

1 Ras-mediated homeostatic control of front-back signaling dictates cell polarity

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18 Abstract

19 Studies in the model systems, *Dictyostelium* amoebae and HL-60 neutrophils, have shown
20 that local Ras activity directly regulates cell motility or polarity. Localized Ras activation on the
21 membrane is spatiotemporally regulated by its activators, RasGEFs, and inhibitors, RasGAPs,
22 which might be expected to create a stable ‘front’ and ‘back’, respectively, in migrating cells.
23 Focusing on C2GAPB in amoebae and RASAL3 in neutrophils, we investigated how Ras activity
24 along the cortex controls polarity. Since existing gene knockout and overexpression studies can be
25 circumvented, we chose optogenetic approaches to assess the immediate, local effects of these Ras
26 regulators on the cell cortex. In both cellular systems, optically targeting the respective RasGAPs
27 to the cell front extinguished existing protrusions and changed the direction of migration, as might
28 be expected. However, when the expression of C2GAPB was induced globally, amoebae polarized
29 within hours. Furthermore, within minutes of globally recruiting either C2GAPB in amoebae or
30 RASAL3 in neutrophils, each cell type polarized and moved more rapidly. Targeting the RasGAPs
31 to the cell backs exaggerated these effects on migration and polarity. Overall, in both cell types,
32 RasGAP-mediated polarization was brought about by increased actomyosin contractility at the
33 back and sustained, localized F-actin polymerization at the front. These experimental results were
34 accurately captured by computational simulations in which Ras levels control front and back
35 feedback loops. The discovery that context-dependent Ras activity on the cell cortex has
36 counterintuitive, unanticipated effects on cell polarity can have important implications for future
37 drug-design strategies targeting oncogenic Ras.

38 **Keywords:** cancer metastasis, biochemical excitability, actin cytoskeleton, optogenetics, signaling

39 Introduction

40 Ras GTPases play a vital role in transmitting signals within cells, influencing growth and
41 survival, and activating mutations in Ras genes are found in ~30% of all cancers¹⁻³. As a result,
42 targeting mutant Ras has become a major focus in cancer drug development. While extensive
43 research has been dedicated to understanding the role of Ras mutations in promoting cancer
44 growth, the impact of these mutations on cell migration and metastasis has received relatively less
45 attention. Nevertheless, studies conducted in the model systems, *Dictyostelium* amoebae and
46 human neutrophils, suggest that local Ras activity plays a direct and immediate role in cell polarity
47 and motility⁴⁻⁹.

48 Biochemical and genetic investigations have revealed that Ras is activated both by
49 stimulation of G-protein coupled receptors and spontaneously at protrusions^{6,10-13}. Ras function is
50 spatiotemporally controlled by its activators, RasGEFs, and inhibitors, RasGAPs, which might be
51 expected to create 'front' and 'back', respectively, during cell migration^{11,14-19}. However, Ras
52 activity must be carefully balanced, as constitutively active Ras expression can lead to hyper-
53 activation of the PI3K/TORC2-PKB pathways, resulting in significant cell spreading and defective
54 migration^{5,20,21}. Additionally, knockout/knockdown studies of RasGAPs, such as NF1, C2GAP1,
55 C2GAP2, and CAPRI have demonstrated their significant impact on Ras and protrusive
56 activities^{8,11,17,18,22,23}. For example, knockout of NF1 in *Dictyostelium* causes increased
57 macropinocytosis, and moreover, human genetic deletion of NF1 results in severe
58 neurofibromatosis type 1²⁴⁻²⁶.

59 Understanding the physiological relevance of manipulating Ras activity is of paramount
60 importance, but knockout studies of Ras proteins have proven relatively ineffective, possibly due
61 to redundancy. For example, *Dictyostelium* cells lacking Ras isoforms continue to grow and
62 directed migration remains little affected²⁷⁻²⁹. Furthermore, while new treatments with small
63 molecule inhibitors targeting constitutively active KRasG12C show promise, they also pose
64 significant challenges³⁰⁻³². A potentially powerful alternative route of inhibiting Ras activity would
65 be to activate the RasGAP proteins. Indeed, a recent study showed that locally activating a
66 RasGAP can inhibit Ras and halt random and directed migration in immune cells⁴.

67 We designed a series of studies to test the capacity of RasGAPs to manipulate Ras activity.
68 Two different RasGAP proteins, C2GAPB and RASAL3, were able to inhibit Ras activity in
69 *Dictyostelium* and differentiated HL-60 neutrophils, respectively, but surprisingly, under certain
70 conditions, these cells become highly polarized and migrate rapidly. Our evidence suggests that
71 the polarization was due to an increase in suppression of Ras activity leading to increased
72 contraction at the back. Taken together with previous observations of hyper-activated Ras activity,
73 our study shows that there is not a direct linear relationship between Ras activation and migration,
74 and an optimal level of activated Ras leads to polarity and migration. Our study has important
75 implications for interventions, such as small molecule inhibitors, designed to simply reduce Ras
76 activity as a reduction could cause unanticipated effects such as exaggerated metastasis.

77 Results

78 RasGAPs inhibit Ras and PI3K activities to shut off cellular protrusions

79 To investigate the impact of Ras activity on cell migration and polarity, we examined the
80 effects of RasGAP, C2GAPB, in modulating Ras activity and downstream PI3K activity. Since we

81 encountered challenges in expressing RasGAP proteins using standard expression methods, we
82 developed a doxycycline-inducible system to improve C2GAPB expression. This system not only
83 facilitated efficient expression of RasGAP proteins but also allowed for convenient comparison of
84 results in the absence or presence of RasGAP expression. We first compared Ras or PI3K activities
85 by expressing fluorescence-labeled biosensors, RBD or PHcrac, respectively, with or without
86 C2GAPB expression. Confocal imaging of the midline of cells revealed broad patches of RBD and
87 PIP3 at the protrusions in the absence of C2GAPB. However, upon overnight induction of
88 C2GAPB expression, these patches reduced in size (Figure 1A-C). Previous studies have shown
89 that the Ras/PIP3 patches underlie protrusions which mediate cell movement and
90 macropinocytosis^{11,33,34}. Uptake measurements showed that macropinocytosis was significantly
91 reduced upon C2GAPB expression (Figure S1A and B).

92 Activities of Ras and PI3K have been extensively documented to form broad, propagating waves
93 on the basal surface of electrofused, giant *Dictyostelium* cells^{12,13,35-40}. Thus, we further
94 investigated the effects of C2GAPB on Ras/PIP3 waves and found that both RBD and PHcrac
95 waves became smaller and more transient upon expressing C2GAPB (Figure 1D and G; Videos
96 S1-4). Quantitative analysis revealed that in the absence of C2GAPB expression, the area covered
97 by RBD or PHcrac waves at steady-state varied between 35% and 80% of the total cell area, but
98 with induction of C2GAPB, the wave area decreased to less than 20% (Figure 1E and H). The
99 mean duration of a wave also decreased from 5 minutes to less than a minute (Figure 1F and I).
100 Therefore, inducible C2GAPB expression led to a significant reduction of RBD and PHcrac
101 patches and waves (Figure 1C and J). Conversely, in C2GAPB null cells, wide, propagating RBD
102 waves were observed, validating that C2GAPB expression suppresses Ras activity (Figure S1C)¹¹.

103 To further investigate the functional implications of C2GAPB on cellular protrusions and
104 cytoskeleton remodeling, we utilized optogenetics to transiently recruit C2GAPB to the plasma
105 membrane. Based on our observations that activated Ras and PIP3 levels decreased with C2GAPB
106 expression, we hypothesized that the recruitment of C2GAPB could inhibit cellular protrusions. In
107 our system, upon 488 nm laser stimulation, mRFPmars-SspB fused C2GAPB is recruited to
108 membrane anchor N150-illD (Figure 2A). When C2GAPB was recruited to the cellular protrusions
109 at the leading edge (indicated with white arrows), mature protrusions quickly vanished causing the
110 cell to contract, while new protrusions emerged at the former back end of the cell (shown with
111 pink arrows). Consequently, the cell moved in the opposite direction (Figure 2A and C). Angular
112 histogram analyses revealed that the probability of nascent protrusion formation was highest at
113 ~120-150 degrees away from the C2GAPB recruitment area (Figure 2D). In contrast, although
114 there was a slight effect of light, recruitment of the empty control vector did not block the
115 production of new protrusions, and the cell continued to move close to its original direction (Figure
116 2E). Angular histogram showed that the highest probability of new protrusion formation occurred
117 at ~0-40 degrees from the C2GAPB recruitment area (Figure 2F).

118 Since C2GAPB had a strong effect on cell behavior, we checked whether it was having this effect
119 by reducing Ras and PI3K activities. While unrecruited cells displayed multiple, small RBD or
120 PHcrac patches ('00:00' in Figure 2G and H), once C2GAPB was rapidly recruited it caused a
121 simultaneous reduction in activated Ras and PIP3 levels at the protrusions ('00:15'-2:30' in Figure
122 2G and H). Similarly, we observed a strong reduction in activated Ras and PIP3 propagating waves
123 upon C2GAPB recruitment (Figure 2I and J; Videos S5 and S6). Once we switched off the blue
124 light, RBD waves recovered within a minute (Video S5).

125 We extended our investigation to differentiated HL-60 neutrophils to assess the conservation of
126 RasGAP function on cytoskeletal remodeling. As previously reported, we developed a recruitable
127 RASAL3 by fusing it with CRY2-mCherry, allowing for recruitment to the membrane anchor,
128 CIBN-CAAX (Figure 2B)⁴. Recruiting RASAL3 to the F-actin-rich front, marked by LifeAct,
129 caused mature protrusions to immediately disappear. Simultaneously, a new broad front emerged
130 at the opposite end of the cell, causing it to migrate away (Figure 2K; Video S7). Angular
131 histogram analysis confirmed that new protrusions consistently appeared at ~130-170 degrees
132 from the RASAL3 recruitment site (Figure 2L).

133 These observations, both in *Dictyostelium* and human neutrophils, demonstrate that locally
134 recruiting RasGAPs resulted in the immediate shutdown of front signaling, and consequently
135 cellular protrusions, and thereby reversed the existing front-back polarity.

136 Inhibitory function of RasGAPs induce polarity and enhance random cell migration

137 Given the results from Figures 1 and 2, we expected that ectopic global expression of
138 RasGAPs would completely inhibit cellular activity and largely decrease cell migration.
139 Surprisingly, inducing C2GAPB expression resulted in a more polarized phenotype (Figure 3A,
140 also see Figure 1A and B). Moreover, polarized C2GAPB-expressing cells exhibited accelerated
141 movement compared to control cells expressing empty vector, as shown in time series images and
142 color-coded temporal overlay profiles (Figure 3B and C; Video S8). Quantification revealed a 2-
143 fold increase in average cell speed and aspect ratio, a proxy for cell polarity, as compared to
144 uninduced control (Figure 3D and E). Although the C2GAPB-expressing cells changed shape, we
145 did not observe a significant increase in basal cell area (Figure 3F).

146 To further characterize this polarization phenotype induced by C2GAPB expression, we examined
147 the localization pattern of two front-back indicators for cytoskeletal activities, LimE, a biosensor
148 for F-actin, and myosin II. Vegetative, wildtype *Dictyostelium* typically displayed multiple,
149 transient, small F-actin fronts, whereas C2GAPB null cells demonstrated an unpolarized
150 morphology with wide fronts (Figures 3G and S1D). However, in C2GAPB-expressing cells, there
151 was a single, broad, polarized LimE-rich protrusion persistently localized at the front of the
152 migrating cell (Figure 3G). Similarly, C2GAPB expression caused myosin II to localize
153 predominantly at the back of the cells, while a more diffused distribution was generally observed
154 in uninduced cells (Figure 3K). These observations were supported by linescan and kymograph
155 analyses (Figure 3H-J and L-N), demonstrating that after C2GAPB induction, actin polymerization
156 and myosin II were localized to a single front and back while being suppressed elsewhere (Figure
157 3O). Although the acquisition of polarity is a defining characteristic of differentiation in
158 *Dictyostelium*, C2GAPB-induced polarization did not require development or GPCR signaling.
159 C2GAPB expression induced similar polarization in Gβ null cells, which are typically unpolarized
160 with low motility, and improved their migration speed (Figure S2A-E)⁴¹⁻⁴³.

161 Given the surprising polarizing effect of C2GAPB expression, we checked whether
162 recruiting C2GAPB to the cell membrane, in closer association with Ras, would exaggerate its
163 polarization effects. We noticed that expressing the recruitable C2GAPB protein, fused to SspB,
164 only moderately induced polarity (Figure 4B), as compared to the native C2GAPB (Figures 1 and
165 3). The SspB tag may be responsible for a weaker C2GAPB function, but this was not explored in
166 our study. However, the global recruitment of C2GAPB triggered a robust and ‘instant
167 polarization’ response within a minute resulting in a 2-fold increase in average migration speed
168 and aspect ratio, but not basal cell area (Figure 4A-E; Videos S9 and S10). However, once the blue

169 laser was switched off, cells reverted to their original morphology within minutes (Figure 4F-H;
170 Video S10). There were several F-actin rich fronts in unrecruited cells ('00:00' in Figure 4I).
171 C2GAPB recruitment first reduced LimE signals everywhere on the membrane, then as the cell
172 polarized, a single and persistent actin polymerization site grew at the leading front (Figure 4I and
173 J). Interestingly, we also successfully polarized G β null cells with C2GAPB recruitment (Figure
174 S2F and G, Video S11). Taken together, these observations also imply that polarity establishment
175 does not require changes in gene and protein profiles which do not take place within a minute.

176 Next, we validated these C2GAPB-induced effects on LimE wave patterns on the basal surface of
177 electrofused cells. Like single cells, expressing recruitable C2GAPB modestly affected wave
178 patterns (Figure 4K). However, upon recruitment, F-actin waves largely disappeared throughout
179 the cell except for one region on the basal membrane where LimE signal remained strong and did
180 not propagate (Figure 4K and L; Video S12). Thus, our data shows that both signaling, and
181 cytoskeletal waves are largely extinguished but cytoskeletal activity remains persistently confined
182 to one location on the membrane, presumably indicating sustained polarity.

