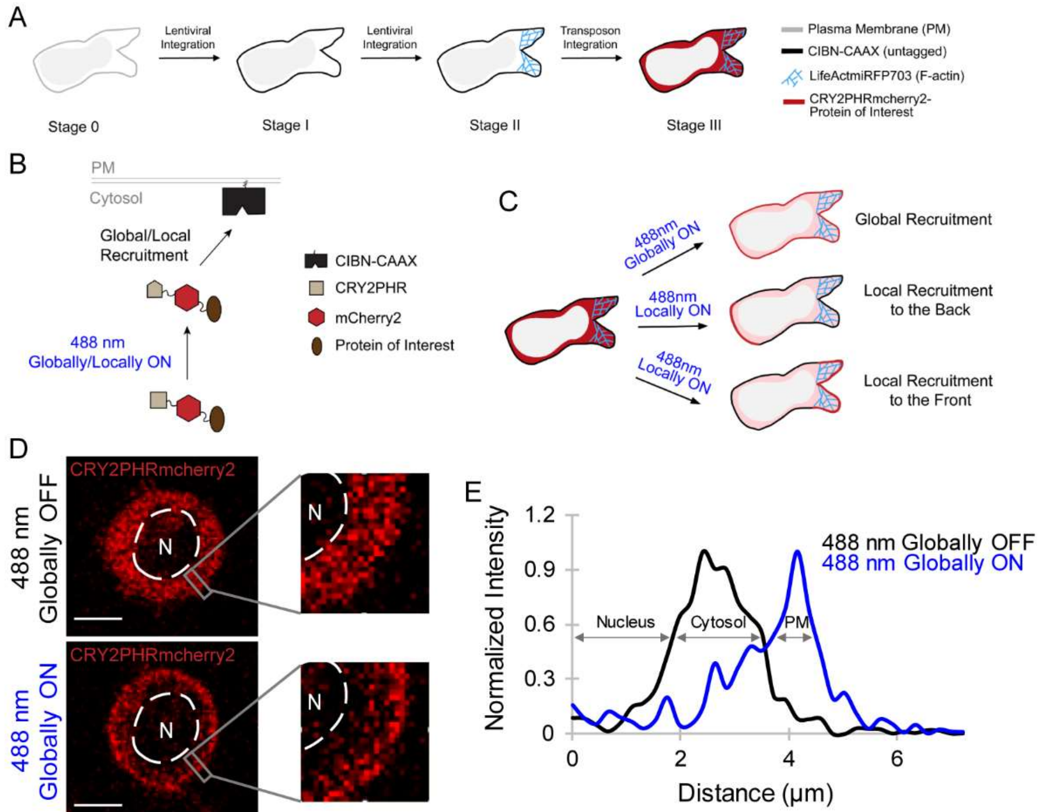
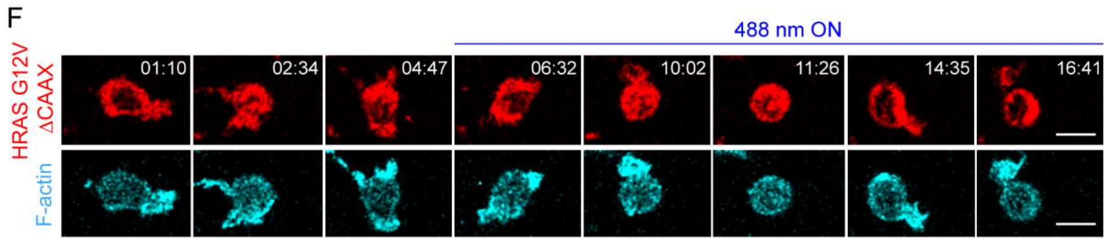
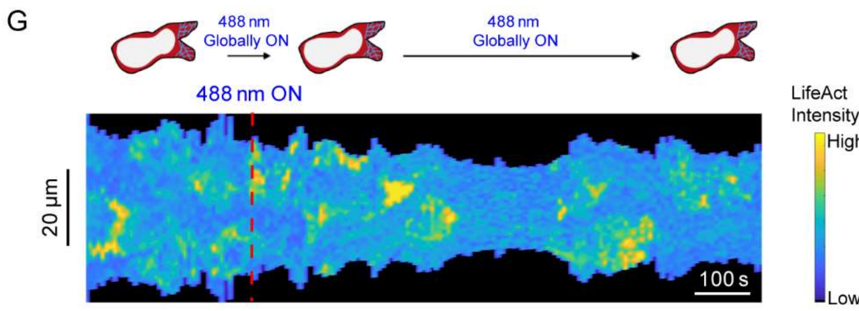
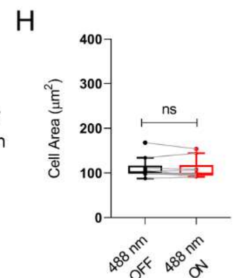
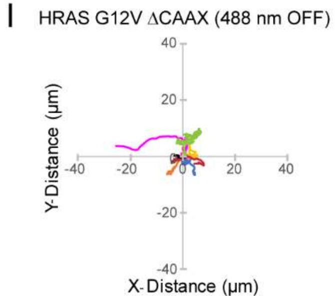
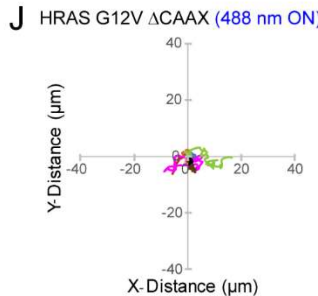

A
Lentiviral Integration
Lentiviral Integration
Transposon Integration
Stage 0
Stage I
Stage II
Stage III
Plasma Membrane (PM)
CIBN-CAAX (untagged)
LifeActmiRFP703 (F-actin)
CRY2PHRmcherry2-
Protein of Interest
B
PM
Cytosol
Global/Local
Recruitment
488 nm
Globally/Locally ON
CIBN-CAAX
CRY2PHR
mCherry2
Protein of Interest
C
488nm Globally ON
488nm Locally ON
488nm Locally ON
Global Recruitment
Local Recruitment to the Back
Local Recruitment to the Front
D
488 nm Globally OFF
488 nm Globally ON
CRY2PHRmcherry2
N
E
Normalized Intensity
488 nm Globally OFF
488 nm Globally ON
Nucleus
Cytosol
PM
Distance (μm)

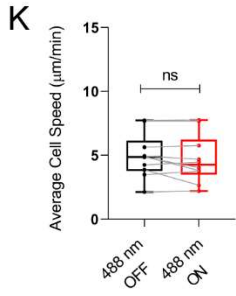

F
488 nm ON
HRAS G12V ΔCAAX
F-actin
01:10
02:34
04:47
06:32
10:02
11:26
14:35
16:41


G
488 nm Globally ON
488 nm Globally ON
488 nm ON
20 μm
100 s
LifeAct Intensity
High
Low


H
Cell Area (μm²)
ns
488 nm OFF
488 nm ON


I
HRAS G12V ΔCAAX (488 nm OFF)
Y-Distance (μm)
X-Distance (μm)


J
HRAS G12V ΔCAAX (488 nm ON)
Y-Distance (μm)
X-Distance (μm)


K
Average Cell Speed (μm/min)
ns
488 nm OFF
488 nm ON

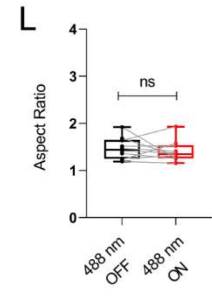

L
Aspect Ratio
ns
488 nm OFF
488 nm ON

Figure S1. Construction of CRY2-CIBN optogenetic system and cytosolic form of constitutively active HRas does not affect neutrophil polarity and migration. Related to STAR Methods and Figure 1. **(A)** Cartoon showing stepwise introduction of CRY2-CIBN optogenetics in HL-60 cells. In wild type HL-60 cells (Stage 0), CIBN-CAAX membrane anchor (Stage I) and F-actin polymerization biosensor, LifeActmiRFP703 (Stage II), were introduced sequentially by lentiviral integration. These cells were used to express cytosolic CRY2PHR-mcherry2 fused with protein of interest by transposon-based integration (Stage III). **(B)** Schematic demonstrating recruitment of cytosolic CRY2PHR-mcherry2 fused with protein of interest to the membrane CIBN-CAAX by local or global illumination with 488 nm laser. **(C)** Depending on the region of the membrane where 488 nm laser was applied, CRY2PHR-mcherry2-protein of interest fusion was recruited all over the cell periphery (global recruitment), or specifically to the back or front of the cell. **(D)** Representative cell showing CRY2PHR-mcherry2 in the cytosol when 488 nm laser was off (top panel). Once light was switched on, CRY2PHR-mcherry2 translocated evenly to the periphery (bottom panel). The circular dashed line region with 'N' denotes the large nucleus in these cells. Images are representative of many cells from atleast three independent experiments. Scale bars represent 5 μm. **(E)** Linescan across the cytosol and membrane of the cell (denoted by white box in D), before (black) or after (blue) 488 nm laser was switched on globally. There is a distinct shift in CRY2PHR-mcherry2 intensity peak from cytosol to plasma membrane (PM) when laser was on. **(F)** Time-lapse confocal images of differentiated HL-60 neutrophil expressing CRY2PHR-mcherry2-HRas G12V ΔCAAX (red; upper panel) and LifeActmiRFP703 (cyan; lower panel), before or after 488 nm laser was switched on globally. No appreciable HRas G12V ΔCAAX recruitment was observed presumably due to low expression of CIBN-CAAX membrane anchor. Time in min:sec format. Scale bars represent 5 μm. **(G)** Representative kymograph of cortical LifeAct intensity in HRas G12V ΔCAAX-expressing neutrophil before or after 488 nm laser was turned on. A linear color map shows that blue is the lowest LifeAct intensity whereas yellow is the highest. Duration of the kymograph is 17 mins. Cartoon depicts recruitment, F-actin polymerization or cell shape status corresponding to the kymograph. **(H)** Box-and-whisker plot of neutrophil cell area before (black) or after (red) 488 nm laser was turned on. n_c=10 from atleast 3 independent experiments; ns denotes $P>0.05$ (Wilcoxon-Mann-Whitney rank sum test). Centroid tracks of neutrophils (n_c=10) showing random motility before **(I)** or after **(J)** 488 nm laser was turned on. Each track lasts atleast 5 mins and was reset to same origin. Box-and-whisker plots of neutrophil speed **(K)** and aspect ratio **(L)** before (black) or after (red) 488 nm laser was switched on. n_c=10 from atleast 3 independent experiments; ns denotes $P>0.05$ (Wilcoxon-Mann-Whitney rank sum test).

