14
- 2381 Representative live-cell time-lapse confocal images of *Dictyostelium* AX2 co-expressing RBD-  
2382 GFP (biosensor for activated Ras) or PHcrac-YFP (biosensor for PIP3) and doxycycline-inducible  
2383 PI5K without DOX induction, or with 2h DOX incubation, or with overnight DOX induction

2384 (rounded), or with overnight DOX induction (spiky). Top left corner shows time in min:sec format.  
2385 Scale bars represent 5  $\mu\text{m}$ .

2386 Video S15

2387 Representative live-cell time-lapse confocal images of *Dictyostelium* AX2 co-expressing RBD-  
2388 GFP (biosensor for activated Ras) and doxycycline-inducible PI5K without DOX induction, or with  
2389 overnight DOX induction during ventral wave propagation. Top left corner shows time in min:sec  
2390 format. Scale bars represent 10  $\mu\text{m}$ .

2391 Video S16

2392 Representative live-cell time-lapse confocal images of *Dictyostelium* AX2 co-expressing Pak1-  
2393 GFP (biosensor for Rac1), or ArpC-GFP, or LimE-mCherry and doxycycline-inducible PI5K  
2394 without DOX induction, or with overnight DOX induction (rounded) or with overnight DOX  
2395 induction (spiky). Top left corner shows time in min:sec format. Scale bars represent 5  $\mu\text{m}$ .

2396 Video S17

2397 Representative live-cell time-lapse confocal images of *Dictyostelium abnABC*- cells co-  
2398 expressing RBD-GFP and doxycycline-inducible PI5K without DOX induction, or with overnight  
2399 DOX induction. Top left corner shows time in min:sec format. Scale bars represent 5  $\mu\text{m}$ .

2400 Video S18

2401 Representative live-cell time-lapse confocal images of *Dictyostelium* AX2 co-expressing RBD-  
2402 RFP, or LimE-RFP and PI5K (K681N, K682N). Top left corner shows time in min:sec format. Scale  
2403 bars represent 5  $\mu\text{m}$ .

2404 Video S19

2405 Representative live-cell time-lapse confocal images of *Dictyostelium pi5k*- cells expressing  
2406 doxycycline-inducible PI5K C2GAPB without DOX induction, or with overnight DOX induction, or  
2407 *Dictyostelium* AX2 co-expressing doxycycline-inducible PI5K C2GAPB and doxycycline-inducible  
2408 PI5K with overnight DOX induction. Top left corner shows time in min:sec format. Scale bars  
2409 represent 10  $\mu\text{m}$  for first 2 videos, and 5  $\mu\text{m}$  for last video.

2410 Video S20

2411 Representative live-cell time-lapse confocal images of *Dictyostelium* AX2 co-expressing RBD-  
2412 GFP (biosensor for activated Ras), or PHcrac-YFP (biosensor for PIP3) and doxycycline-inducible  
2413 PI5K without DOX induction, or with overnight DOX induction upon Latrunculin A treatment. Top  
2414 left corner shows time in min:sec format. Scale bars represent 5  $\mu\text{m}$ .

2415 Video S21

2416 Representative live-cell time-lapse confocal images of responses of PH-Akt-mCherry to global  
2417 stimulation C5aR agonist in RAW 264.7 WT cells, or cells overexpressing PIP5K1C at at 0.1  $\mu\text{M}$ -  
2418 100  $\mu\text{M}$ . To start global stimulation, C5a agonist was added at '00:53', or '06:09', or '05:45', or  
2419 '06:40', or '16:42', or '03:42', or '06:21', or '05:55', or '12:52', once '+ C5a' appears at the top of  
2420 the video. Scale bar represents 5  $\mu\text{m}$ .

2421 Video S22

2422 Representative live-cell time-lapse confocal images of *Dictyostelium Gb*- cells co-expressing  
2423 RBD-GFP and doxycycline-inducible PI5K without DOX induction, or with overnight DOX  
2424 induction. Top left corner shows time in min:sec format. Scale bars represent 5  $\mu\text{m}$ .



2425 Video S23

2426 Representative live-cell time-lapse confocal images of vegetative *Dictyostelium* AX2 expressing  
2427 doxycycline-inducible PI5K without DOX, or with overnight DOX induction, chemotaxing to 10 mM  
2428 folic acid-filled micropipette. The white box is where the center of the chemoattractant source. Top  
2429 left corner shows time in min:sec format. Scale bars represent 20  $\mu$ m.

2430 Video S24

2431 Representative live-cell time-lapse confocal images of *Dictyostelium* AX2 co-expressing CAR1-  
2432 FKBP-FKBP, mCherry-FRB-MHCKC, and doxycycline-inducible PI5K with overnight DOX  
2433 induction before and after 5  $\mu$ M rapamycin treatment. Top left corner shows time in min:sec format.  
2434 To start recruitment, Rapamycin was added at '14:03', once '+ Rapamycin' appears at the top of  
2435 the video. Scale bars represent 10  $\mu$ m.

2436 Videos S25

2437 **(e)** Representative live-cell time-lapse images of *Dictyostelium* cells coexpressing RBD-GFP and  
2438 doxycycline-inducible PI5K with overnight DOX induction during ventral wave propagation, before  
2439 and after 50  $\mu$ M blebbistatin treatment. Top left corner shows time in min:sec format. Blebbistatin  
2440 was added at '06:33', once '+ Blebbistatin' appears at the top of the video. Scale bars represent  
2441 10  $\mu$ m.

2442 Videos S26

2443 Representative live-cell time-lapse confocal images of differentiated HL-60 macrophage  
2444 expressing untagged CIBN-CAAX, CRY2PHR-mCherry-Mypt169 (magenta), or differentiated HL-  
2445 60 neutrophil and macrophage expressing untagged CIBN-CAAX, CRY2PHR-mCherry-Mypt169  
2446 (magenta), and PIP5K1B-GFP (green), before or after 488 nm laser was switched on globally.  
2447 Top left corner shows time in min:sec or hour:min:sec format. To start recruitment (magenta), the  
2448 laser was switched on at '03:41', or '00:05:55', or '02:51', once '488 nm ON' appears at the top of  
2449 the video. Cell was not exposed to chemoattractant during the experiment. Scale bar represents  
2450 10  $\mu$ m for the first video, and 5  $\mu$ m for the rest two videos.

2451 Video S27

2452 Representative live-cell time-lapse confocal images of *Dictyostelium* AX2 co-expressing ABD120-  
2453 GFP (green), LimE-Halo (magenta), and doxycycline-inducible PI5K without DOX induction, or  
2454 with overnight DOX induction. Top left corner shows time in min:sec format. Scale bars represent  
2455 5  $\mu$ m.

2456 Video S28

2457 Representative live-cell time-lapse images of *Dictyostelium* cells coexpressing LimE-mCherry  
2458 and doxycycline-inducible PI5K with overnight DOX induction, before and after 100  $\mu$ M CK666  
2459 treatment. Top left corner shows time in min:sec format. CK666 was added at '12:23', once '+  
2460 CK666' appears at the top of the video. Scale bars represent 5  $\mu$ m.

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