183 **Localization of recruited RasGAP to the back of the cell led to even stronger polarization**

184 Previously, we showed that recruiting RASAL3 to the front in neutrophils extinguished
185 mature protrusions and made the cells move away (Figure 2K and L). When we continued to
186 extinguish any new protrusions, the cell rounded up completely and did not move (Figure 5A).
187 However, once we stopped extinguishing new protrusions, the cell continued to move away
188 persistently from the last recruitment area (Figure 5A and B; Video S13). Strikingly, RASAL3
189 simultaneously rearranged itself to the back region, presumably dragging CIBN-CAAX membrane
190 anchor with it. The previously non-polarized, migration-incompetent cell now had a stable back
191 and a sustained front which allowed it to migrate away (Figure 5A-C).

192 This spontaneous 'back' rearrangement of RASAL3 made it necessary to repeatedly extinguish
193 new fronts to prevent cell movement, since when RASAL3 was globally recruited over the entire
194 cell periphery, it rapidly localized to the rear and polarized the cell. This self-arranged back
195 localization was dependent on cytoskeletal dynamics (Figure S3A and B) and was mediated
196 through the C-terminal tail of RASAL3 (Figure S3C-E). As a result of this strong back localization,
197 neutrophils developed long adhesive uropods at the RASAL3-enriched regions, became highly
198 polarized and migrated (Figure 5D and E; Video S14). Although F-actin levels increased at the
199 front with RASAL3 recruitment, it did not appear to be as high as F-actin changes induced with
200 recruitment of constitutively active Ras or RasGEF shown previously⁴. Across the population,
201 upon RASAL3 back localization, cells moved more extensively with a 1.4-fold increase in average
202 cell speed (Figure 5G-I). Generally, RASAL3 recruitment also induced 1.44- and 1.26-fold
203 increase in cell area and polarity, respectively (Figure 5J and K). A similar polarization was
204 induced upon recruiting the GAP domain fused to its C-terminal tail (Figure S3F-H). No such
205 phenotypic change was observed when the CRY2PHR component, without being fused to
206 RASAL3, was recruited to the cell membrane (Figure S4A-C; Video S15; also Figure S3H-N in⁴).
207 Similarly, when we recruited C2GAPB locally to the back of *Dictyostelium*, the cell moved away
208 persistently (Figure S4D and E). Altogether, these results suggest that reduction in Ras activity
209 can polarize cells, but the localization of RasGAPs to the back can lead to a strong polarization of
210 cells.

211

212 **RasGAPs polarize cells by increasing contractility at the back**

213 We were interested to check if the RasGAP-induced protrusions were derived from Arp2/3-
214 mediated actin. We used Arp2/3 inhibitor, CK-666, which completely removed all protrusions in
215 neutrophils (Figure S5A)^{4,44}. When we globally recruited RASAL3 and it localized to the back,
216 interestingly, cells made long, thin protrusions which did not display LifeAct biosensor at the tips.
217 This led us to believe that these structures are long blebs which were forming presumably through
218 hydrostatic pressure elsewhere. Rather, the biosensor appeared to be along the lateral edges of
219 these narrow protrusions (Figure S5A and B). Similarly, long blebs were induced upon C2GAPB
220 expression in CK-666-treated *Dictyostelium* cells, but not in control cells where C2GAPB was
221 absent (Figure S5C and D). These observations suggested that the polarizing effects of the
222 RasGAPs were due to increased contraction.

223 To test whether this contraction was mediated by actomyosin, we induced C2GAPB
224 expression in myosin II heavy chain null (*mhcA*⁻) mutant of *Dictyostelium*, and also recruited
225 C2GAPB to the membrane. The *mhcA*⁻ cells, as previously reported, are generally flattened,
226 unpolarized, and multinucleated cells⁴⁵⁻⁴⁸. Expression of C2GAPB had little effect on improving
227 this existing phenotype in this cell line (Figure 6A). Furthermore, robust recruitment of C2GAPB
228 that typically polarizes wild type cells, failed to do so in the *mhcA*⁻ cells (Figure 6B). Similarly, in
229 C2GAPB-recruited polarized cells, blebbistatin (myosin II inhibitor) could diminish polarity
230 although it was not as effective as a complete loss of myosin in the *mhcA*⁻ cells (Figure S6A)⁴⁹.

231 We tested whether myosin-based contractility was needed in neutrophils by treating them with
232 blebbistatin (Figure 6C). Treatment with the myosin II inhibitor resulted in complete stoppage of
233 cell movement and loss of polarity (Figures 6D and S6B)⁵⁰⁻⁵². When RASAL3 was globally
234 recruited, it moved to a particular region which became the new back. This local suppression of
235 Ras activity in the region overcame blebbistatin inhibition and caused increased contraction. The
236 polarized cells began to move rapidly, although the blebbistatin-treated cells looked and behaved
237 differently from untreated, RASAL3-recruited cells (Figure 6D; Video S15). With RASAL3
238 recruitment, compared to untreated cells, blebbistatin treated cells migrated faster with largely
239 diminished uropods and bigger F-actin-driven fronts (Figure 6D and E, G-I; Video S16).
240 Representative kymograph showed increased LifeAct intensity and cell area (Figure 6F). Overall,
241 blebbistatin-treatment improved migration speed and cell area of RASAL3-recruited cells by over
242 50% and 25%, respectively (Figures 6G-J and 5G-J), whereas aspect ratio was reduced to half of
243 what was seen for untreated, recruited cells (Figures 6K and 5K). We validated our results by
244 treating neutrophils with the ROCK inhibitor, Y27632, which stalled basal motility (Figure S6C
245 and D)⁵³⁻⁵⁵. RASAL3 recruitment and its back localization mostly overcame the effects of ROCK
246 inhibition; cells polarized and migrated with huge F-actin rich fronts and relatively short uropods
247 (Figure S6E-G; Video S17). Generally, RASAL3 recruitment induced 1.91-, 1.53- and 1.46-fold
248 increase in cell speed, cell area and polarity, respectively (Figure S6H-L). While the inability of
249 C2GAPB to polarize *Dictyostelium* *mhcA*⁻ cells suggests that myosin is required, it is unclear
250 whether myosin is dispensable for RASAL3 to polarize neutrophils. It is possible that the
251 inhibitors used are not completely effective. Nevertheless, our data suggests that myosin does
252 contribute to the rear contraction since RASAL3-induced uropods are much weaker in the presence
253 of blebbistatin or Y27632⁵⁶.

255 **RasGAPs polarize cells by localizing actin polymerization at the front**

256 Previously, we observed that back localization of recruited RASAL3 induced F-actin to
257 localize to a stable front which caused neutrophils to polarize and migrate persistently (Figure 5D-
258 K). To confirm that RASAL3 at the back is directly affecting protrusive activity at the front, we
259 pharmacologically targeted front signaling pathways in neutrophils before recruiting RASAL3.
260 First, we inhibited PI3K/PIP3 signaling using pan-PI3K inhibitor, LY294002, which stalled
261 neutrophil polarity and basal motility (Figure 7A and B)^{4,57}. Within 5 minutes of applying blue
262 light globally, RASAL3 moved to the back region of LY294002-treated cells and polarized them
263 to move by generating broad F-actin-rich lamellipodium but without an appreciable uropod (Figure
264 7B and C, F and G; Video S18). The kymograph shows that the LifeAct level increased
265 considerably along with an increase in cell area (Figure 7D). Across the entire population,
266 RASAL3 recruitment caused 1.75-, 1.49, or 1.43-fold improvement in average cell speed, basal
267 cell area or aspect ratio (Figure 7E, H and I). We observed a similar recovery in polarity and
268 random motility in presence of a PI3K γ inhibitor, AS605240, with RASAL3 recruitment (Figure
269 S7; Video S19)^{4,58,59}.

270 Next, we inhibited mTORC2 signaling in neutrophils using the established mTOR inhibitor, PP242
271 (Figure 7J)⁶⁰. Inhibitor treatment caused cells to round up, which is due to mTORC2 complex
272 inhibition and not mTORC1 (Figure 7K)^{4,61}. Once RASAL3 recruitment was initiated after turning
273 on the blue laser, cells displayed weak, intermittent polarity (Figure 7K and L). Time-lapse
274 imaging and migration tracks showed that recruited RASAL3, which goes to the back, moved
275 PP242-treated cells slightly, but not persistently (Figure 7K, O and P; Video S20). As a result,
276 although we noticed a 1.3-fold improvement in average cell speed, we did not see any significant
277 change in cell area or polarity (Figure 7M and N, Q and R).

278 **Increasing RasGAP in simulations of the Signal Transduction Excitable Network lead to
279 reduced Ras activity but cells can still polarize**

280 To determine the role of RasGAP on the level of Ras on the signaling network, we turned
281 to a model of the excitable behavior that has been used to recreate the observed wave behavior in
282 cells^{37,62,63}. As shown in Figure 8A, the model incorporates complementary inhibition between Ras
283 and PIP2 which acts as a positive feedback loop^{11,64}. There is a competing negative feedback loop
284 involving Ras and PKB^{37,64}. These three elements form the core of the Signal Transduction
285 Excitable Network (STEN). Additionally, we incorporate two further feedbacks to capture the
286 polarizing behavior that is observed over time^{62,65}. Note that here we have implemented one of the
287 polarity loops as a positive loop emanating from back events in STEN, such as PIP2, rather than
288 as negative feedback from F-actin. Simulations based on this model display characteristic excitable
289 behavior, including propagating waves that annihilate when they come together (Figure 8B; Video
290 S21). We considered the effect of increasing/decreasing RasGAP activity. Whereas simulations in
291 which RasGAP was reduced showed increased Ras activity and resulted in large, fast wave patterns
292 compared to WT levels of RasGAP, those with increased RasGAP showed small, slow waves
293 (Figure 8B; Video S21). These observations were confirmed by summing all Ras activity
294 throughout the simulations (Figure 8C). The two-dimensional simulations described above can
295 model the basal surface of large, electrofused cells. We also carried out simulations of a single
296 cell, in which activity is measured along the perimeter (Figure 8D). Since polarity is more obvious
297 in these cells, the strength of the feedback loops was increased. In these simulations, we saw great
298 increases in activity before changes in the RasGAP level. Halfway through the simulation, this

299 was increased. This led to an overall decrease in Ras activity. Thereafter, single streaks
300 representing one or two stationary waves were observed (Figure 8D). These observations are
301 similar to the experiment in Figure 3M and 3N.

302 Discussion

303 Using optogenetic activation techniques on RasGAPs, C2GAPB and RASAL3, expressed
304 in *Dictyostelium* and neutrophils, respectively, our study yielded significant insights. Notably,
305 locally dampening Ras activity could extinguish cellular protrusions and impede migration.
306 However, contrary to expectations, a global reduction in Ras activity heightened both polarity and
307 motility. Furthermore, in *Dictyostelium*, targeting C2GAPB to the cell back amplified migration
308 and polarity, whereas in neutrophils, spontaneous movement of RASAL3 to the back generated
309 uropods and localized F-actin protrusions at the front. Thus, surprisingly, polarity can be achieved
310 by suppressing Ras activity at the rear of the cell. Although Ras and its downstream pathways are
311 typically associated with longer-term growth control, this investigation establishes the immediate
312 and spatially defined function of Ras activity at the cell cortex in governing cell polarity and
313 motility.

314 How can Ras inhibition improve polarity? We propose that a global decrease in Ras
315 activity raises the threshold for initiating protrusive activity but this is not enough to block all
316 protrusions. Our observation on the effects on RBD or PHcrac wave activity with RasGAP strongly
317 suggests that we are raising the activation threshold of our system. The increased threshold causes
318 typically broad, propagating cortical waves to break up into smaller, short-lived waves in
319 electrofused cells. Analogously, in single cells, RasGAPs suppress multiple, co-existing
320 protrusions all around the periphery and confine them to a single protrusion. This singular
321 protrusion appeared to be further enhanced by some positive feedback. Obviously, if all
322 protrusions were shut down, that would block migration; however, we were unable to achieve this
323 in our study presumably because our RasGAPs were not strong enough to raise the activation
324 threshold enough. In our model, Ras plays a central role in regulating two strong opposing
325 feedback loops consisting of molecules, such as myosin and PI(4,5)P₂ that typically form the back,
326 and PIP3 and F-actin that generate the front. This model faithfully recapitulates the results
327 presented here as well as predicting the previously observed increased cell spreading and front
328 activity upon knocking out RasGAPs^{8,11,18,22,23}. It also predicts a presumed ability of a further
329 reduction of Ras activity to eliminate cell movement.

330 Previous studies have suggested that increases in actomyosin contraction at the back,
331 occurring spontaneously or locally triggered by optogenetic RhoA or RGS4, can initiate polarity
332 and cause cells to move away⁶⁶⁻⁷⁴. Our study demonstrates that spatiotemporal regulation of Ras
333 signaling pathways not only controls F-actin protrusive activity at the front, but also directly
334 coordinates contraction at the back. Even when Arp2/3 was inhibited, the ability of back
335 suppression to polarize a cell could be observed. Since F-actin was absent, instead of making
336 normal protrusions, suppressing Ras activity at the back led to formation of long bleb-like
337 structures.

338 In addition to increasing contractility at the back, Ras inhibition at the back appeared to
339 trigger increased F-actin polymerization at the front. This was evidenced by the fact that while
340 inhibitors of signaling downstream of Ras suppressed protrusions and stopped cells from moving,
341 the inhibition could be partially overcome by recruiting RasGAP to the back. These RasGAP-

342 mediated long-range effects on the cell front were mediated primarily through mTORC2, rather
343 than PI3K/PIP3. Traditionally, these possibilities have been difficult to distinguish since
344 conventional chemotactic gradient studies lack sufficient spatiotemporal resolution to determine
345 chronology of front and back formation during symmetry breaking^{71,72,74,75}. However, our results
346 revealed that suppression at the back can trigger polarization, which may simultaneously activate
347 the front.