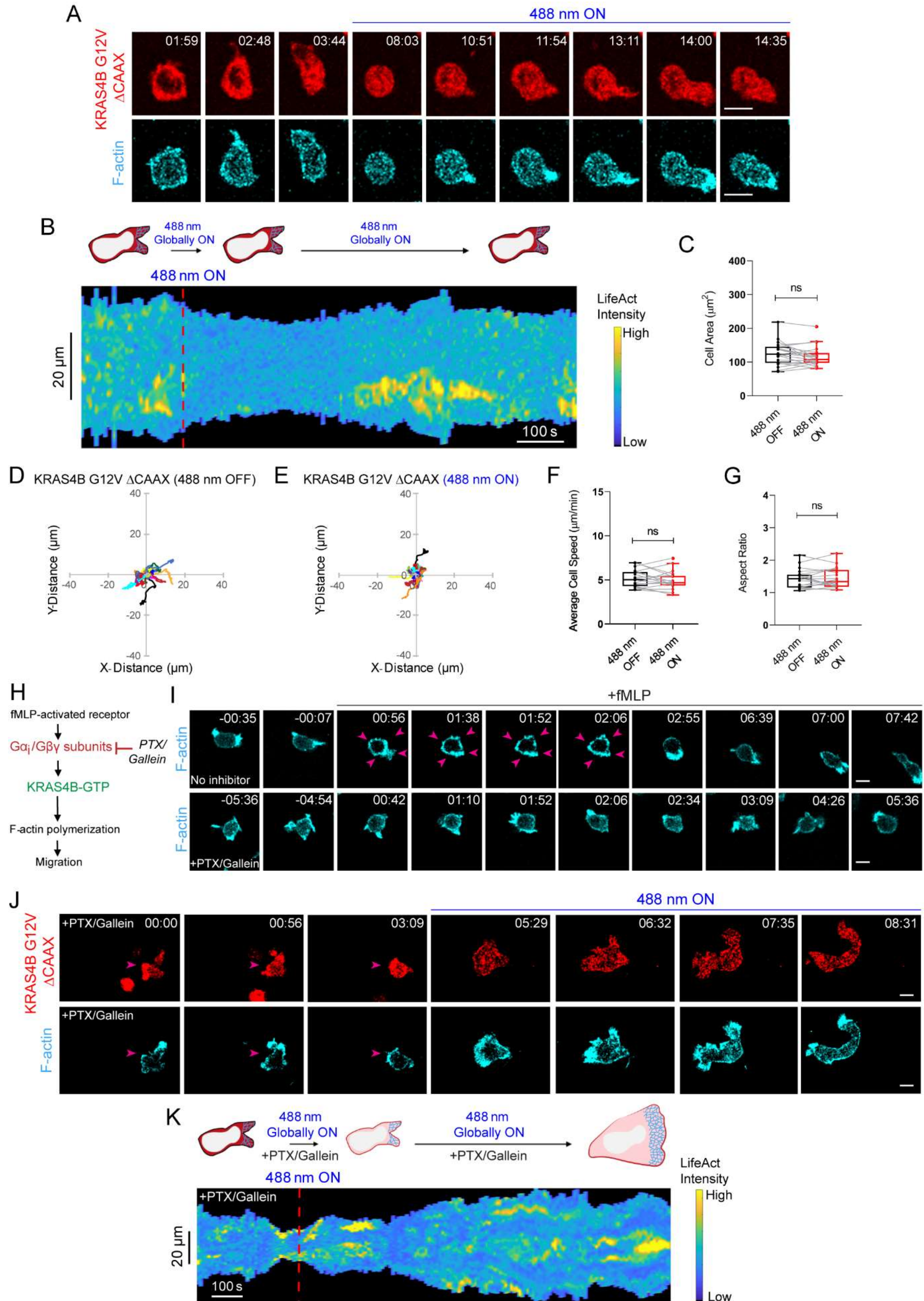
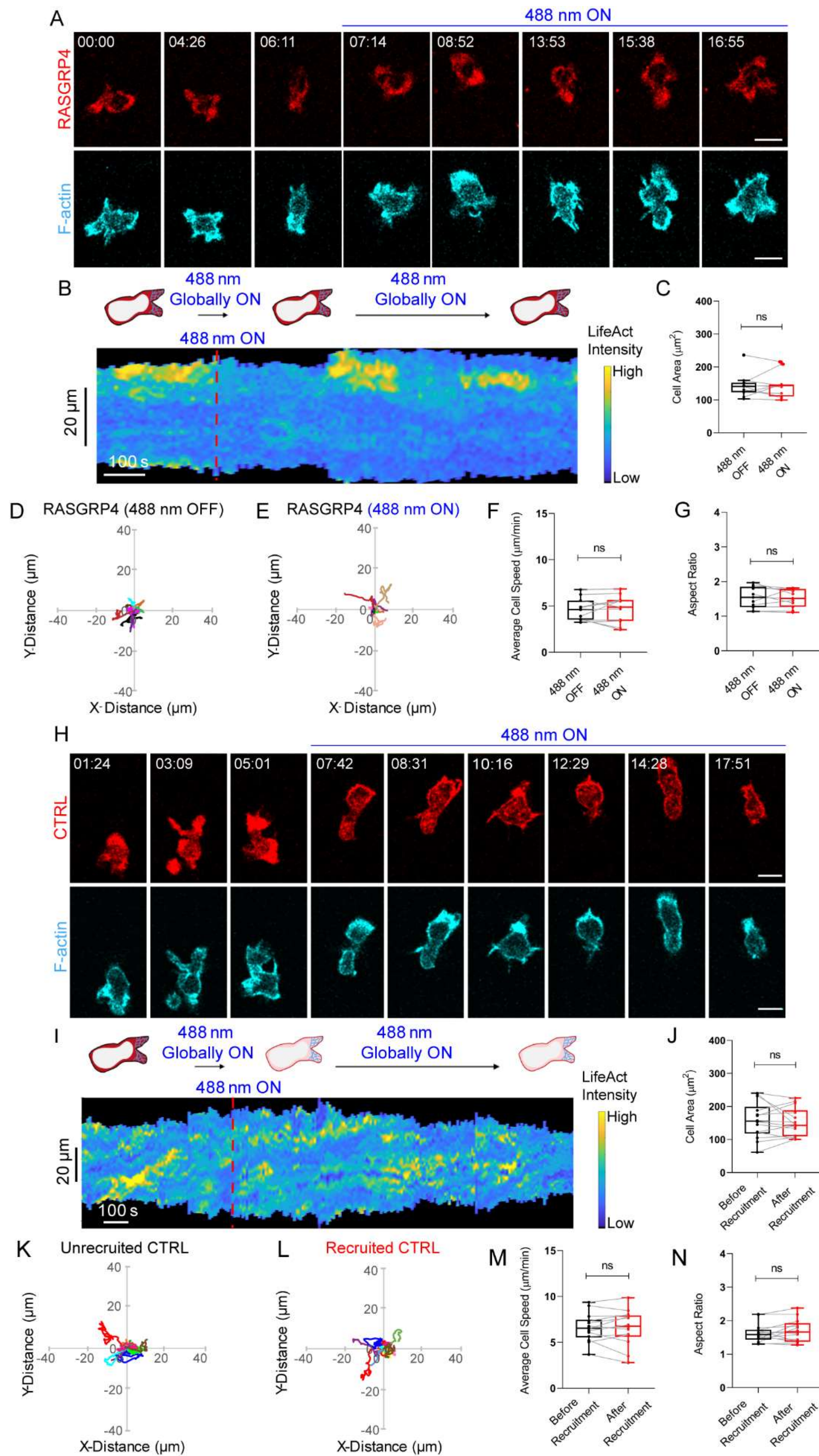
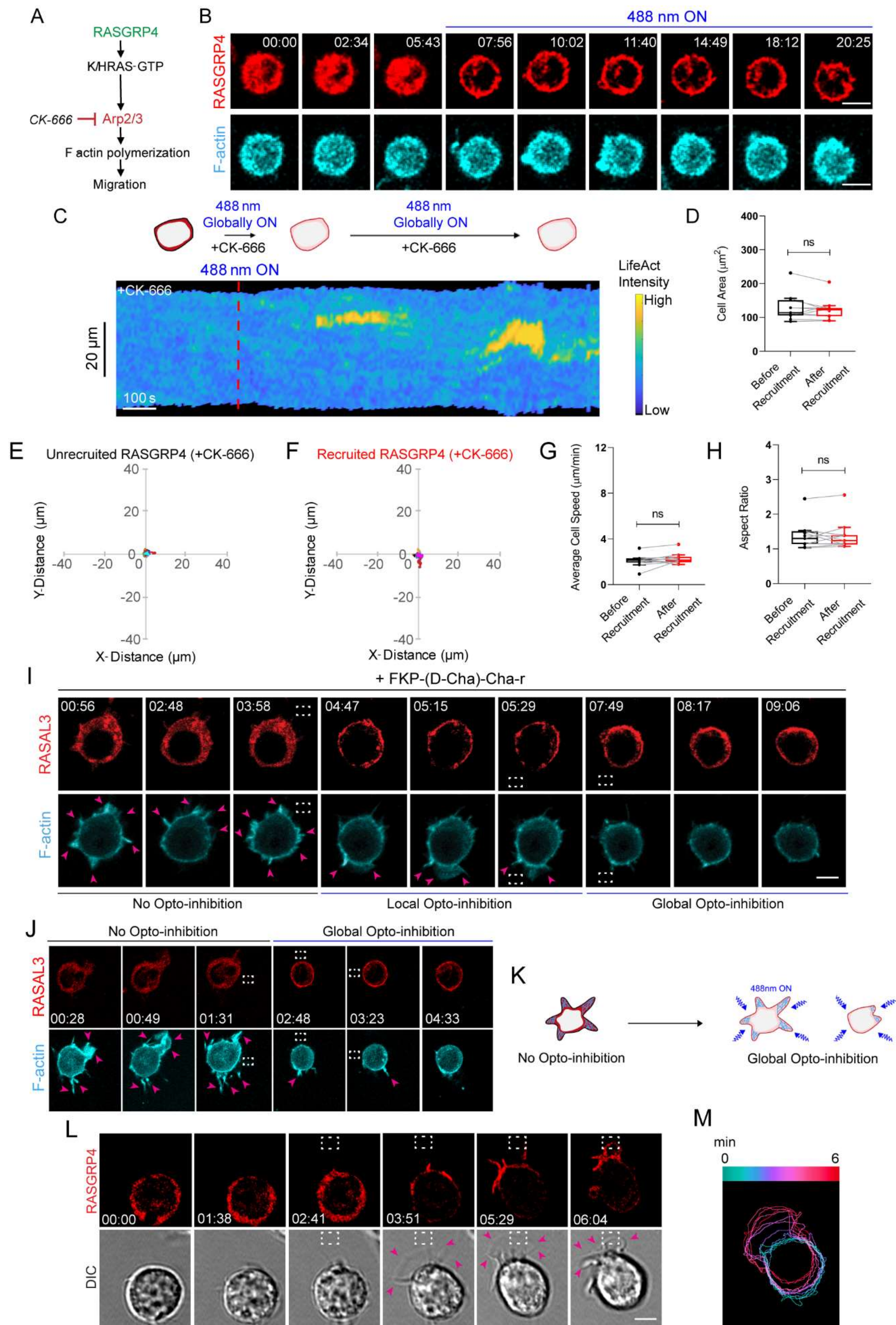