348 Our study sheds light on the complexity of Ras function in cells. Previous reports have
349 shown that expression of oncogenic Ras leads to excessive protrusions in multiple different cell
350 types^{4,20,61}. The extraneous protrusions impair cell movement. Our recent findings clearly show
351 that local activation or inhibition of Ras at the cell perimeter can quickly elicit or extinguish
352 protrusions, indicating that Ras activity is necessary and sufficient⁴. It was, therefore, surprising
353 that global inhibition of Ras activity quite effectively polarized both amoebae and neutrophils.
354 Polarization occurred even though in some of the amoebae experiments Ras activity was reduced
355 to undetectable levels in single cells. Small RBD puncta were still visible in giant, electrofused
356 cells indicating Ras was not completely suppressed with C2GAPB recruitment. Altogether, the
357 studies suggest that, for effective motility, there is an optimal level of Ras activity which is very
358 low. Cells move most effectively when maintaining persistent Ras activation at a narrow location
359 and strongly inhibit on the remainder of the perimeter. Consistently, our modeling showed that
360 polarity was best achieved when Ras activity was strongly reduced. Of course, in the simulation a
361 complete loss of Ras activity could stop movement; we were unable to achieve this experimentally.

362 Such findings have important implications for cancer treatment, where targeting Ras or
363 constitutively active Ras mutations may not always be beneficial. Caution must be exercised to
364 avoid, while attempting to abrogate cell proliferation, forcing cells into a more polarized migratory
365 state. The increased migration could, for example, induce cells to exit epithelium and metastasize.
366 Consistently, it is well-known that metastasizing cells do not readily divide⁷⁶⁻⁷⁹. A deeper
367 understanding of the different roles that Ras activities play in cell migration versus cell growth is
368 essential for developing effective therapeutic strategies.

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380 Author Contributions

381 DSP, YL, and PND conceived and designed the project; YL and DSP engineered
382 constructs/stable cell lines, designed and executed experiments, and performed majority of data
383 analyses with input from other authors; PB and PAI devised and conducted computational

384 simulations; TB and GQ executed kymograph and some MATLAB/Python analyses; YD
385 performed uptake assays; JB made some constructs; DSP, PND, and YL extensively revised
386 ChatGPT-generated initial drafts, which were developed from bullet points supplied by authors,
387 and wrote final version of the manuscript with help from other authors; DSP and PND supervised
388 the study.

389

390 Competing Interests

391 The authors declare no competing interests.

392

393 Materials and Methods

394 Reagents and inhibitors

395 200 µg/mL fibronectin stock (Sigma-Aldrich; F4759-2MG) was prepared in sterile water,
396 followed by dilution in PBS. 20 mM AS605240 (Sigma-Aldrich; #A0233), 20 mM PP242 (EMD
397 Millipore; #475988), 50 mM LY294002 (Thermo Fisher; #PHZ1144), 50 mM CK-666 (EMD
398 Millipore; #182515), 50 mM blebbistatin (Peprotech; #8567182), 10 mM latrunculin B (Sigma-
399 Aldrich; #428020), or 5 mM Y27632 (Sigma-Aldrich; #688001) stock solution was made in
400 DMSO (Sigma Aldrich; #D2650). Jasplakinolide (Sigma-Aldrich; #420127) was available as a
401 ready-made 1 mM stock. Hygromycin B (Thermo Fisher Scientific; #10687010) or G418 sulphate
402 (Thermo Fisher Scientific; #10131035) was purchased as 50 mg/mL stock solution whereas
403 blasticidine S (Sigma-Aldrich; #15205) or puromycin (Sigma-Aldrich; #P8833) was dissolved in
404 sterile water to make stock solutions of 10 mg/mL or 2.5 mg/mL, respectively. Doxycycline
405 hyclate (Sigma; #D9891-1G) was dissolved in sterile water to make a stock of 5 mg/mL. 50 mg/mL
406 TRITC-dextran (Sigma-Aldrich; #T1162) was made in sterile water. All stock solutions were
407 aliquoted and stored at -20°C. According to experimental requirements, further dilutions were
408 made in development buffer (DB), PBS, or growth medium before adding to cells.

409 Plasmid construction

410 All DNA oligonucleotides were purchased from Sigma-Aldrich. *Dictyostelium* C2GAPB
411 (RasGAP2, RG2) gene was previously cloned in KF2 expression plasmid¹¹. Using this construct,
412 we subcloned C2GAPB into doxycycline-inducible pDM335 plasmid (DictyBase #523) using
413 BglII/SpeI restriction digestion to generate mRFPmars-C2GAPB/pDM335 and GFP-
414 C2GAPB/pDM335 constructs. SspB R73Q ORF was amplified from tgRFPt-SspB R73Q plasmid
415 (Addgene #60416) and then subcloned into C2GAPB/pDM335 at the BglII site to generate the
416 optogenetically-recruitable C2GAPB construct, mRFPmars-SspB R73Q-C2GAPB⁸⁰. Similarly,
417 tgRFPt-SspB R73Q ORF was introduced into pDM335 to generate tgRFPt-SspB R73Q-Ctrl
418 construct. The construct for the membrane anchor, N150-Venus-iLID/pDM358, was made
419 previously⁴. This construct was used to subclone PHcrac-YFP, LimE_{Δcoil}-YFP, or RBD-YFP ORF
420 to generate dual expressing N150-Venus-iLID/PHcrac-YFP, N150-Venus-iLID/LimE-YFP, or
421 N150-Venus-iLID/RBD-YFP construct, respectively. The shuttle vector, pDM344 (DictyBase
422 #551), was used for this purpose. Constructs for GFP-RBD and GFP- LimE_{Δcoil} were procured
423 from R. Firtel lab (UCSD) and G. Marriott lab (University of Wisconsin-Madison), respectively,
424 whereas myosin-GFP/pDRH was obtained from D. Robinson lab (School of Medicine, JHU)^{5,81,82}.

425 Mammalian constructs, CRY2PHR-mCherry-RASAL3/pPB, CIBN-CAAX/pLJM1, and
426 LifeAct-miRFP703/pLJM1 were generated previously⁴. DNA sequences (2064-3036 or 1276-
427 3036 bases) encoding the last 322 or 585 amino acids of RASAL3 were PCR amplified and cloned
428 into BspEI/NotI sites of the PiggyBac™ transposon plasmid to generate the CRY2PHR-mCherry-
429 RASAL3₆₈₉₋₁₀₁₁/pPB or CRY2PHR-mCherry-RASAL3₄₂₆₋₁₀₁₁/pPB construct, respectively
430 (PiggyBac™ transposon system was gifted by S Collins lab, UC Davis)^{83,84}.

431 All constructs were verified by diagnostic restriction digestion and sequenced at the JHMI
432 Synthesis and Sequencing Facility.

433 Cell culture

434 Wild-type *Dictyostelium discoideum* cells of the AX2 strain, obtained from the R. Kay lab
435 (MRC Laboratory of Molecular Biology, UK), were used in this study. The Gβ-null (Gβ⁻) cells
436 were created in our lab previously⁴¹. Myosin heavy chain-null strain (mhcA⁻) was obtained from
437 D. Robinson laboratory (School of Medicine, JHU)⁴⁷. C2GAPB-null strain (C2GAPB⁻) was
438 generated in our lab¹¹. All cell lines were cultured axenically in HL5 medium supplemented with
439 300 µg/mL streptomycin (GoldBio; #S-150-100) at 22°C. Growth-stage cells were used for all
440 imaging experiments and all strains were used within 2 months of thawing from frozen stocks⁸⁵.

441 Female human neutrophil-like HL-60 cell line was obtained from Orion Weiner lab, UCSF,
442 and subcultured in RPMI 1640 medium (Gibco #22400-089) supplemented with 15% heat-
443 inactivated fetal bovine serum (FBS; Thermo Fisher Scientific #16140071)^{4,86}. To obtain
444 migration-competent cells for experimentation, wildtype or stable cell lines were differentiated in
445 presence of 1.3% DMSO (Sigma #D2650) over 5-7 days^{4,86}. Upon differentiation, these cells can
446 be used as an effective model to study human neutrophils⁸⁷. Stable cell lines were maintained in
447 selection antibiotics which were removed during differentiation and experimentation. Cells were
448 grown in humidified conditions at 5% CO₂ and 37°C. Experiments were performed with cells at
449 low passage.

450

451 Stable cell line generation

452 0.5-1×10⁷ wildtype cells were harvested, washed twice, and finally resuspended in 100 µL
453 chilled H-50 buffer (20 mM HEPES, 1 mM MgSO₄, 50 mM KCl, 5 mM NaHCO₃, 10 mM NaCl,
454 and 1 mM NaH₂PO₄; pH 7.0). Next, 2 µg total DNA was added to the cell suspension which was
455 subsequently transferred to an ice-cold 0.1-cm electroporation cuvette (BioRad; #1652089), and
456 electroporated at 0.85 kV/25 µF twice with 5 secs between pulses. The cuvette was then incubated
457 on ice for 10 min and cells were transferred to a 10-cm Petri dish (Greiner Bio-One #664160)
458 containing 10 mL HL5 medium supplemented with heat-killed *Klebsiella aerogenes* (lab stock).
459 Next day, 50 µg/mL hygromycin and/or 20 µg/mL G418 sulfate was added, and cells were selected
460 over a period of 3-4 weeks^{4,88}.

461 HL-60 cell line stably co-expressing optically recruitable RASAL3 and LifeAct-
462 miRFP703 was generated previously using a combination of lentiviral- and transposon integration-
463 based approaches^{4,88}. In this study, we stably co-expressed recruitable RASAL3₆₈₉₋₁₀₁₁ or
464 RASAL3₄₂₆₋₁₀₁₁ along with LifeAct-miRFP703 in HL-60 cells using the same protocol.

465

466 **Preparation of electrofused giant *Dictyostelium***

467 Growth-phase cells were harvested, washed, and resuspended in Soerensen buffer (SB; 15
468 mM KH₂PO₄ and 2 mM Na₂HPO₄, pH 6.0) at a density of 1.5×10⁷ cells/mL. 7–10 mL cell
469 suspension was put into a 50-mL conical tube and rolled gently for 30 min to promote visible cell
470 cluster formation. 800 µL of ‘rolled cells’ were gently transferred to a 4 mm-gap electroporation
471 cuvette (BioRad; #1652088). Electroporation was then performed using the following settings:
472 1,000 V, 3 µF once, and 1,000 V, 1 µF thrice, with 1–2 sec between pulses. Next, 50 µL cell
473 suspension was transferred to the center of a well in an 8-well chamber (Lab-Tek, #155409 PK)
474 and left for 5 min. 450 µL SB supplemented with 2 mM CaCl₂ and 2 mM MgCl₂ was added to the
475 well and cells were resuspended gently. Medium along with unadhered cells was removed and 450
476 µL fresh supplemented SB was gently added to the well. Cells were allowed to recover for 1 hr
477 before imaging^{35,36}.

478 **Microscopy**

479 Vegetative *Dictyostelium* in HL5 medium were placed in an 8-well coverslip chamber and
480 allowed to adhere for 40 min. Differentiated HL-60 cells, pre-treated with heat-killed *Klebsiella*
481 *aerogenes*, were allowed to adhere to fibronectin-coated 8-well coverslip chamber for 40 mins^{4,88}.
482 Next, non-adherent cells were washed off, 300–450 µL DB or fresh RPMI 1640 medium was added
483 to the attached *Dictyostelium* or differentiated HL-60 cells, respectively, and used for imaging. To
484 induce C2GAPB expression in *Dictyostelium*, doxycycline (50 µg/mL) was added 8 hr prior to
485 imaging time. For macropinocytosis assay, cells were incubated with TRITC-dextran for 4 mins,
486 washed thrice with DB, and imaged subsequently^{33,89}. All time-lapse imaging in *Dictyostelium* or
487 HL-60 cells was acquired with 0.3–0.5% or 1.7–6% laser intensity, respectively, using the
488 following microscopes: (1) Zeiss LSM780-FCS single-point, laser scanning confocal microscope
489 with 780-Quasar; 34-channel spectral, high-sensitivity gallium arsenide phosphide detectors
490 supported with ZEN Black software, and (2) Zeiss LSM800 GaAsP single-point laser scanning
491 confocal microscope with wide-field camera supported with ZEN Blue software. All images of
492 *Dictyostelium* or HL-60 cells were acquired with 63X/1.40 PlanApo oil or 40X/1.30 PlanNeofluar
493 oil DIC objective, respectively, along with digital zoom. In single *Dictyostelium* or HL-60 cells,
494 confocal imaging was performed at a middle plane of the cells, whereas in giant *Dictyostelium*
495 cells, laser was focused near the bottom surface of cells to visualize cortical wave
496 propagation^{4,35,88,90}. For inhibitor experiments, differentiated HL-60 cells were treated with 20 µM
497 AS605240, 20 µM PP242, 50 µM LY294002, 50 µM CK-666, 50 µM blebbistatin, or 10 µM
498 Y27632 for atleast 10 mins before imaging. For pre-treatment with JLY cocktail, HL-60 cells were
499 incubated with 10 µM Y27632 for 10 min. These cells were then treated with 8 µM jasplakinolide
500 and 5 µM latrunculin B without changing the final concentration of Y27632^{91,92}. In *Dictyostelium*,
501 50 µM blebbistatin or CK-666 was added during imaging.

502 Optogenetic experiments with vegetative *Dictyostelium* or differentiated HL-60 cells were
503 done in absence of any chemoattractant. Throughout image acquisition, solid state laser (561 nm
504 excitation and 579–632 nm emission) was used for visualizing proteins or recruitable effectors
505 fused to mCherry, mRFPmars, or tgRFPT tag whereas a diode laser (633 nm excitation and 659–
506 709 nm emission) was used to capture miRFP703 expression. Images were acquired for 5–10 mins,
507 after which 450/488 nm excitation laser was switched on globally to activate recruitment. Image
508 acquisition and photoactivation was done at ~7 sec intervals. Using the T-PMT associated with the

509 red channel, we acquired DIC images. We took advantage of the interactive photobleaching
510 module on Zeiss LSM800 to perform local recruitment experiments. A small region of interest was
511 placed in front or back of migrating cells, and bleached with 488 nm laser (laser power of ~0.5%
512 or 7% for *Dictyostelium* or HL-60 cells, respectively) in multiple iteration. Time interval of
513 photoactivation and image acquisition was ~10 secs. For imaging HL-60 cells, both microscopes
514 were equipped with a temperature-controlled chamber set at 5% CO₂ and 37°C^{4,35,88,90}.

515 **SDS-PAGE and Western blotting**

516 Wildtype or mRFPmars-C2GAPB-expressing *Dictyostelium* was starved and developed,
517 and 6×10⁶ cells were collected every hour, upto 8 hours, for western blot analysis^{88,93}. Each sample
518 was resuspended in pre-chilled 1× RIPA buffer (supplemented with protease inhibitor cocktail;
519 Thermo Scientific, # 89900) and lysed on ice for 20 mins. Next, 3× Laemmili sample buffer (lab
520 stock) was added and samples were incubated at RT for 5 mins. Finally, sample equivalent to
521 1.5×10⁶ cells were loaded into pre-cast 4–15% polyacrylamide gel and was run at 120 V for 1 hour.
522 Immunoblotting was performed as per standardized lab protocol^{4,88}. cAR1 expression (~44 kDa)
523 was detected by incubating PVDF membrane (Bio-Rad; 162-0262) with rabbit anti-cAR1 antibody
524 (1:1000 dilution; generated in our lab⁹⁴) overnight at 4°C, followed by goat anti-rabbit IRDye
525 680RD-conjugated secondary antibody (1:10,000 dilution; Li-Cor; #925-68071) for an hour in the
526 dark. We used Odyssey CLx imaging system (Li-Cor) to detect the near-infrared signal from the
527 blot.

528 **Image analysis**

529 All images were analyzed with ImageJ (NIH) and MATLAB 2019b (MathWorks, Natick,
530 MA) software⁹⁵. We utilized GraphPad Prism 8 (GraphPad software, CA, USA) and Microsoft
531 Excel (Microsoft, WA, USA) for plotting our results^{4,35,88}.

532 To get the ratio of wave area to cell area in Figure 1E and H, the image was first binarized
533 using ImageJ, by adjusting the threshold to cover all pixels of the wave or cell. The range was not
534 reset and the ‘Calculate threshold for each image’ option was unchecked. Subsequently, using the
535 “Analyze Particle” function, a size-based thresholding was applied (to exclude non-cell particles)
536 and cell masks were generated. Next, the ‘Fill holes’, ‘Erode’ and ‘Dilate’ options were applied,
537 sequentially and judiciously, to obtain the proper binarized mask for waves or cells^{4,35}. Next, the
538 ratio of wave area to cell area for each frame was obtained and plotted with time. Duration of
539 waves for Figure 1F and I was obtained by counting the number of frames from when a wave starts
540 to when it ends, and then multiplying it with the time interval.

541 Cell outline overlays (Figures 3C, 4B, 4F, 5B, 5E, 6A, 6E, 7C, 7L, S2C, S2E, S2G, S4B,
542 S6F, S7C) were obtained by first segmenting the cell using ‘Threshold’ option of Fiji/ImageJ to
543 obtain a binary image which properly covered all pixels of the cell. Subsequently, using the
544 “Analyze Particle” option, cell masks were generated. For optimizing these masks, we used ‘Fill
545 holes’, ‘Erode’ and ‘Dilate’ functions. Finally, the ‘Outline’ command was operated on binarized
546 cells, and ‘Temporal-Color Code’ was used^{4,35}.

547 For membrane kymographs (Figures 3I-J, 3M-N, 5F, 6F, 7D, 7M, S4C, S6G, S7D), cells
548 were segmented against the background following standard image-processing steps with custom
549 code written in MATLAB^{4,35,88}. Next, kymographs were created from the segmented cells by
550 aligning consecutive lines over time by minimizing the sum of the Euclidean distances between

551 the coordinates in two consecutive frames using a custom-written MATLAB function⁶⁵. A linear
552 colourmap was used for the normalized intensities in the kymographs; the lowest intensity is
553 indicated by blue and the highest with yellow.

554 For linescan analysis (Figure 3H, L), a straight-line segment (width of 5 pixels) was drawn,
555 using the ‘Straight’ tool in Fiji/ImageJ, across the cellular protrusion region to obtain an average
556 intensity value. The intensity values along that particular line were obtained using the ‘Plot Profile’
557 option. These values were plotted along the distance of the line in Microsoft Excel^{35,88}.

558 Local protrusion (Figure 2D, F, L) and cell migration (Figures 5G-K, 6G-K, 7E-I, 7N-R,
559 S6H-L, S7E-I) analyses were performed as described previously^{4,35,88}. For cell speed, area and
560 aspect ratio quantifications (in Figures 3D-F, 4C-E, 4G-H), cells were segmented against the
561 background following standard image-processing steps with custom code written in Python based
562 on package scikit-image, Trackpy and PyImageJ. Human supervision was involved during the
563 process with an integration to Fiji, and we obtained the X-position, Y-position, cell area, and aspect
564 ratio for each frame of each cell. Distance between adjacent frames was calculated from X-position
565 and Y-position, and was divided by time interval to obtain an instantaneous velocity. The average
566 speed of cells was calculated by taking an average of all instantaneous velocities. For Figure S1B,
567 macropinocytosis uptake was analyzed by outlining each cell and quantifying the total TRITC
568 fluorescence signal within each outline divided by the cell area⁸⁹. For Figure S4B, numbers of
569 blebs for each cell, before or after recruitment, were quantified by summing them over a min⁹⁶.

570 Simulations

571 The simulations are based on a model, previously described, in which three interacting
572 species, RasGTP, PIP2, and PKB, form an excitable network³⁷. To simplify the notation, we
573 describe the concentration of these three species using the symbols: F , B and R , respectively,
574 which represent the fact that RasGTP and PIP2 are front- and back-associated molecules,
575 respectively, and that PKB acts to provide the refractory element of the excitable network behavior.

576 The concentrations of each of these molecules is described by stochastic, reaction-diffusion partial
577 differential equations:

$$\begin{aligned} 578 \frac{\partial F}{\partial t} &= -(a_1 + a_2 R)F + \frac{a_3}{1 + a_4^2 B^2} + a_5 + w_F + D_F \nabla^2 F \\ 579 \frac{\partial B}{\partial t} &= -(b_1 + b_2 F)B + b_3 + w_B + D_B \nabla^2 B \\ 580 \frac{\partial R}{\partial t} &= -c_1 R + c_2 F + w_R + D_R \nabla^2 R \end{aligned}$$

581 In each of these equations, the final term represents the diffusion of the species, where D_* is the
582 respective diffusion coefficient and ∇^2 is the spatial Laplacian (in one or two dimensions). The
583 second-to-last terms represent the molecular noise. Our model assumes a Langevin approximation
584 in which the size of the noise is based on the reaction terms⁹⁷. For example, in the case of PKB
585 (R), the noise is given by

$$586 w_R(t) = \alpha \sqrt{c_1 R + c_2 F} w(t)$$

587 where $w(t)$ is a zero mean, unit variance Gaussian Brown noise process. In the simulations, the
588 size of this noise was adjusted with the empirical parameter α .

589 In addition to the EN dynamics described above, we incorporated two other terms related to cell
590 polarization^{37,62}. These feedback loops come from B and R :

$$\begin{aligned} 592 \quad \frac{\partial P_F}{\partial t} &= -p_1 P_F + p_2 R + w_{P_F} + D_{P_F} \nabla^2 P_F \\ 593 \quad \frac{\partial P_B}{\partial t} &= -p_3 P_B + p_4 B + w_{P_B} + D_{P_B} \nabla^2 P_B \end{aligned}$$

591
592 They modify the equation for Ras:

$$593 \quad (a_1 + a_2 R + a_{P_B} P_B) F \mapsto \frac{(a_1 + a_2 R + a_{P_B} P_B) F}{1 + a_{P_F} P_F}$$

594 having the effect of increasing front and back contributions. Lastly, we note that changes in
595 RasGAP contributions are modeled as changes in the parameters a_1 and a_2 .

596 Simulations were run on MATLAB 2023a (Mathworks, Natick, MA) on custom-code based on
597 the Ito solution in the Stochastic Differential Equation toolbox (<http://sdetoolbox.sourceforge.net>).
600 Two-dimensional simulations were used to recreate the observed wave patterns of larger
601 electrofused cells, and so assume a grid 40 $\mu\text{m} \times 40 \mu\text{m}$ with a spacing of 0.4 $\mu\text{m} \times 0.4 \mu\text{m}$ per
602 grid point (i.e., 100 \times 100 points) and zero flux boundary conditions. The one-dimensional
603 simulations aim to recreate the membrane fluorescence observed in single-cell confocal images.
604 The dimension is therefore smaller, assuming a cell radius of 5 μm and a spacing of 0.25 μm ,
605 resulting in $2\pi \times 5 / 0.25 \approx 126$ points along the perimeter, and periodic boundary conditions.

606 Parameter values

Parameter	Value	Units	Parameter	Value	Units
a_1	1	s^{-1}	b_1	0.06	s^{-1}
a_2	37	$\mu\text{M}^{-1} \text{s}^{-1}$	b_2	8880	$\mu\text{M}^{-1} \text{s}^{-1}$
a_3	12	s^{-1}	b_3	8.20	$\mu\text{M} \text{s}^{-1}$
a_4	444	μM^{-1}	c_1	0.15	$\mu\text{M}^{-1} \text{s}^{-1}$
a_5	5.4×10^{-3}	s^{-1}	c_2 (1D)	1.50	$\mu\text{M}^{-1} \text{s}^{-1}$
D_F	0.15	$\mu\text{m}^2/\text{s}$	c_2 (2D)	0.75	$\mu\text{M}^{-1} \text{s}^{-1}$
D_B	0.15	$\mu\text{m}^2/\text{s}$	p_1	0.05	$\mu\text{M}^{-1} \text{s}^{-1}$
p_2	0.05	$\mu\text{M}^{-1} \text{s}^{-1}$	p_3	0.05	$\mu\text{M}^{-1} \text{s}^{-1}$
p_4	0.05	$\mu\text{M}^{-1} \text{s}^{-1}$	D_R (1D)	1.50	$\mu\text{m}^2/\text{s}$
D_R (2D)	0.25	$\mu\text{m}^2/\text{s}$	D_{P_F}	0.0125	$\mu\text{m}^2/\text{s}$

D_{P_B}	0.0125	$\mu\text{m}^2/\text{s}$	a_{P_F}	2	μM^{-1}
a_{P_B}	1	$\mu\text{M}^{-1} \text{ s}^{-1}$	α (1D)	0.0833	
α (2D)	0.0417				

607

608 **Statistical analysis**

609 Statistical analyses were executed using unpaired or paired 2-tailed non-parametric tests
610 on GraphPad Prism 8. Results are expressed as mean \pm SD from at least 3 independent
611 experiments. ns denotes $P > 0.05$, * denotes $P \leq 0.05$, ** denotes $P \leq 0.01$, *** denotes $P \leq 0.001$,
612 **** denotes $P \leq 0.0001$.

613 **Data availability**

614 All data are provided in the main or supplementary text. Requests for additional
615 information on this work are to be made to the corresponding authors.

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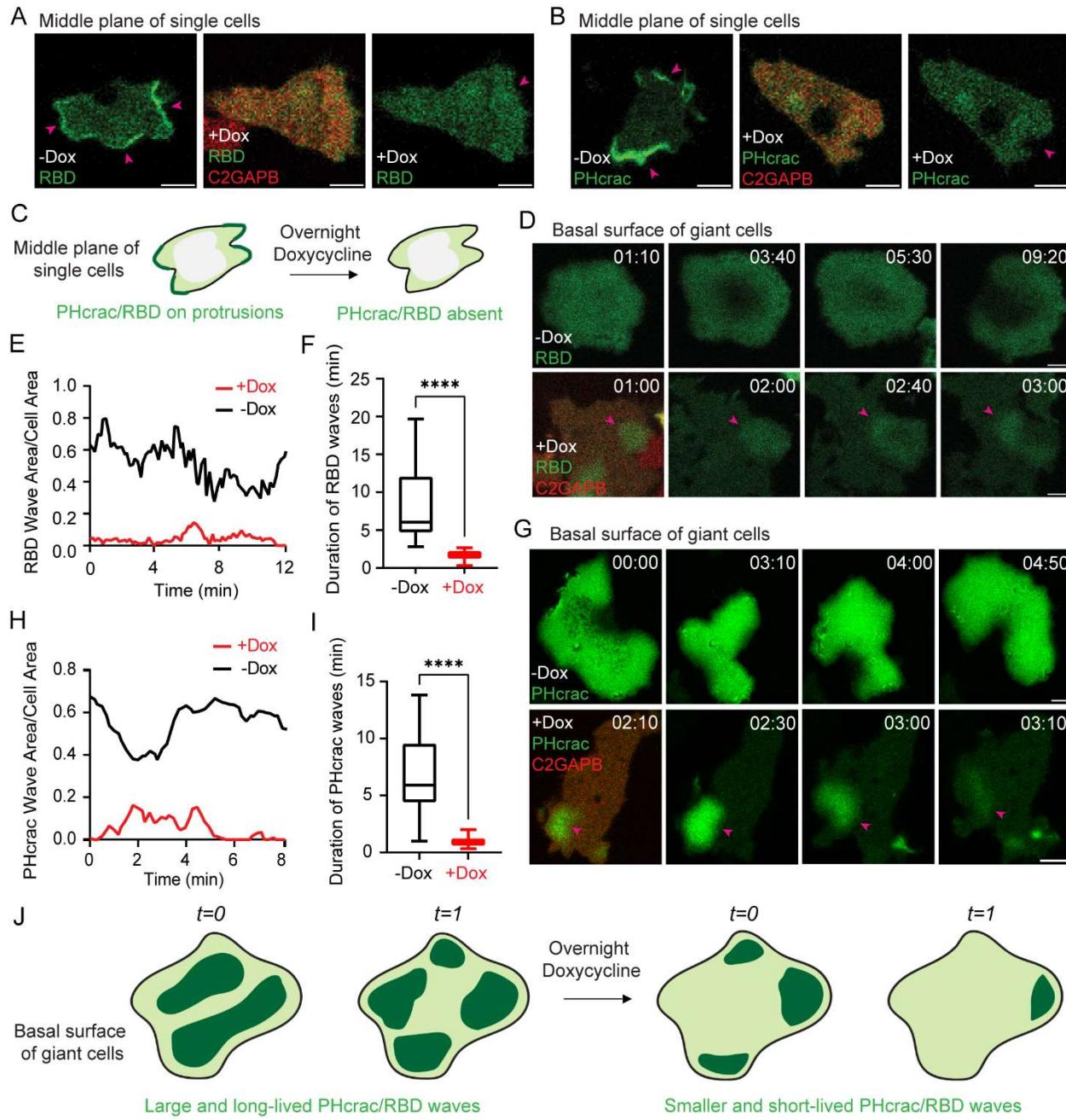
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907 **Main Figures**