A
488 nm ON
KRAS4B G12V ΔCAAX
F-actin
01:59
02:48
03:44
08:03
10:51
11:54
13:11
14:00
14:35
B
488 nm Globally ON
488 nm ON
20 µm
100 s
LifeAct Intensity
High
Low
C
Cell Area (µm²)
ns
488 nm OFF
488 nm ON
D
KRAS4B G12V ΔCAAX (488 nm OFF)
E
KRAS4B G12V ΔCAAX (488 nm ON)
Y-Distance (µm)
X-Distance (µm)
F
Average Cell Speed (µm/min)
G
Aspect Ratio
H
fMLP-activated receptor
Gαi/Gβγ subunits
PTX/ Gallein
KRAS4B-GTP
F-actin polymerization
Migration
I
+fMLP
No inhibitor
+PTX/Gallein
J
K
+PTX/Gallein

Figure S2. Constitutively active KRas4B, only when recruited on cell membrane, promotes neutrophil polarity and migration even in absence of G-protein signaling. Related to Figure 1. **(A)** Time-lapse confocal images of differentiated neutrophil expressing CRY2PHR-mcherry2-KRas4B G12V ΔCAAX (red; upper panel) and LifeActmiRFP703 (cyan; lower panel), before or after 488 nm laser was switched on globally. No appreciable KRas4B G12V ΔCAAX recruitment was observed presumably due to low expression of CIBN-CAAX membrane anchor. Time in min:sec format. Scale bars represent 5 μm. **(B)** Representative kymograph of cortical LifeAct intensity in KRas4B G12V ΔCAAX -expressing neutrophil before or after 488 nm laser was turned on. A linear color map shows that blue is the lowest LifeAct intensity whereas yellow is the highest. Duration of the kymograph is 15 mins. Cartoon depicts recruitment, F-actin polymerization or cell shape status corresponding to the kymograph. **(C)** Box-and-whisker plot of neutrophil cell area before (black) or after (red) 488 nm laser was turned on. n_c=20 from atleast 3 independent experiments; ns denotes P>0.05 (Wilcoxon-Mann-Whitney rank sum test). Centroid tracks of neutrophils (n_c=20) showing random motility before **(D)** or after **(E)** 488 nm laser was turned on. Each track lasts atleast 5 mins and was reset to same origin. Box-and-whisker plots of neutrophil speed **(F)** and aspect ratio **(G)** before (black) or after (red) 488 nm laser was switched on. n_c=20 from atleast 3 independent experiments; ns denotes P>0.05 (Wilcoxon-Mann-Whitney rank sum test). **(H)** Experimental scheme testing if KRas4B G12V ΔCAAX activity, in absence of G-protein signaling, promotes F-actin polymerization and migration. A combination of Gαi and Gβγ inhibitors, pertussis toxin (PTX) and gallein respectively, were used to test this. **(I)** Time-lapse confocal images of untreated (upper panel) or PTX/gallein-treated (lower panel) HL-60 neutrophil expressing LifeActmiRFP703 (cyan), before or after a uniform stimulus of 100 nM fMLP was applied. Within a minute of fMLP addition, we observed a global burst of F-actin polymerization, as indicated with increased LifeActmiRFP703 on the membrane (shown with pink arrows), in untreated cells. This did not occur in cells pre-treated with Gαi/ Gβγ inhibitors. Images are representative of many cells from atleast three independent experiments. Time in min:sec format. Scale bars represent 5 μm. **(J)** Time-lapse confocal images of PTX/gallein-treated HL-60 neutrophil expressing CRY2PHR-mcherry2-KRas4B G12V ΔCAAX (red; upper panel) and LifeActmiRFP703 (cyan; lower panel), before or after 488 nm laser was turned on globally. Pink arrows denote representative cell. Images are representative of many cells from atleast three independent experiments. Time in min:sec format. Scale bars represent 5 μm. **(K)** Representative kymograph of cortical LifeAct intensity in PTX/gallein-treated KRas4B G12V ΔCAAX-expressing neutrophil before or after 488 nm laser was turned on. A linear color map shows that blue is the lowest LifeAct intensity whereas yellow is the highest. Duration of the kymograph is 10 mins. Cartoon depicts membrane recruitment, actin polymerization or cell shape status corresponding to the kymograph.



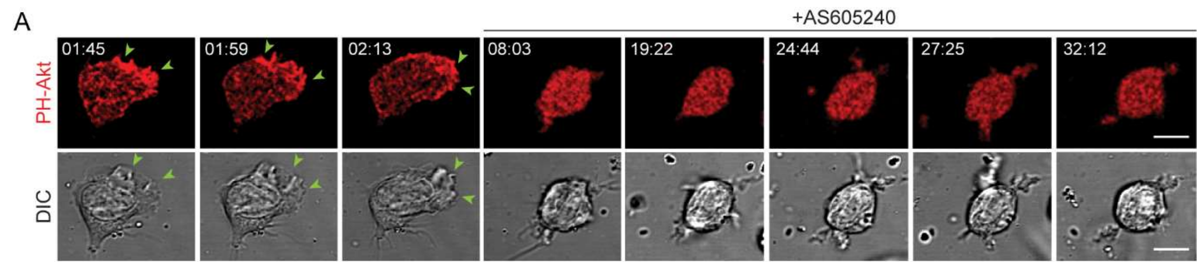
A
488 nm ON
00:00
04:26
06:11
07:14
08:52
13:53
15:38
16:55
RASGRP4
F-actin
B
488 nm
Globally ON
488 nm
Globally ON
488 nm ON
LifeAct
Intensity
High
Low
20 µm
100 s
C
ns
Cell Area (µm²)
488 nm OFF
488 nm ON
D
RASGRP4 (488 nm OFF)
Y-Distance (µm)
X-Distance (µm)
E
RASGRP4 (488 nm ON)
F
Average Cell Speed (µm/min)
G
Aspect Ratio
H
488 nm ON
01:24
03:09
05:01
07:42
08:31
10:16
12:29
14:28
17:51
CTRL
F-actin
I
J
Before Recruitment
After Recruitment
K
Unrecruited CTRL
L
Recruited CTRL
M
N