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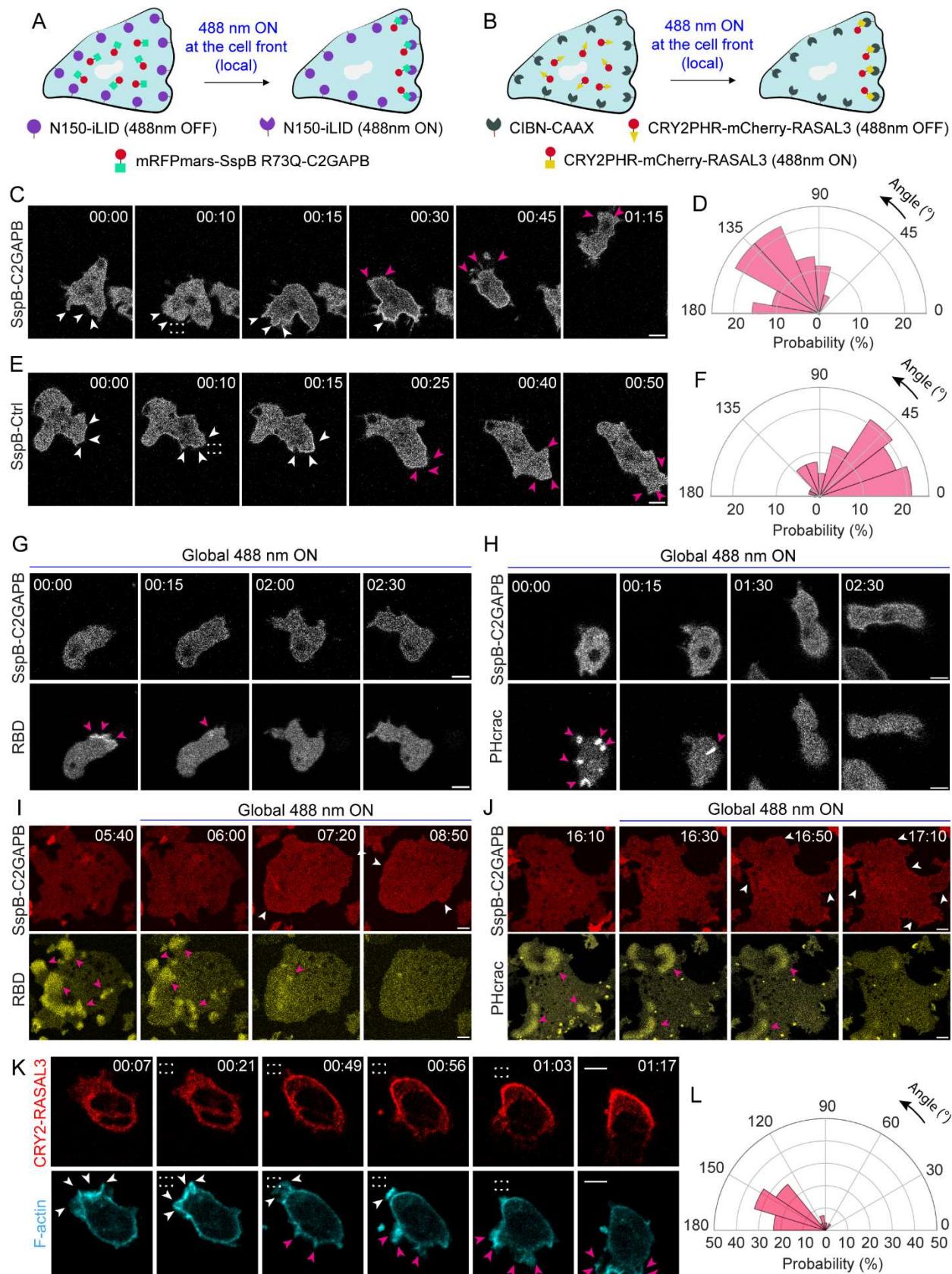
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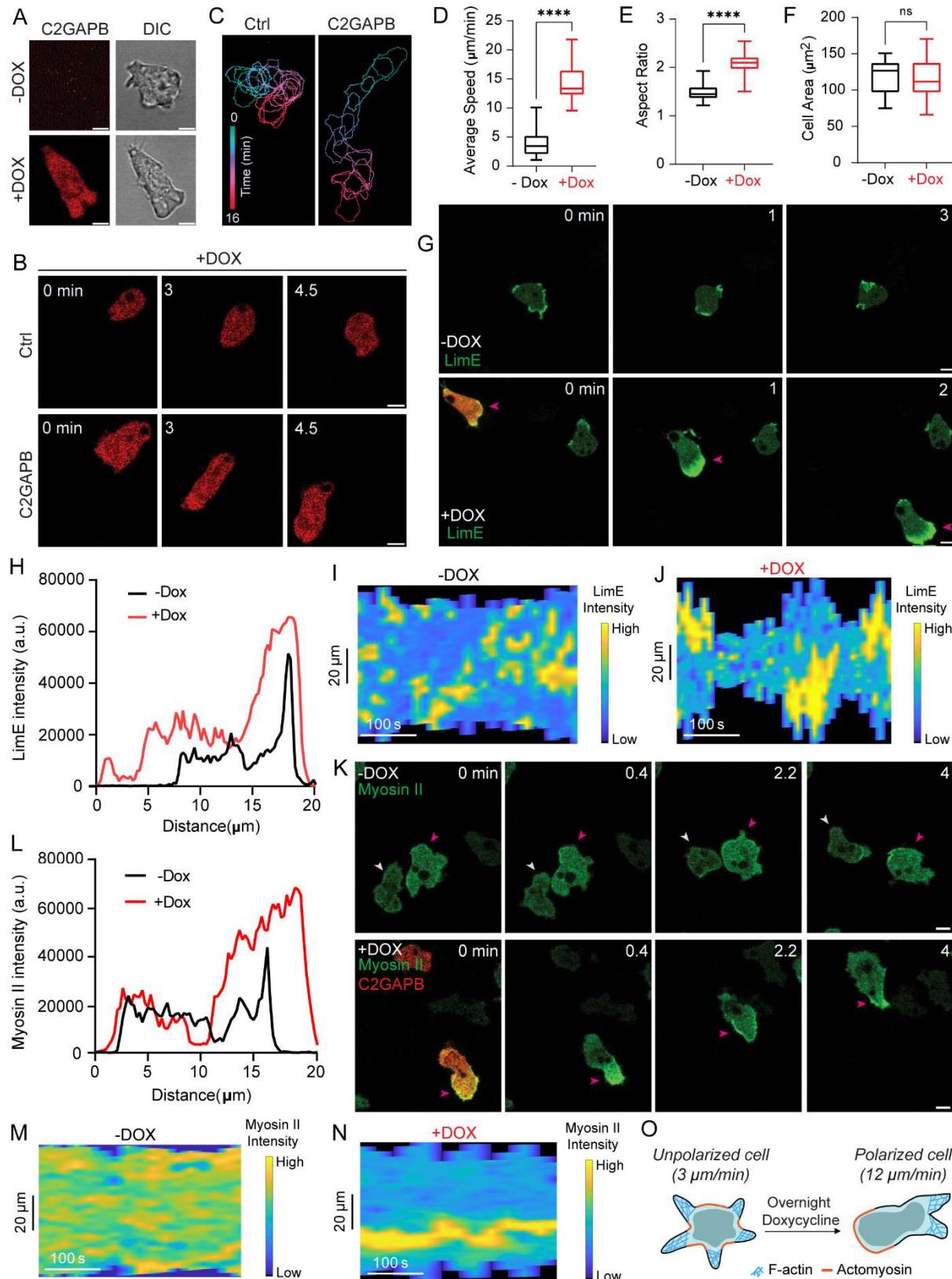
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914 **Figure 1. C2GAPB inhibits Ras and PI3K activities in single and electrofused *Dictyostelium*.**
915 Confocal images of vegetative *Dictyostelium* single cells expressing (**A**) GFP-RBD (biosensor for
916 activated Ras; green) or (**B**) PHcrac-YFP (biosensor for PIP3; green) before and after doxycycline-
917 induced mRFPmars-C2GAPB (red) expression. Pink arrows highlight fronts of these cells. Scale
918 bars represent 5 μ m. (**C**) Cartoon summarizes our observations in (A) and (B) that both Ras
919 activation and PIP3 level on the cell membrane significantly reduce with C2GAPB expression.
920 Time-lapse confocal images of vegetative *Dictyostelium* electrofused or ‘giant’ cells expressing
921 (**D**) GFP-RBD (green) or (**G**) PHcrac-YFP (green) before and after doxycycline-induced
922 mRFPmars-C2GAPB (red) expression. Pink arrows point at reduced RBD or PHcrac waves in
923 presence of C2GAPB. Time in min:sec format. Scale bars represent 5 μ m. RBD or PHcrac wave
924 area (**E or H**) and duration (**F or I**) before (‘-Dox’; black) and after (‘+Dox’; red) doxycycline-
925 induced mRFPmars-C2GAPB expression. (**J**) Cartoon depicting reduction in size and duration of
926 RBD and PHcrac waves on the basal surface of giant *Dictyostelium* cells, after overnight
927 doxycycline induction of C2GAPB.



929 **Figure 2. Recruitment of C2GAPB and RASAL3 shuts off protrusions by inhibiting Ras and**
930 **PI3K activities in *Dictyostelium* and neutrophils.** Cartoons illustrating mechanism of (A) opto-
931 C2GAPB or (B) opto-RASAL3 recruitment to the cell front with help of SspB-iLID or CRY2-
932 CIBN optogenetic system, respectively. Time-lapse confocal images of vegetative *Dictyostelium*
933 single cells expressing (C) mRFPmars-SspB R73Q-C2GAPB or (E) tgRFPt-SspB R73Q-Ctrl
934 (control without C2GAPB). C2GAPB or Ctrl is recruited to the front of the migrating cell by
935 applying 488 nm laser near it, as shown by the dashed white box. White arrows denote existing
936 older protrusions whereas pink arrows highlight emerging newer protrusions. Time in min:sec
937 format. Scale bars represent 5 μ m. Polar histogram demonstrates higher probability of fresh
938 protrusion formation away from C2GAPB recruitment area ($n_c=11$ and $n_p=45$) (D) whereas for
939 Ctrl, new protrusions form in the same direction as the old ones, near or at the Ctrl recruitment
940 area ($n_c=13$ and $n_p=76$) (F). (G, H) Time-lapse confocal images of vegetative *Dictyostelium* single
941 cells expressing mRFPmars-SspB R73Q-C2GAPB (upper panel) and (G) RBD-YFP or (H)
942 PHcrac-YFP (both lower panels) after 488 nm laser was switched on globally. RBD or PHcrac
943 status at ‘00:00’ is considered as a control timepoint since C2GAPB has not been recruited yet.
944 Pink arrows denote RBD or PHcrac patches in the cells. Time in min:sec format. Scale bars
945 represent 5 μ m. (I, J) Time-lapse confocal images of *Dictyostelium* electrofused giant cells
946 expressing mRFPmars-SspB R73Q-C2GAPB (upper panel; red) and (I) RBD-YFP or (J) PHcrac-
947 YFP (both lower panels; yellow) before and after 488 nm laser was switched on globally. White
948 arrows highlight C2GAPB recruitment in the red channel whereas pink arrows denote RBD or
949 PHcrac waves near the bottom surface of these cells. Time in min:sec format. Scale bars represent
950 10 μ m. (K) Time-lapse confocal images of differentiated HL-60 neutrophil expressing CRY2PHR-
951 mCherry-RASAL3 (red; upper panel) and LifeAct-miRFP703 (cyan; lower panel). RASAL3 was
952 recruited to the cell front by applying 488 nm laser near it, as shown by the dashed white box.
953 White arrows denote existing older protrusions whereas pink arrows highlight emerging newer
954 protrusions. Time in min:sec format. Scale bars represent 5 μ m. (L) Polar histogram demonstrates
955 higher probability of fresh protrusion formation away from RASAL3 recruitment area; $n_c=12$ and
956 $n_p=30$.