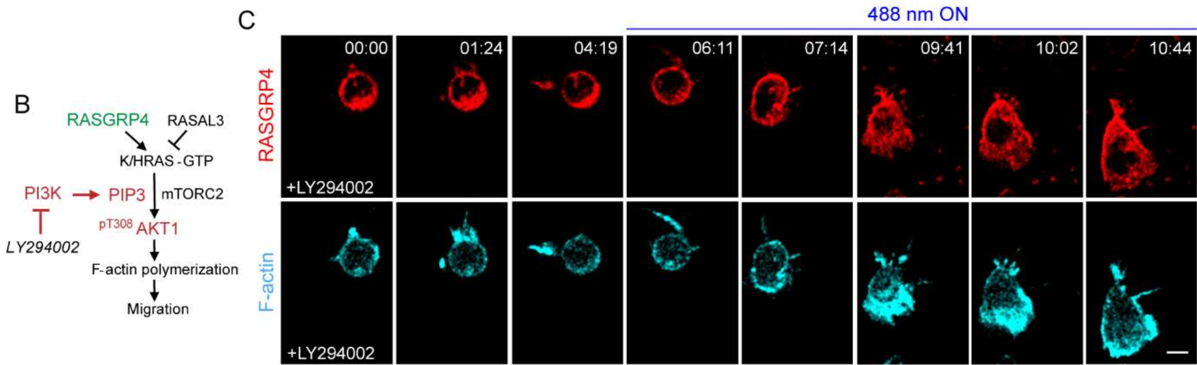
Figure S3. Cytosolic RasGRP4 or empty CRY2PHR recruitment does not improve polarity and migration. Related to Figures 1 and 2. **(A)** Time-lapse confocal images of differentiated HL-60 neutrophil expressing CRY2PHR-mcherry2-RasGRP4 (red; upper panel) and LifeActmiRFP703 (cyan; lower panel), before or after 488 nm laser was switched on globally. No appreciable RasGRP4 recruitment was observed presumably due to low expression of CIBN-CAAX membrane anchor. Time in min:sec format. Scale bars represent 5 µm. **(B)** Representative kymograph of cortical LifeAct intensity in RasGRP4-expressing neutrophil before or after 488 nm laser was turned on. A linear color map shows that blue is the lowest LifeAct intensity whereas yellow is the highest. Duration of the kymograph is 17 mins. Cartoon depicts recruitment, F-actin polymerization or cell shape status corresponding to the kymograph. **(C)** Box-and-whisker plot of neutrophil cell area before (black) or after (red) 488 nm laser was turned on. n_c=11 from atleast 3 independent experiments; ns denotes $P>0.05$ (Wilcoxon-Mann-Whitney rank sum test). Centroid tracks of neutrophils (n_c=11) showing random motility before **(D)** or after **(E)** 488 nm laser was turned on. Each track lasts atleast 5 mins and was reset to same origin. Box-and-whisker plots of neutrophil speed **(F)** and aspect ratio **(G)** before (black) or after (red) 488 nm laser was switched on. n_c=11 from atleast 3 independent experiments; ns denotes $P>0.05$ (Wilcoxon-Mann-Whitney rank sum test). **(H)** Time-lapse confocal images of differentiated HL-60 neutrophil expressing CRY2PHR-mcherry2-CTRL (control; red; upper panel) and LifeActmiRFP703 (cyan; lower panel), before or after 488 nm laser was switched on globally. Time in min:sec format. Scale bars represent 5 µm. **(I)** Representative kymograph of cortical LifeAct intensity in CTRL-expressing neutrophil before or after 488 nm laser was turned on. A linear color map shows that blue is the lowest LifeAct intensity whereas yellow is the highest. Duration of the kymograph is 18 mins. Cartoon depicts membrane recruitment, actin polymerization or cell shape status corresponding to the kymograph. **(J)** Box-and-whisker plot of cell area before (black) or after (red) CTRL membrane recruitment. n_c=14 from atleast 3 independent experiments; ns denotes $P>0.05$ (Wilcoxon-Mann-Whitney rank sum test). Centroid tracks of neutrophils (n_c=14) showing random motility before **(K)** or after **(L)** CTRL membrane recruitment. Each track lasts atleast 5 mins and was reset to same origin. Box-and-whisker plots of neutrophil speed **(M)** and aspect ratio **(N)** before (black) or after (red) CTRL recruitment. n_c=14 from atleast 3 independent experiments; ns denotes $P>0.05$ (Wilcoxon-Mann-Whitney rank sum test).

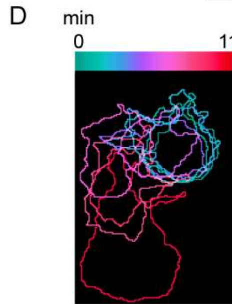


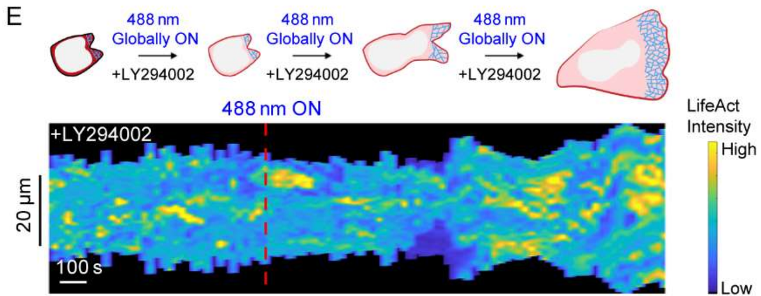
A
RASGRP4
K/HRAS-GTP
CK-666
Arp2/3
F actin polymerization
Migration
B
488 nm ON
00:00
02:34
05:43
07:56
10:02
11:40
14:49
18:12
20:25
RASGRP4
F-actin
C
488 nm Globally ON
+CK-666
488 nm Globally ON
+CK-666
488 nm ON
+CK-666
20 µm
100 s
LifeAct Intensity
High
Low
D
ns
Cell Area (µm2)
Before Recruitment
After Recruitment
E
Unrecruited RASGRP4 (+CK-666)
Y-Distance (µm)
X-Distance (µm)
F
Recruited RASGRP4 (+CK-666)
Y-Distance (µm)
X-Distance (µm)
G
Average Cell Speed (µm/min)
ns
Before Recruitment
After Recruitment
H
Aspect Ratio
ns
Before Recruitment
After Recruitment
I
+ FKP-(D-Cha)-Cha-r
00:56
02:48
03:58
04:47
05:15
05:29
07:49
08:17
09:06
RASAL3
F-actin
No Opto-inhibition
Local Opto-inhibition
Global Opto-inhibition
J
No Opto-inhibition
Global Opto-inhibition
RASAL3
F-actin
00:28
00:49
01:31
02:48
03:23
04:33
K
488nm ON
No Opto-inhibition
Global Opto-inhibition
L
RASGRP4
DIC
00:00
01:38
02:41
03:51
05:29
06:04
M
min
0
6

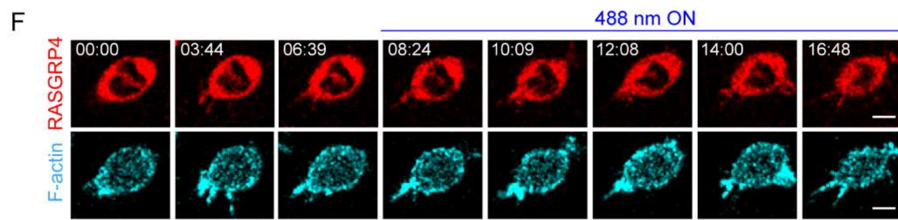
Figure S4. Global and local recruitment of RasGRP4 or RASAL3 activates or inhibits Arp2/3-mediated actin-based protrusions and motility in neutrophils and macrophages. Related to Figures 3 and 4. **(A)** Experimental scheme to test whether RasGRP4 activation promotes F-actin polymerization and migration through Arp2/3 complex. CK-666 was the inhibitor used to test this. **(B)** Time-lapse confocal images of CK-666-treated HL-60 neutrophil expressing CRY2PHR-mcherry2-RasGRP4 (red; upper panel) and LifeActmiRFP703 (cyan; lower panel), before or after 488 nm laser was turned on globally. Time in min:sec format. Scale bars represent 5 μm. **(C)** Representative kymograph of cortical LifeAct intensity in CK-666-treated RasGRP4-expressing neutrophil before or after 488 nm laser was turned on. A linear color map shows that blue is the lowest LifeAct intensity whereas yellow is the highest. Duration of the kymograph is 21 mins. Cartoon depicts membrane recruitment, actin polymerization or cell shape status corresponding to the kymograph. **(D)** Box-and-whisker plot of cell area before (black) or after (red) RasGRP4 membrane recruitment. n_c=11 from atleast 3 independent experiments; ns denotes P>0.05 (Wilcoxon-Mann-Whitney rank sum test). Centroid tracks of CK-666-treated neutrophils (n_c=11) showing random motility before **(E)** or after **(F)** RasGRP4 membrane recruitment. Each track lasts atleast 5 mins and was reset to same origin. Box-and-whisker plots of speed **(G)** and aspect ratio **(H)** before (black) or after (red) RasGRP4 recruitment in CK-666-treated neutrophils. n_c=11 from atleast 3 independent experiments; ns denotes P>0.05 (Wilcoxon-Mann-Whitney rank sum test). **(I)** Time-lapse confocal images of FKP-(D-Cha)-Cha-r-treated RAW 264.7 macrophage expressing CRY2PHR-mcherry2-RASAL3 (red; upper panel) and LifeAct-Halo (cyan; lower panel). Activated macrophage had multiple F-actin-rich protrusions (marked by LifeAct) around its perimeter, as shown by pink arrows (No Opto-inhibition). Next, RASAL3 was recruited to protrusions by applying 488 nm laser near it, as shown by the dashed white box. Protrusions disappeared at site of recruitment and only formed at the cortex where RASAL3 was mostly absent (Local Opto-inhibition). Pink arrows highlight these protrusions. Finally, RASAL3 was recruited over the entire periphery, after 488 nm light was applied all around the cell. It shrank the cell and LifeAct-containing protrusions soon disappeared (Global Opto-inhibition). Images are representative of many cells from atleast three independent experiments. Time in min:sec format. Scale bars represent 5 μm. **(J)** Time-lapse confocal images of HL-60 neutrophil expressing CRY2PHR-mcherry2-RASAL3 (red; upper panel) and LifeActmiRFP703 (cyan; lower panel). Unpolarized, non-migratory neutrophil had multiple F-actin-rich protrusions around its perimeter, as shown by pink arrows (No Opto-inhibition). RASAL3 recruitment over the entire periphery, after 488 nm light was applied all around the cell, caused the cell to shrink and LifeAct-containing protrusions soon disappeared (Global Opto-inhibition). Pink arrows highlight cellular protrusions. Region of blue light illumination is shown by the dashed white box. Images are representative of many cells from atleast three independent experiments. Time in min:sec format. Scale bars represent 5 μm. **(K)** Cartoon illustrating RASAL3-mediated phenomenon seen in (A, 'Global Opto-inhibition') and (B). **(L)** Time-lapse confocal images of quiescent RAW264.7 macrophage expressing CRY2PHR-mcherry2-RasGRP4 (red; upper panel). RasGRP4 is recruited continuously to one side of the unpolarized cell by applying 488 nm laser near it, as shown by the dashed white box. Pink arrows highlight RasGRP4-induced new protrusions which make the cell move towards the light source (DIC). Time in min:sec format. Scale bars represent 5 μm. **(M)** Color-coded (at 30 sec intervals) outlines of the cell shown in (A).

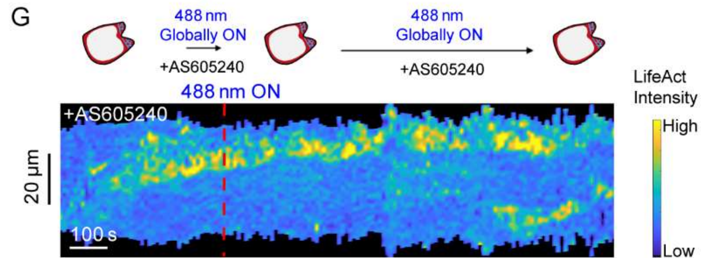

A
+AS605240
PH-Akt
DIC
01:45
01:59
02:13
08:03
19:22
24:44
27:25
32:12

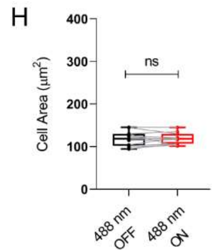

C
488 nm ON
RASGRP4
F-actin
+LY294002
00:00
01:24
04:19
06:11
07:14
09:41
10:02
10:44
B
RASGRP4
RASAL3
K/HRAS-GTP
PI3K
PIP3
mTORC2
pT308 AKT1
LY294002
F-actin polymerization
Migration

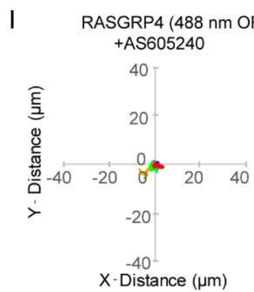

D
min
0
11

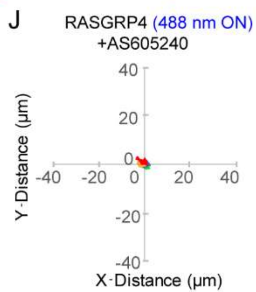

E
488 nm Globally ON
+LY294002
488 nm ON
LifeAct Intensity
High
Low
20 μm
100 s

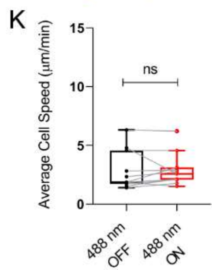

F
488 nm ON
RASGRP4
F-actin
00:00
03:44
06:39
08:24
10:09
12:08
14:00
16:48


G
488 nm Globally ON
+AS605240
488 nm ON
LifeAct Intensity
High
Low
20 μm
100 s


H
Cell Area (μm²)
ns
488 nm OFF
488 nm ON


I
RASGRP4 (488 nm OFF)
+AS605240
Y-Distance (μm)
X-Distance (μm)


J
RASGRP4 (488 nm ON)
+AS605240
Y-Distance (μm)
X-Distance (μm)


K
Average Cell Speed (μm/min)
ns
488 nm OFF
488 nm ON

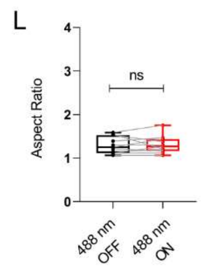
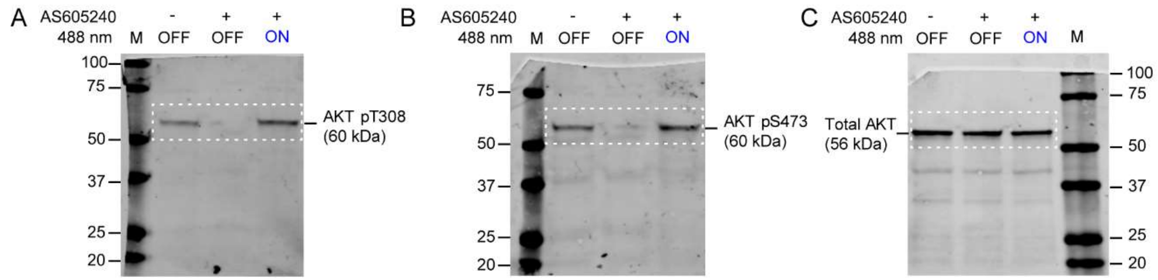
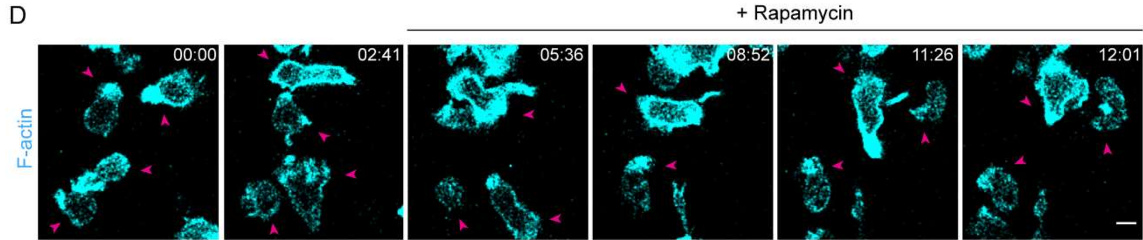
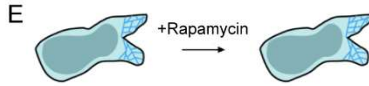
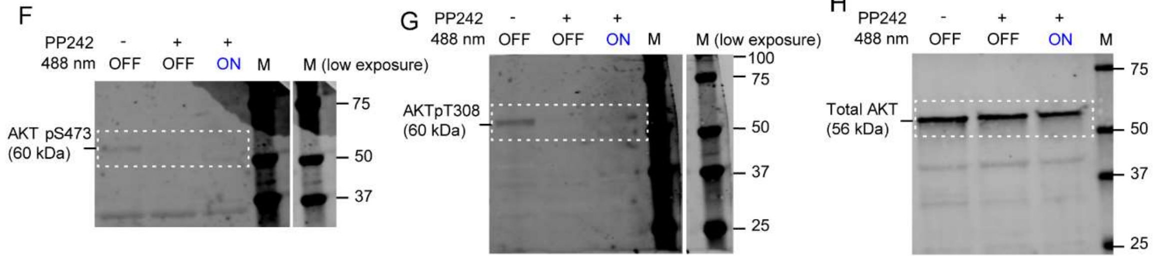

L
Aspect Ratio
ns
488 nm OFF
488 nm ON

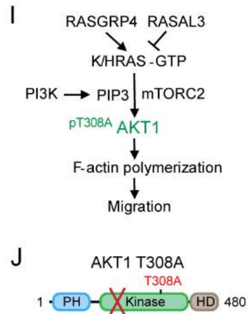
Figure S5. Global RasGRP4 recruitment can activate neutrophil in presence of multiple PI3K inhibitors. Related to Figure 5. **(A)** Time-lapse confocal images of AS605240-treated HL-60 neutrophil expressing RFP-PH-Akt (red). Green arrows show PH-Akt localization or PIP3 accumulation on protrusions (DIC). Images are representative of many cells from atleast three independent experiments. Time in min:sec format. Scale bars represent 5 μm. **(B)** Experimental scheme to test whether RasGRP4-mediated Ras activation can promote F-actin polymerization and migration in presence of pan-PI3K inhibitor, LY294002. **(C)** Time-lapse confocal images of LY294002-treated HL-60 neutrophil expressing CRY2PHR-mcherry2-RasGRP4 (red; upper panel) and LifeActmiRFP703 (cyan; lower panel), before or after 488 nm laser was switched on globally. Time in min:sec format. Scale bars represent 5 μm. **(D)** Color-coded (at 1 min intervals) outlines of the cell shown in (B). **(E)** Representative kymograph of cortical LifeAct intensity in LY294002-treated RasGRP4-expressing neutrophil before or after 488 nm laser was turned on. A linear color map shows that blue is the lowest LifeAct intensity whereas yellow is the highest. Duration of the kymograph is 11 mins. Cartoon depicts recruitment, F-actin polymerization or cell shape status corresponding to the kymograph. **(F)** Time-lapse confocal images of AS605240-treated HL-60 neutrophil expressing CRY2PHR-mcherry2-RasGRP4 (red; upper panel) and LifeActmiRFP703 (cyan; lower panel), before or after 488 nm laser was switched on globally. No appreciable RasGRP4 recruitment was observed presumably due to low expression of CIBN-CAAX membrane anchor. Time in min:sec format. Scale bars represent 5 μm. **(G)** Representative kymograph of cortical LifeAct intensity in AS605240-treated RasGRP4-expressing neutrophil before or after 488 nm laser was turned on. A linear color map shows that blue is the lowest LifeAct intensity whereas yellow is the highest. Duration of the kymograph is 17 mins. Cartoon depicts recruitment, F-actin polymerization or cell shape status corresponding to the kymograph. **(H)** Box-and-whisker plot of neutrophil cell area before (black) or after (red) 488 nm laser was turned on. n_c=11 from atleast 3 independent experiments; ns denotes $P>0.05$ (Wilcoxon-Mann-Whitney rank sum test). Centroid tracks of AS605240-treated neutrophils (n_c=11) showing random motility before **(I)** or after **(J)** 488 nm laser was turned on. Each track lasts atleast 5 mins and was reset to same origin. Box-and-whisker plots of neutrophil speed **(K)** and aspect ratio **(L)** before (black) or after (red) 488 nm laser was switched on. n_c=11 from atleast 3 independent experiments; ns denotes $P>0.05$ (Wilcoxon-Mann-Whitney rank sum test).