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960 **Figure 3. C2GAPB expression polarizes *Dictyostelium* and improves random cell migration.**

961 (A) Confocal images demonstrating doxycycline-induced mRFPmars-C2GAPB expression (red)
962 polarize vegetative *Dictyostelium* cell (shown in DIC). Scale bars represent 5 μm . (B) Time-lapse
963 confocal images of vegetative *Dictyostelium* cells expressing tgRFPt-Ctrl (empty vector control,
964 red; top panel) or mRFPmars-C2GAPB (red; bottom panel) after overnight doxycycline treatment.
965 Time in ‘min’ format. Scale bars represent 5 μm . (C) Color-coded (at 1-min interval) outlines of
966 the Ctrl- or C2GAPB-expressing cell shown in (B). Box-and-whisker plots of (D) average cell
967 speed, (E) aspect ratio, and (F) cell area before (black; -DOX) or after (red; +DOX) overnight
968 doxycycline induction of C2GAPB. $n_c=34$ from atleast 3 independent experiments; asterisks
969 indicate significant difference, *** $P \leq 0.0001$ and ns denotes $P>0.05$ (Wilcoxon-Mann-Whitney
970 rank sum test). Time in min:sec format. Scale bars represent 5 μm . Time-lapse confocal images of
971 vegetative *Dictyostelium* cells expressing (G) GFP-LimE $_{\Delta\text{coil}}$ (LimE, F-actin biosensor; green) or
972 (K) myosin II-GFP (green), before (-DOX) and after (+DOX) overnight doxycycline-induction of
973 C2GAPB (red; not shown here). White or pink arrows denote cells of interest. Time in ‘min’
974 format. Scale bars represent 5 μm . Representative linescan of (H) LimE or (L) myosin II intensity
975 of cells in (G) or (K) respectively. Representative kymograph of cortical (I, J) LimE or (M, N)
976 myosin II intensity in cells in (G) or (K) respectively. A linear color map shows that blue is the
977 lowest LimE or myosin II intensity whereas yellow is the highest. (O) Cartoon summarizing the
978 polarizing effects of expressing C2GAPB in *Dictyostelium*. Unpolarized cells with small, transient
979 protrusions become polarized with a distinct F-actin front and a myosin II-labelled back after
980 C2GAPB is expressed.

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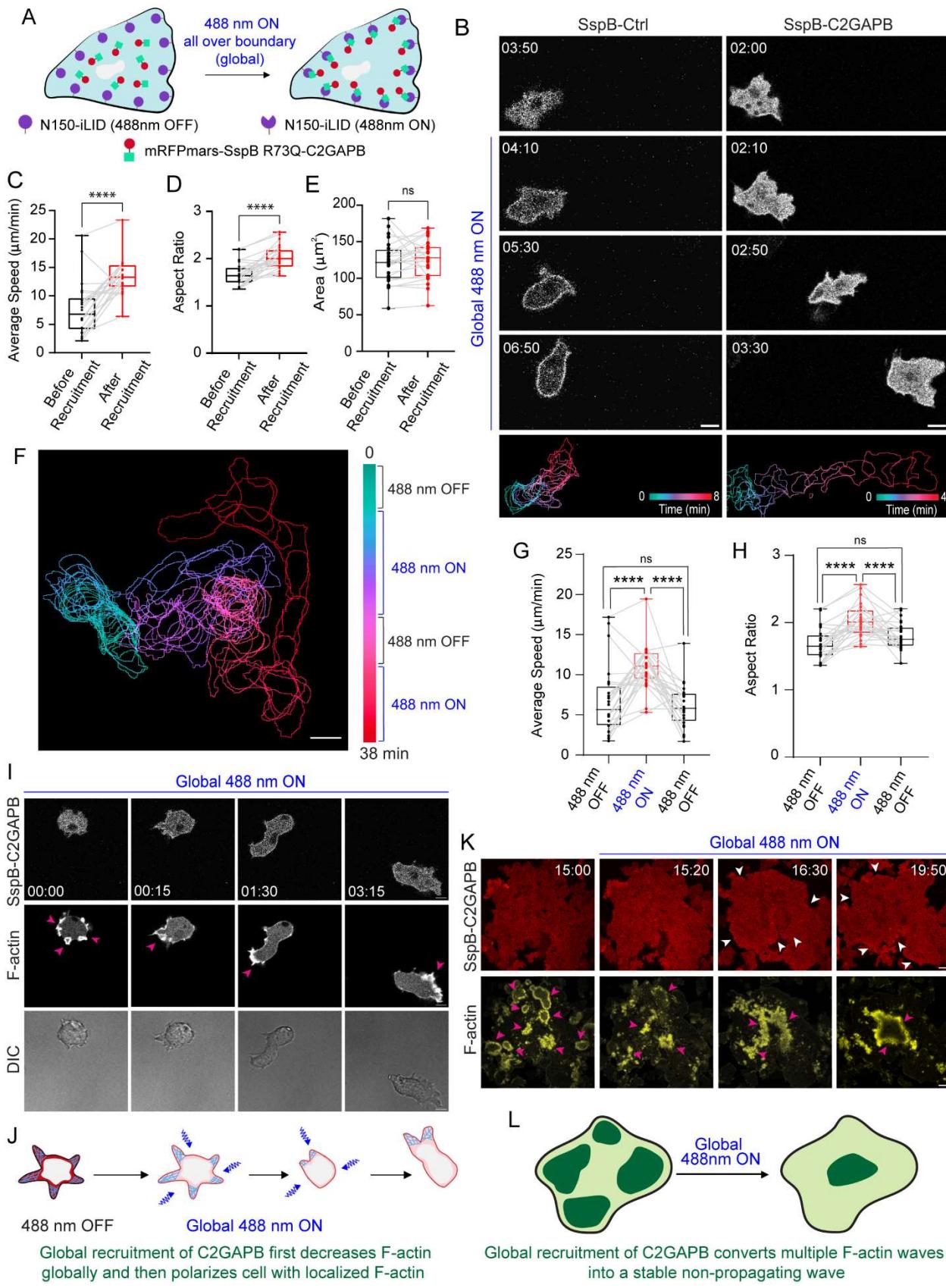
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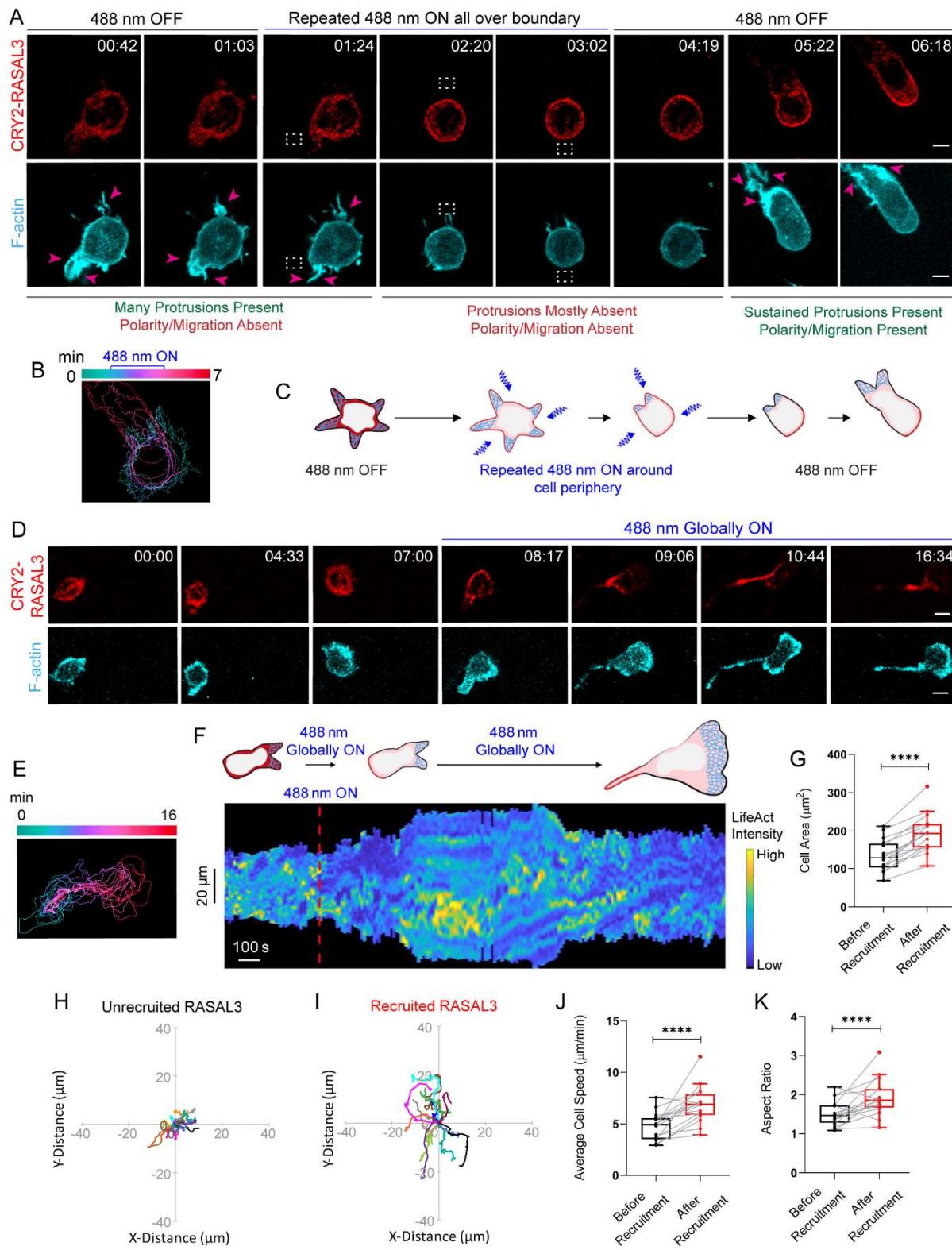


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997 **Figure 4. Global C2GAPB recruitment polarizes *Dictyostelium* and enhances cell migration.**

998 (A) Cartoon illustrating mechanism of opto-C2GAPB global recruitment on *Dictyostelium* cell
999 membrane with help of SspB-iLID optogenetic system. (B) Time-lapse confocal images of
1000 vegetative *Dictyostelium* cell expressing tgRFPt-SspB R73Q-Ctrl (control without C2GAPB; left
1001 panels) or mRFPmars-SspB R73Q-C2GAPB (right panels), before or after 488 nm laser was
1002 switched on globally. Bottom panels demonstrate color-coded (at 1-min interval) outlines of the
1003 Ctrl- or C2GAPB-recruited cell. Time in min:sec format. (F) Color-coded (1-min interval) outlines
1004 of a representative cell in presence of intermittent 488 nm light. Scale bars represent 10 μ m. Box-
1005 and-whisker plots of (C, G) average cell speed, (D, H) aspect ratio, and (E) cell area, before (black)
1006 or after (red) C2GAPB global recruitment. For plots in G and H, blue laser was switched on or off
1007 multiple times. $n_c=25$ from atleast 3 independent experiments; asterisks indicate significant
1008 difference, *** $P \leq 0.0001$, and ns denotes $P>0.05$ (Wilcoxon-Mann-Whitney rank sum test). (I)
1009 Time-lapse confocal images of vegetative *Dictyostelium* single cells co-expressing mRFPmars-
1010 SspB R73Q-C2GAPB (upper panel) and LimE-YFP (middle panel) after 488 nm laser was
1011 switched on globally. LimE patches are highlighted with pink arrows. ‘00:00’ is considered as
1012 control timepoint since C2GAPB has not been recruited yet. DIC channel (bottom panel) shows
1013 change in cell polarity with C2GAPB recruitment. Time in min:sec format. Scale bars represent 5
1014 μ m. (J) Cartoon illustrates phenomenon seen in cell in (I). (K) Time-lapse confocal images of
1015 electrofused *Dictyostelium* giant cells co-expressing mRFPmars-SspB R73Q-C2GAPB (upper
1016 panel; red) and Lime-YFP (lower panel; yellow) before or after 488 nm laser was switched on
1017 globally. White arrows highlight C2GAPB recruitment in the red channel whereas pink arrows
1018 denote LimE propagating waves near the bottom surface of these cells. Time in min:sec format.
1019 Scale bars represent 10 μ m. (L) Cartoon illustrates phenomenon seen in giant cell in (K).