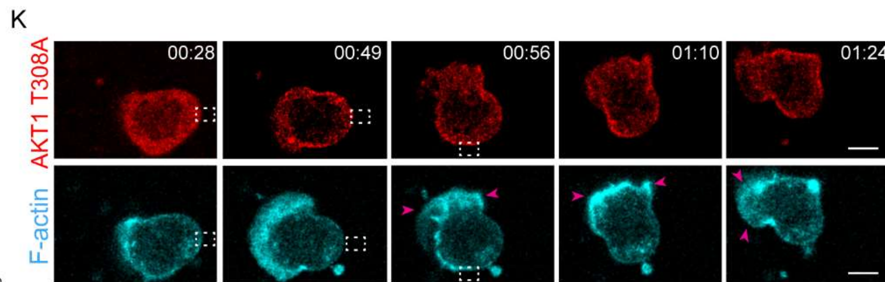

A
AS605240 - + +
488 nm M OFF OFF ON
100
75
50
37
25
20
AKT pT308
(60 kDa)
B
AS605240 - + +
488 nm M OFF OFF ON
75
50
37
25
20
AKT pS473
(60 kDa)
C
AS605240 - + +
488 nm OFF OFF ON M
100
75
50
37
25
20
Total AKT
(56 kDa)

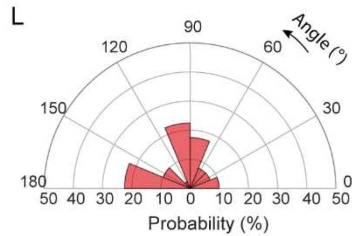

D
+ Rapamycin
F-actin
00:00
02:41
05:36
08:52
11:26
12:01


E
+Rapamycin


F
PP242 - + +
488 nm OFF OFF ON M M (low exposure)
AKT pS473
(60 kDa)
75
50
37
G
PP242 - + +
488 nm OFF OFF ON M M (low exposure)
AKTpT308
(60 kDa)
100
75
50
37
25
H
PP242 - + +
488 nm OFF OFF ON M
Total AKT
(56 kDa)
75
50
37
25


I
RASGRP4 RASAL3
K/HRAS-GTP
PI3K PIP3 mTORC2
pT308A AKT1
F-actin polymerization
Migration
J
AKT1 T308A
T308A
1 PH Kinase HD 480


K
AKT1 T308A
F-actin
00:28
00:49
00:56
01:10
01:24


L
Angle (°)
90
120
60
150
30
180
0
50 40 30 20 10 0 10 20 30 40 50
Probability (%)

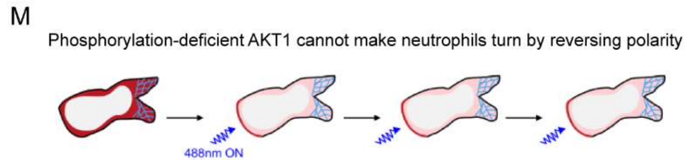
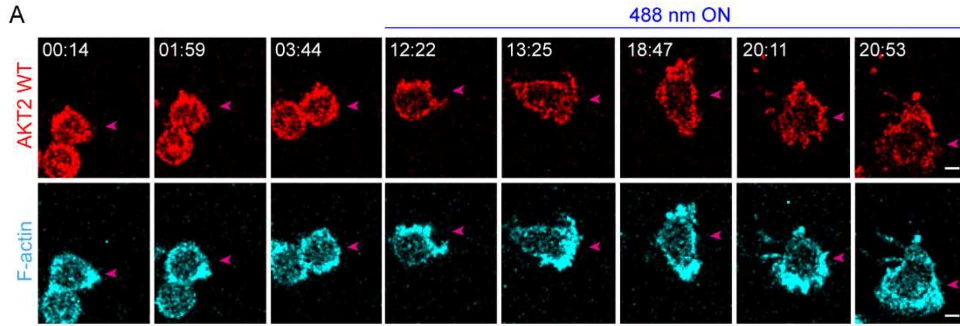
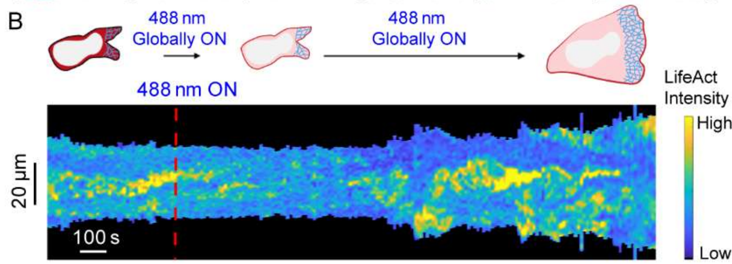
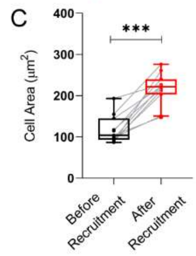
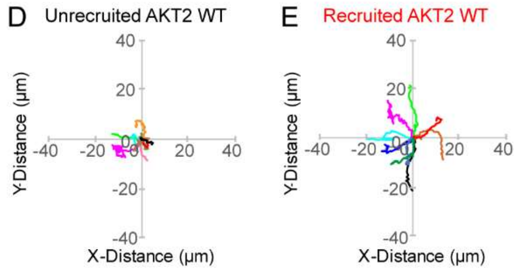

M
Phosphorylation-deficient AKT1 cannot make neutrophils turn by reversing polarity
488nm ON

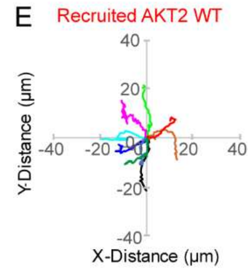
Figure S6. RasGRP4 activates Akt which rearranges pre-existing polarity via mTorC2 complex, and not mTorC1. Related to Figures 5 and 6. Uncropped western blots showing **(A)** phospho-Akt at T308 (60 kDa), **(B)** phospho-Akt at S473 (60 kDa), and **(C)** total Akt (56 kDa) status in untreated/unrecruited, AS605240-treated/unrecruited or AS605240-treated/RasGRP4-recruited whole cell lysates. Molecular weight markers indicated for each. White dotted boxes indicate cropped regions shown in Figure 5I. **(D)** Time-lapse confocal images of rapamycin-treated HL-60 neutrophils expressing LifeActmiRFP703 (cyan). Pink arrows show representative cells which have not lost polarity or show any deformity in shape or size after rapamycin treatment. Images are representative of many cells from atleast three independent experiments. Time in min:sec format. Scale bars represent 5 μm. **(E)** Cartoon depicting rapamycin treatment does not affect neutrophil shape or F-actin polymerization. Uncropped western blots showing **(F)** phospho-Akt at S473 (60 kDa), **(G)** phospho-Akt at T308 (60 kDa), and **(H)** total Akt (56 kDa) status in untreated/unrecruited, PP242-treated/unrecruited or PP242-treated/RasGRP4-recruited whole cell lysates. Molecular weight markers indicated for each. White dotted boxes indicate cropped regions shown in Figure 5R. **(I)** Experimental scheme testing if phospho-dead $Akt1_{T308A}$ optical recruitment can promote F-actin polymerization and migration. **(J)** Cartoon of $Akt1_{T308A}$ protein sequence with PH, kinase, and hypervariable domains. Due to T308A mutation, phosphorylation and kinase activity are absent. **(K)** Time-lapse confocal images of differentiated HL-60 neutrophil expressing CRY2PHR-mcherry2-$Akt1_{T308A}$ (red; upper panel) and LifeActmiRFP703 (cyan; lower panel). $Akt1_{T308A}$ is recruited exclusively to the cell rear by shining 488 nm laser near it, as indicated by the dashed white box. Pink arrows indicate new protrusions forming at the opposite end. Time in min:sec format. Scale bars represent 5 μm. **(L)** Polar histogram demonstrates $Akt1_{T308A}$ recruitment does not bias new protrusion formation; n_c=15 and n_p=40. **(M)** Cartoon illustrating that optically recruiting phosphorylation-deficient, kinase-dead $Akt1_{T308A}$ at the cell back has no effect on neutrophil polarity.