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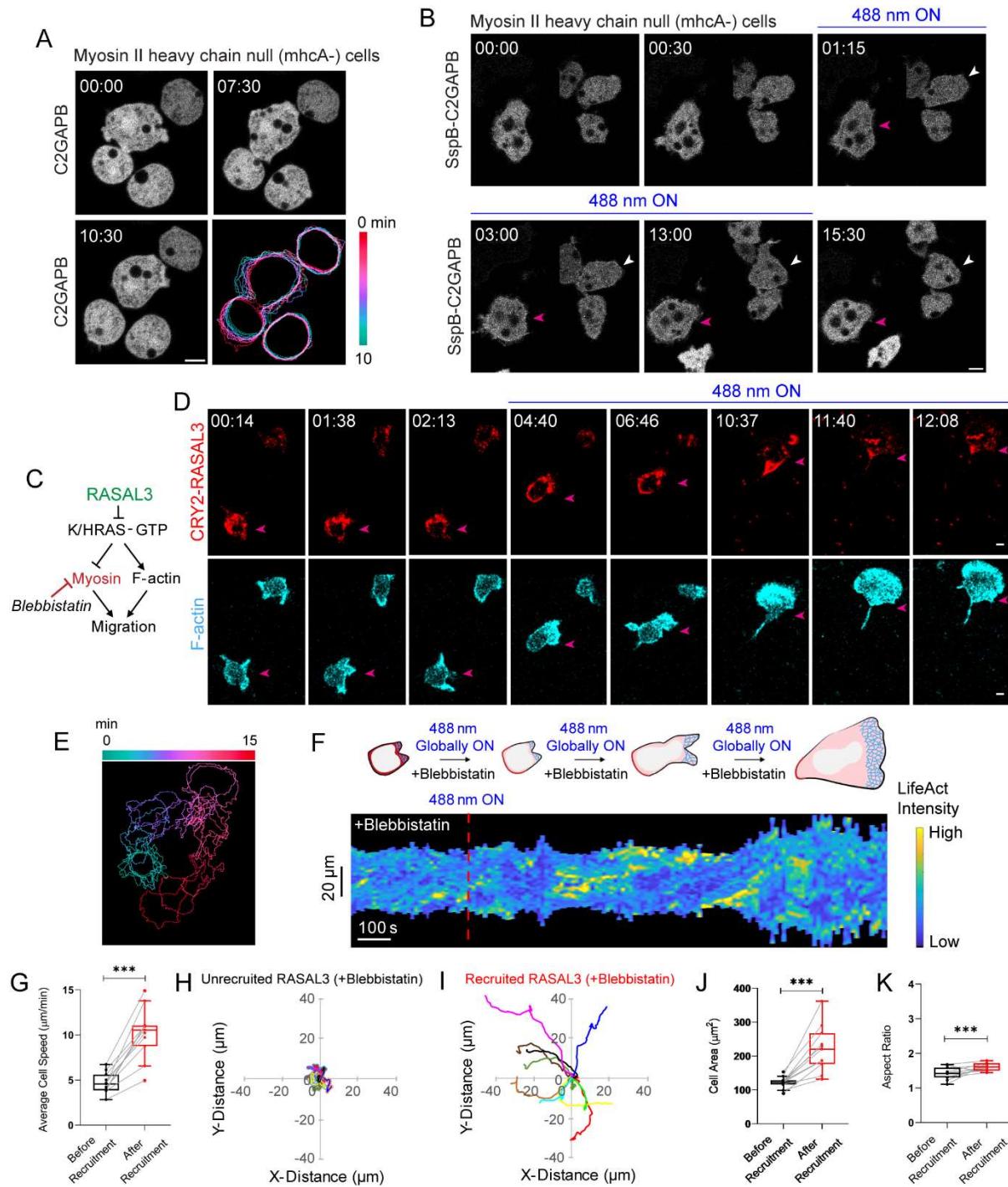


1023 **Figure 5. Localization of recruited RASAL3 to the back of the neutrophil led to even stronger**
1024 **polarization.** (A) Time-lapse confocal images of HL-60 neutrophil expressing CRY2PHR-
1025 mCherry-RASAL3 (red; upper panel) and LifeAct-miRFP703 (cyan; lower panel). Unpolarized,
1026 non-migratory neutrophil had multiple F-actin-rich protrusions around its perimeter, as shown by
1027 pink arrows. RASAL3 recruitment over the entire periphery, after 488 nm light was repeatedly
1028 applied all around the cell, caused cell to shrink and LifeAct-containing protrusions soon
1029 disappeared. Once blue laser was switched off, RASAL3 self-arranged to the back causing cell to
1030 polarize, form LifeAct-rich protrusions, and migrate away. Pink arrows highlight cellular
1031 protrusions. Region of blue light illumination is shown by the dashed white box. Time in min:sec
1032 format. Scale bars represent 5 μ m. (B) Color-coded (at 1 min intervals) outlines of the cell shown
1033 in (A). (C) Cartoon illustrating RASAL3-mediated phenomenon seen in (A). (D) Time-lapse
1034 confocal images of differentiated HL-60 neutrophil expressing CRY2PHR-mCherry-RASAL3
1035 (red; upper panel) and LifeAct-miRFP703 (cyan; lower panel), before or after 488 nm laser was
1036 switched on globally. Time in min:sec format. Scale bars represent 5 μ m. (E) Color-coded (at 1
1037 min intervals) outlines of the cell shown in (D). (F) Representative kymograph of cortical LifeAct
1038 intensity in RASAL3-expressing neutrophil before or after 488 nm laser was turned on. A linear
1039 color map shows that blue is the lowest LifeAct intensity whereas yellow is the highest. Duration
1040 of the kymograph is 24 mins. Cartoon depicts membrane recruitment, F-actin polymerization or
1041 cell shape status corresponding to the kymograph. Box-and-whisker plots of (G) cell area, (J) cell
1042 speed, and (K) aspect ratio, before (black) or after (red) RASAL3 membrane recruitment. $n_c=16$
1043 from atleast 3 independent experiments; asterisks indicate significant difference, *** $P \leq 0.0001$
1044 (Wilcoxon-Mann-Whitney rank sum test). Centroid tracks of neutrophils ($n_c=16$) showing random
1045 motility before (H) or after (I) RASAL3 membrane recruitment. Each track lasts atleast 5 mins
1046 and was reset to same origin.

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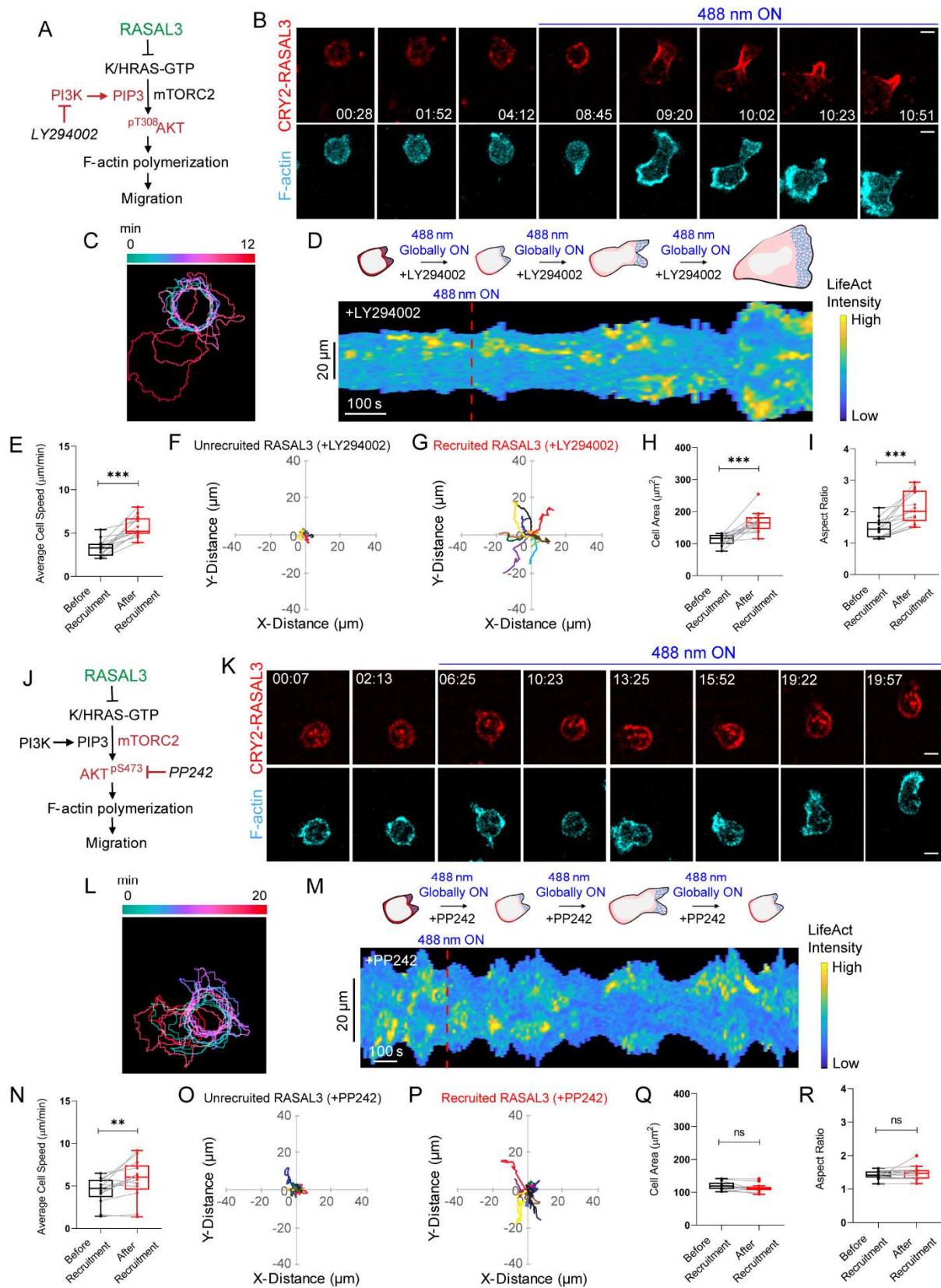
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1055 **Figure 6. RasGAPs polarize cells by increasing actomyosin contractility at the back. (A)**
1056 Time-lapse confocal images of vegetative myosin II heavy chain null ($mhcA^-$) *Dictyostelium* cells
1057 expressing C2GAPB after overnight doxycycline treatment. Time in min:sec format. Scale bars
1058 represent 5 μ m. Color-coded (at 1-min interval) outlines of C2GAPB-expressing cells. **(B)** Time-
1059 lapse confocal images of $mhcA^-$ *Dictyostelium* cell expressing mRFPmars-SspB R73Q-C2GAPB,
1060 before or after 488 nm laser was switched on globally. White and pink arrows denote cells of
1061 interest. Time in min:sec format. Scale bars represent 5 μ m. **(C)** Strategy for testing effects of
1062 myosin inhibitor, blebbistatin, on RASAL3-mediated actomyosin contraction and migration. **(D)**
1063 Time-lapse confocal images of blebbistatin-treated HL-60 neutrophil expressing CRY2PHR-
1064 mCherry-RASAL3 (red; upper panel) and LifeAct-miRFP703 (cyan; lower panel), before or after
1065 488 nm laser was turned on globally. Pink arrows denote cell of interest. Time in min:sec format.
1066 Scale bars represent 5 μ m. **(E)** Color-coded (at 1 min intervals) outlines of the cell shown in (D).
1067 **(F)** Representative kymograph of cortical LifeAct intensity in blebbistatin-treated RASAL3-
1068 expressing neutrophil before or after 488 nm laser was turned on. A linear color map shows that
1069 blue is the lowest LifeAct intensity whereas yellow is the highest. Duration of the kymograph is
1070 15 mins. Cartoon depicts membrane recruitment, actin polymerization or cell shape status
1071 corresponding to the kymograph. Box-and-whisker plots of **(G)** cell speed, **(J)** cell area, and **(K)**
1072 aspect ratio, before (black) or after (red) RASAL3 membrane recruitment. $n_c=12$ from atleast 3
1073 independent experiments; asterisks indicate significant difference, ***P \leq 0.001 (Wilcoxon-
1074 Mann-Whitney rank sum test). Centroid tracks of blebbistatin-treated neutrophils ($n_c=12$) showing
1075 random motility before **(H)** or after **(I)** RASAL3 membrane recruitment. Each track lasts atleast 5
1076 mins and was reset to same origin.

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1080 **Figure 7. RASAL3 polarizes cells by localizing actin polymerization at the front.** Strategy for
1081 testing effects of **(A)** pan-PI3K inhibitor, LY294002, or **(J)** mTOR inhibitor, PP242 on RASAL3-
1082 directed actin polymerization and motility. Time-lapse confocal images of **(B)** LY294002- or **(K)**
1083 PP242-treated HL-60 neutrophil expressing CRY2PHR-mCherry-RASAL3 (red; upper panel) and
1084 LifeAct-miRFP703 (cyan; lower panel), before or after 488 nm laser was turned on globally. Time
1085 in min:sec format. Scale bars represent 5 μ m. **(C, L)** Color-coded (at 1 min intervals) outlines of
1086 the cells shown in **(B)** and **(K)**, respectively. Representative kymographs of cortical LifeAct
1087 intensity in **(D)** LY294002- or **(M)** PP242-treated RASAL3-expressing neutrophil before or after
1088 488 nm laser was turned on. A linear color map shows that blue is the lowest LifeAct intensity
1089 whereas yellow is the highest. Duration of the kymographs are 12 mins and 20 mins, respectively.
1090 Cartoons depict membrane recruitment, actin polymerization or cell shape status corresponding to
1091 the kymographs. Box-and-whisker plots of **(E or N)** cell speed, **(H or Q)** cell area, and **(I or R)**
1092 aspect ratio, before (black) and after (red) RASAL3 recruitment in LY294002- or PP242-treated
1093 cells. $n_c=13$ from atleast 3 independent experiments; asterisks indicate significant difference, ***P
1094 ≤ 0.001 , **P ≤ 0.01 , and ns denotes P>0.05 (Wilcoxon-Mann-Whitney rank sum test). Centroid
1095 tracks of LY294002- or PP242-treated neutrophils ($n_c=13$) showing random motility before **(F or**
1096 **O)** or after **(G or P)** RASAL3 recruitment. Each track lasts atleast 5 mins and was reset to same
1097 origin.