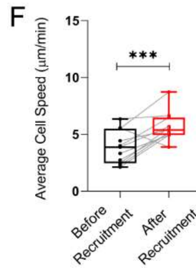

A
488 nm ON
00:14
01:59
03:44
12:22
13:25
18:47
20:11
20:53
AKT2 WT
F-actin

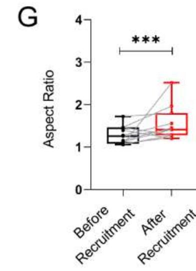

B
488 nm Globally ON
488 nm Globally ON
488 nm ON
LifeAct Intensity
High
Low
20 µm
100 s

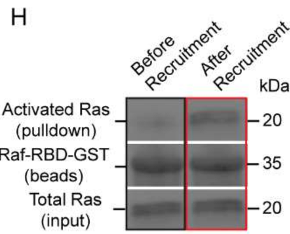

C
Cell Area (µm²)
***
Before Recruitment
After Recruitment

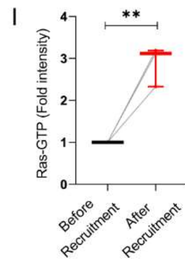

D
Unrecruited AKT2 WT
Y-Distance (µm)
X-Distance (µm)

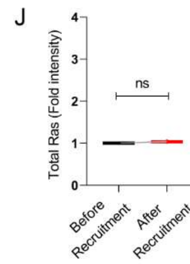

E
Recruited AKT2 WT
Y-Distance (µm)
X-Distance (µm)

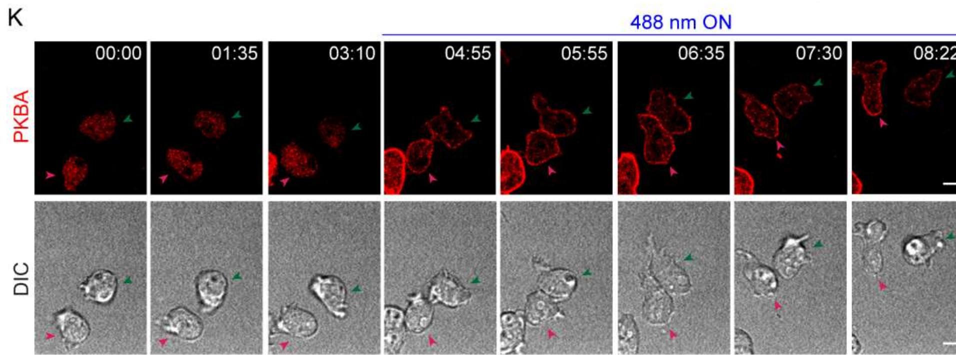

F
Average Cell Speed (µm/min)
***
Before Recruitment
After Recruitment


G
Aspect Ratio
***
Before Recruitment
After Recruitment


H
Before Recruitment
After Recruitment
kDa
Activated Ras (pulldown)
Raf-RBD-GST (beads)
Total Ras (input)
20
35
20


I
Ras-GTP (Fold intensity)
**
Before Recruitment
After Recruitment


J
Total Ras (Fold intensity)
ns
Before Recruitment
After Recruitment


K
488 nm ON
00:00
01:35
03:10
04:55
05:55
06:35
07:30
08:22
PKBA
DIC

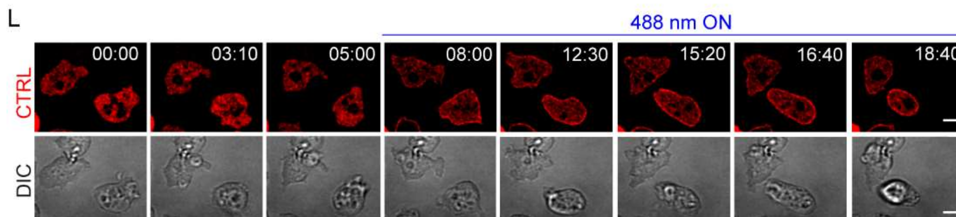

L
488 nm ON
00:00
03:10
05:00
08:00
12:30
15:20
16:40
18:40
CTRL
DIC

Figure S7. Global recruitment of Akt2 or PKBA improves neutrophil polarity and migration. Related to Figure 6. **(A)** Time-lapse confocal images of differentiated HL-60 neutrophil expressing CRY2PHR-mcherry2-AKT2 WT (red; upper panel) and LifeActmiRFP703 (cyan; lower panel), before or after 488 nm laser was switched on globally. Pink arrows denote representative cell. Time in min:sec format. Scale bars represent 5 μm. **(B)** Representative kymograph of cortical LifeAct intensity in Akt2 WT-expressing neutrophil before or after 488 nm laser was turned on. A linear color map shows that blue is the lowest LifeAct intensity whereas yellow is the highest. Duration of the kymograph is 23 mins. Cartoon depicts recruitment, F-actin polymerization or cell shape status corresponding to the kymograph. **(C)** Box-and-whisker plot of neutrophil cell area before (black) or after (red) CRY2PHR-mcherry2-Akt2 WT membrane recruitment. n_c=11 from atleast 3 independent experiments; asterisks indicate significant difference, ***$P \leq 0.001$ (Wilcoxon-Mann-Whitney rank sum test). Centroid tracks of neutrophils (n_c=11) showing random motility before **(D)** or after **(E)** Akt2 WT membrane recruitment. Each track lasts atleast 5 mins and was reset to same origin. Box-and-whisker plots of neutrophil speed **(F)** and aspect ratio **(G)** before (black) or after (red) Akt2 WT membrane recruitment. n_c=11 from atleast 3 independent experiments; asterisks indicate significant difference, ***$P \leq 0.001$ (Wilcoxon-Mann-Whitney rank sum test). **(H)** Representative western blot showing active Ras levels after pulldown (top panel), Raf-RBD-GST in beads (loading control; middle panel) and total Ras (input; lower panel) of unrecruited or Akt2 WT-recruited samples. Fold intensities of **(I)** activated and **(J)** total Ras in unrecruited or Akt2-recruited samples. Data is shown as mean±SD from 3 independent experiments; asterisks indicate significant difference, **$P \leq 0.01$, and ns denotes $P>0.05$ (Wilcoxon-Mann-Whitney rank sum test). **(K)** Time-lapse confocal images of vegetative *Dictyostelium* expressing tgRFPt-SSPB R73Q-PKBA (red; upper panel), before or after 488 nm laser was switched on globally. Within minutes of PKBA recruitment, previously non-polarized cells formed sustained protrusions and started migrating (DIC; lower panel). Pink and green arrows are provided to demarcate the two cells throughout the images. These are representative of many cells from atleast three independent experiments. Time in min:sec format. Scale bars represent 5 μm. **(L)** Time-lapse confocal images of vegetative *Dictyostelium* expressing tgRFPt-SSPB R73Q-CTRL (control, red; upper panel), before or after 488 nm laser was switched on globally. Non-polarized cells do not polarize or migrate once CTRL is recruited to the membrane (DIC; lower panel). These images are representative of many cells from atleast three independent experiments. Time in min:sec format. Scale bars represent 5 μm.

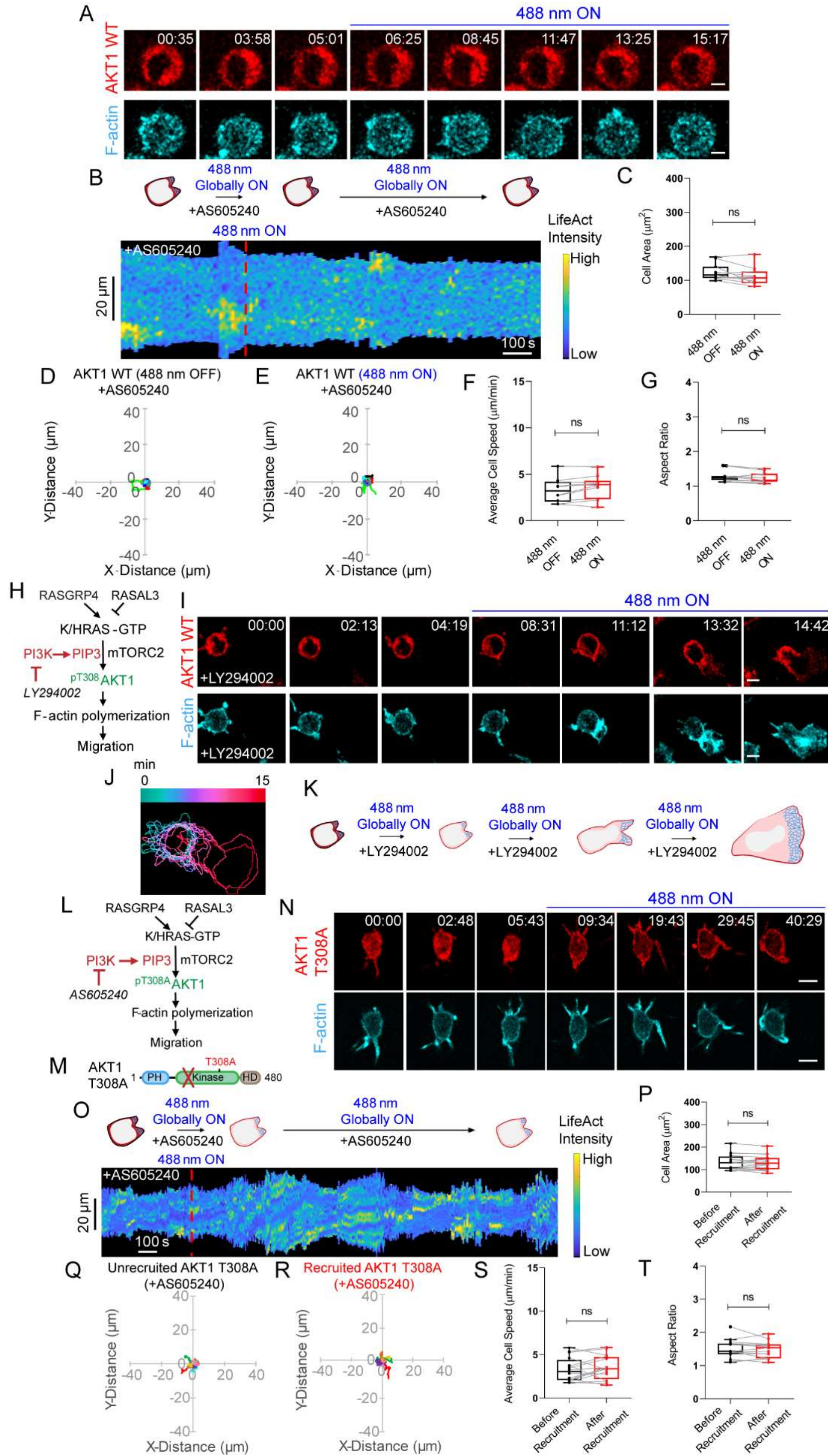

A
488 nm ON
AKT1 WT
F-actin
00:35
03:58
05:01
06:25
08:45
11:47
13:25
15:17
B
488 nm Globally ON
+AS605240
488 nm ON
LifeAct Intensity
High
Low
20 µm
100 s
C
Cell Area (µm2)
ns
488 nm OFF
488 nm ON
D
AKT1 WT (488 nm OFF) +AS605240
E
AKT1 WT (488 nm ON) +AS605240
Y-Distance (µm)
X-Distance (µm)
F
Average Cell Speed (µm/min)
G
Aspect Ratio
H
RASGRP4
RASAL3
K/HRAS-GTP
PI3K
PIP3
mTORC2
pT308 AKT1
LY294002
F-actin polymerization
Migration
I
AKT1 WT
F-actin
+LY294002
00:00
02:13
04:19
08:31
11:12
13:32
14:42
J
min
0
15
K
488 nm Globally ON
+LY294002
L
AS605240
pT308A AKT1
M
AKT1 T308A
PH
Kinase
HD
T308A
480
N
AKT1 T308A
00:00
02:48
05:43
09:34
19:43
29:45
40:29
O
P
Before Recruitment
After Recruitment
Q
Unrecruited AKT1 T308A (+AS605240)
R
Recruited AKT1 T308A (+AS605240)
S
T

Figure S8. Recruited Akt1, through its T308 phosphorylation, polarizes neutrophil migration even in presence of multiple PI3K inhibitors. Related to Figure 6. **(A)** Time-lapse confocal images of AS605240-treated HL-60 neutrophil expressing CRY2PHR-mcherry2-Akt1 WT (red; upper panel) and LifeActmiRFP703 (cyan; lower panel), before or after 488 nm laser was switched on globally. No appreciable Akt1 WT recruitment was observed presumably due to low expression of CIBN-CAAX membrane anchor. Time in min:sec format. Scale bars represent 5 μm. **(B)** Representative kymograph of cortical LifeAct intensity in AS605240-treated Akt1 WT-expressing neutrophil before or after 488 nm laser was turned on. A linear color map shows that blue is the lowest LifeAct intensity whereas yellow is the highest. Duration of the kymograph is 16 mins. Cartoon depicts recruitment, F-actin polymerization or cell shape status corresponding to the kymograph. **(C)** Box-and-whisker plot of neutrophil cell area before (black) or after (red) 488 nm laser was turned on. n_c=10 from atleast 3 independent experiments; ns denotes P>0.05 (Wilcoxon-Mann-Whitney rank sum test). Centroid tracks of AS605240-treated neutrophils (n_c=10) showing random motility before **(D)** or after **(E)** 488 nm laser was turned on. Each track lasts atleast 5 mins and was reset to same origin. Box-and-whisker plots of neutrophil speed **(F)** and aspect ratio **(G)** before (black) or after (red) 488 nm laser was switched on. n_c=10 from atleast 3 independent experiments; ns denotes P>0.05 (Wilcoxon-Mann-Whitney rank sum test). **(H)** Experimental scheme to test whether Akt1 WT recruitment can activate F-actin polymerization and migration in presence of pan-PI3K inhibitor, LY294002. **(I)** Time-lapse confocal images of LY294002-treated HL-60 neutrophil expressing CRY2PHR-mcherry2-Akt1 WT (red; upper panel) and LifeActmiRFP703 (cyan; lower panel), before or after 488 nm laser was switched on globally. Time in min:sec format. Scale bars represent 5 μm. **(J)** Color-coded (at 1 min intervals) outlines of the cell shown in (B). **(K)** Cartoon depicts recruitment, F-actin polymerization or cell shape status corresponding to images in (B). **(L)** Experimental scheme testing if phospho-dead Akt1$_{T308A}$ optical recruitment can overcome PI3Kγ inhibition. **(M)** Cartoon of Akt1$_{T308A}$ protein sequence with PH, kinase, and hypervariable domains. Due to T308A mutation, phosphorylation and kinase activity are absent. **(N)** Time-lapse confocal images of AS605240-treated HL-60 neutrophil expressing CRY2PHR-mcherry2- Akt1$_{T308A}$ (red; upper panel) and LifeActmiRFP703 (cyan; lower panel), before or after 488 nm laser was switched on globally. Time in min:sec format. Scale bars represent 5 μm. **(O)** Representative kymograph of cortical LifeAct intensity in AS605240-treated Akt1$_{T308A}$-expressing neutrophil before or after 488 nm laser was turned on. A linear color map shows that blue is the lowest LifeAct intensity whereas yellow is the highest. Duration of the kymograph is 46 mins. Cartoon depicts recruitment, F-actin polymerization or cell shape status corresponding to the kymograph. **(P)** Box-and-whisker plot of cell area before (black) or after (red) Akt1$_{T308A}$ membrane recruitment. n_c=16 from atleast 3 independent experiments; ns denotes P>0.05 (Wilcoxon-Mann-Whitney rank sum test). Centroid tracks of neutrophils (n_c=16) showing random motility before **(Q)** or after **(R)** Akt1$_{T308A}$ membrane recruitment. Each track lasts atleast 5 mins and was reset to same origin. Box-and-whisker plots of cell speed **(S)** and aspect ratio **(T)** before (black) or after (red) Akt1$_{T308A}$ recruitment. n_c=16 from atleast 3 independent experiments; ns denotes P>0.05 (Wilcoxon-Mann-Whitney rank sum test).

Table S1. Oligonucleotides used in this paper. Related to STAR Methods.

Primer	Primer Sequence (5'- 3')[a]	Purpose
P1	CGC*GCTAGC*CGCCACCATGAATGGAGCTATAGGAGGTGACCTTTTGCTC	Cloning of CIBN-CAAX
P2	GCG*GAATTC*TTACTACATAATTACACACTTTG	
P3	CGC*GCTAGC*GCCACCATGGGCGTGGCCGACTTGATCAAGAA	Cloning of LifeActmiRFP703
P4	GCG*GAATTC*TTAGCTCTCAAGCGCGGTGATCCGCGT	
P5	CGC*CTCGAG*GCCACCATGAAGATGGACAAAAAGACTATAGTTTG	Cloning of CRY2PHR-mcherry2
P6	GCG*GCGGCCGC*CTCCGGAACCCCCTGCCCCAGCCTTGTACAGCTCGTCCATGCC	
P7	CGC*TCCGGA*ACGGAATATAAGCTGGTGGTGGTGGGC	Cloning of HRas G12V ΔCAAX
P8	GCG*GTCGAC*TCACTTGCAGCTCATGCAGCCGGG	
P9	CGC*TCCGGA*ACTGAATATAAACTTGTGGTAGTTGGAGCTGTTGGC	Cloning of KRas4B G12V ΔCAAX
P10	GCG*GTCGAC*TTACTTTGTCTTTGACTTCTTTTTCTTCTTTTTACCATCTTTGC	
P11	CGC*TCCGGA*AACAGAAAAGACAGTAAGAGGAAGTCCCAC	Cloning of RasGRP4
P12	GCG*GTCGAC*TTAGGAATCCAGCTTGGAGGATGCAGT	
P13	CGC*GCGGCCGC*GATCCCCCGTCCCCCTCACG	Cloning of RASAL3
P14	GCG*GCGGCCGC*TTAAGTAGTATCCCCGTTCAGGCACGG	
P15	CGC*TCCGGA*AGCGACGTGGCTATTGTGAAGGAGGG	Cloning of Akt1
P16	GCG*GTCGAC*TCAGGCCGTGCCGCTGGC	
P17	CGC*TCCGGA*AGCGACGTGGCTATTGTGAAGGAGGG	Cloning of $Akt1_{T308A}$
P18	GCG*GTCGAC*TCAGGCCGTGCCGCTGGC	
P19	CGC*TCCGGA*AATGAGGTGTCTGTCATCAAAGAAGGCTGG	Cloning of Akt2
P20	GCG*TCCGGA*TCACTCGCGGATGCTGGCC	
P21	CGC*TGTACA*AGGCTGGGGCAGGGGGTGATCCCCCGTCCCCCTCACG	Cloning of RASAL3
P22	GCG*GCGGCCGC*TTAAGTAGTATCCCCGTTCAGGCACGG	
P23	CGC*TGTACA*AGGCTGGGGCAGGGGGTTCCGGAAACAGAAAAGACAGTAAGAGG AAGTCCCAC	Cloning of RasGRP4
P24	GCG*GCGGCCGC*TTAGGAATCCAGCTTGGAGGATGCAGT	
P25	TGGATCCGCT*GCTAGC*ATGTCAACAGCACCAATTAAACATG	Cloning of PKBA
P26	CCGTCGACA*GCGGCCGC*TTATCTTAAATGTTCAGATTCAGCG	

[a] restriction sites in primer sequences are italicized.