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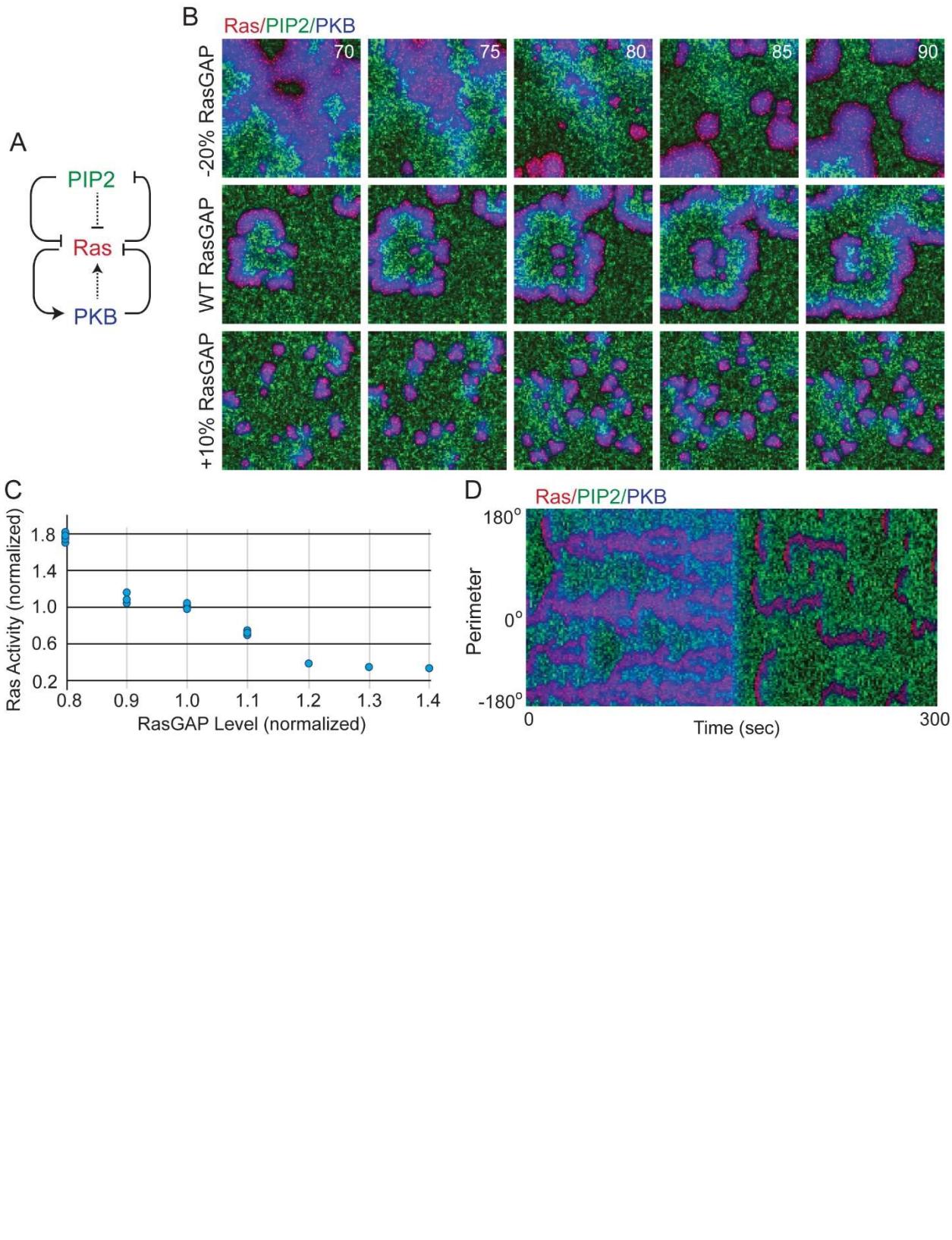
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1125 **Figure 8. Simulations of the Signal Transduction Excitable Network show increasing**
1126 **RasGAP leads to reduced Ras activity but cells still polarize.** (A) An excitable system
1127 incorporating positive and negative feedback loops between Ras, PIP2, and PKBA was simulated.
1128 Also included are two extra loops (dotted lines) described the effect of polarization. (B) The EN
1129 was simulated under varying levels of RasGAP (Methods). Assuming a wild-type level of
1130 RasGAP, firings of the excitable network were seen and led to propagating waves of high Ras
1131 activity (red) moving at speeds of ~10 $\mu\text{m}/\text{min}$. The trailing edge of these wave fronts was marked
1132 by high PKB (blue) activity, and by an absence of PIP2 (green). These waves showed
1133 characteristic excitable medium behavior, including annihilation of fronts following a collision,
1134 wave splitting and spiral activity. In simulations in which the level of RasGAP was lowered by
1135 20%, the size and speed of the wave increased by ~50% and ~100%, respectively. A 10% increase
1136 in the levels of RasGAP had the opposite effect. Firings were still seen, but they broke up
1137 frequently, leading to ~75% smaller and ~40% slower waves. The simulation denotes a square
1138 where each side is 40 μm and the time is in seconds. (C) To quantify the effect of RasGAP activity
1139 in the simulations ($n=5$ for each condition), we computed the total level of Ras over the
1140 simulations, while varying RasGAP from 80-140% of wild type levels. Shown is the mean level
1141 of activity over the whole simulation. Note that after about a 30% increase in RasGAP, no firings
1142 were observed, and hence the level of Ras plateaus. (D) We also carried out simulations in smaller
1143 cells represented by a one-dimensional environment (representative of the perimeter of a cell). The
1144 strength of the RasGAP contribution was increased 50% halfway during the simulation.

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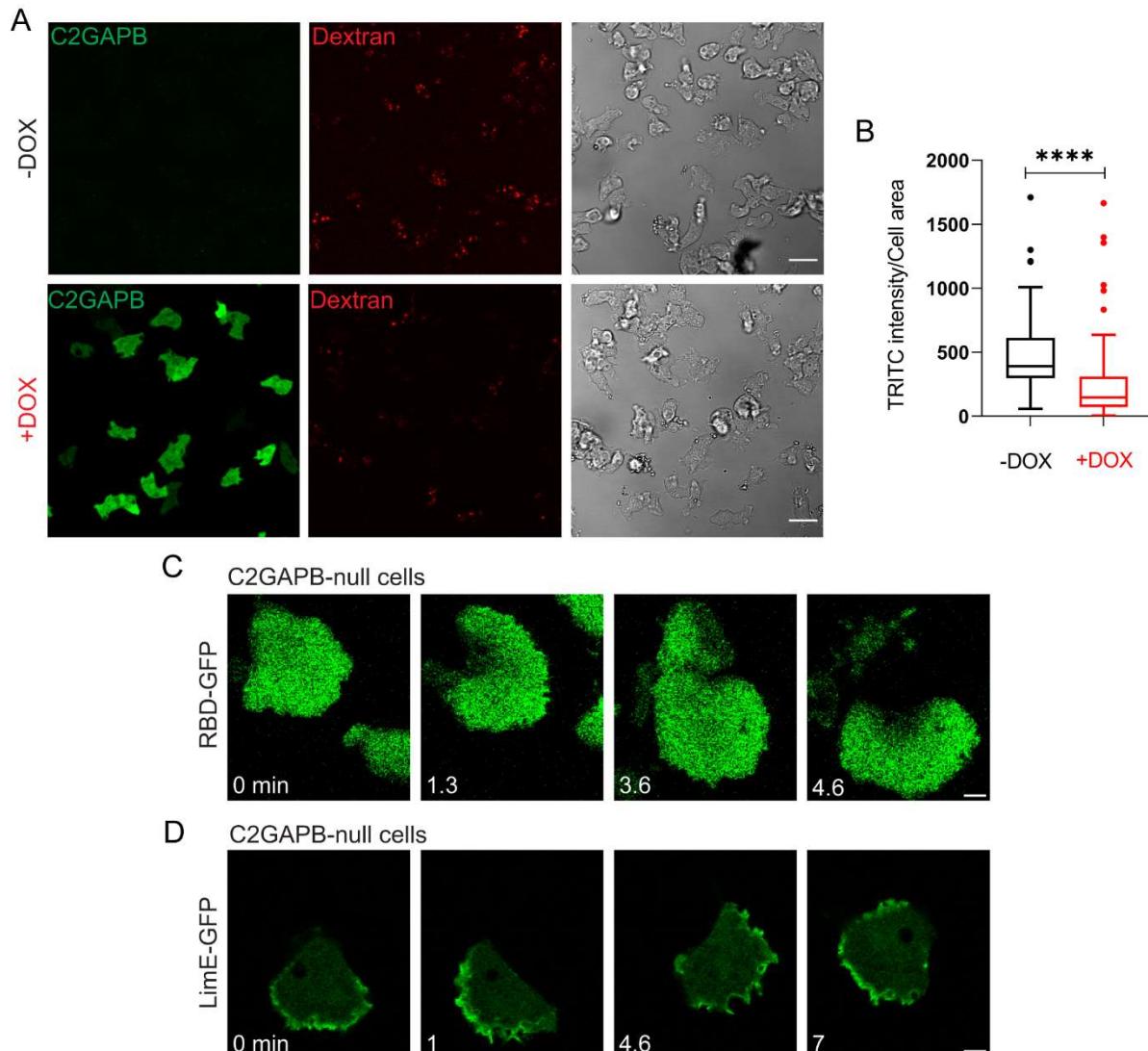
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1159 **Supplementary Information**

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1161 **Supplementary Figures**

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1170 **Figure S1. C2GAPB regulates Ras and protrusive activities.** **(A)** Representative confocal
1171 images of vegetative *Dictyostelium* cells before (top panel, ‘-DOX’) and after (bottom panel,
1172 ‘+DOX’) doxycycline-induced GFP-C2GAPB (green) expression. Cells (DIC) were treated with
1173 TRITC-dextran (red) before imaging. Scale bars represent 10 μm . **(B)** Quantification of
1174 macropinocytosis uptake, before (black) and after (red) C2GAPB expression. $n_c=103$ from atleast
1175 3 independent experiments; asterisks indicate significant difference, *** $P \leq 0.0001$ (Wilcoxon-
1176 Mann-Whitney rank sum test). **(C, D)** Time-lapse confocal images of vegetative C2GAPB-null
1177 *Dictyostelium* cells expressing **(C)** RBD-GFP or **(D)** LimE-GFP. Time in ‘min’ format. Scale bars
1178 represent 5 μm .

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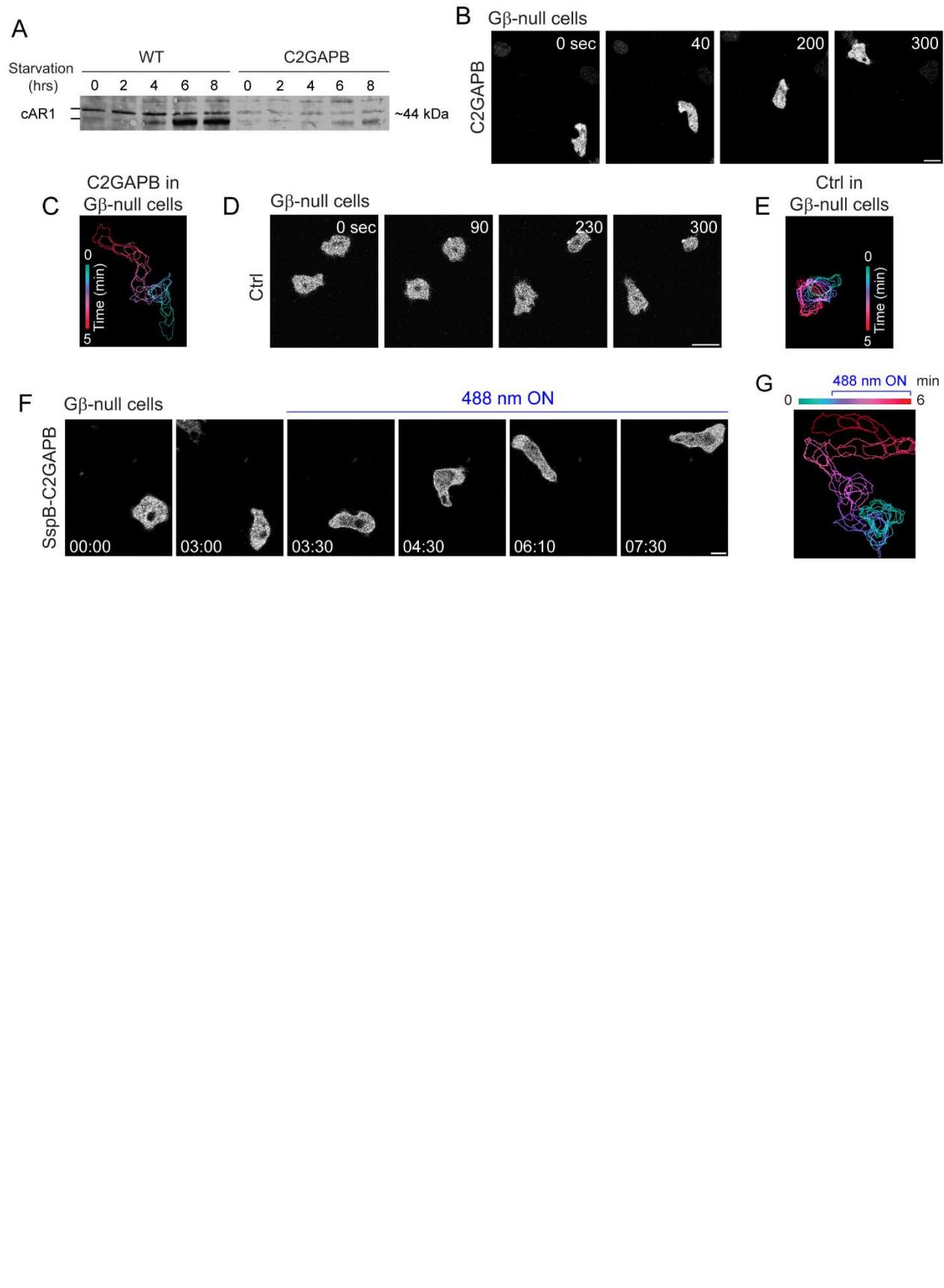
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1209 **Figure S2. C2GAPB-induced polarization does not require development or GPCR signaling.**

1210 **(A)** Representative western blot showing expression of cAR1 (~44 kDa) in developing wildtype
1211 (WT) or C2GAPB-expressing *Dictyostelium* cells during starvation (0-8 hrs). cAR1 appears as a
1212 doublet denoting its two forms, unmodified (lower band) and phosphorylated (upper band). Time-
1213 lapse confocal images of vegetative G β -null *Dictyostelium* cells expressing **(B)** mRFPmars-
1214 C2GAPB or **(D)** tgRFPt-Ctrl (control without C2GAPB) after overnight doxycycline treatment.
1215 Time in ‘sec’ format. Scale bars represent 5 μ m. **(C, E)** Color-coded (at 1-min interval) outlines
1216 of the C2GAPB- or Ctrl-expressing cell shown in (B) or (D), respectively. **(F)** Time-lapse confocal
1217 images of G β -null *Dictyostelium* cell expressing mRFPmars-SspB R73Q-C2GAPB, before or after
1218 488 nm laser was switched on globally. Time in min:sec format. Scale bars represent 5 μ m. **(G)**
1219 Color-coded (at 1-min interval) outlines of the mRFPmars-SspB R73Q-C2GAPB-expressing cell
1220 shown in (F).

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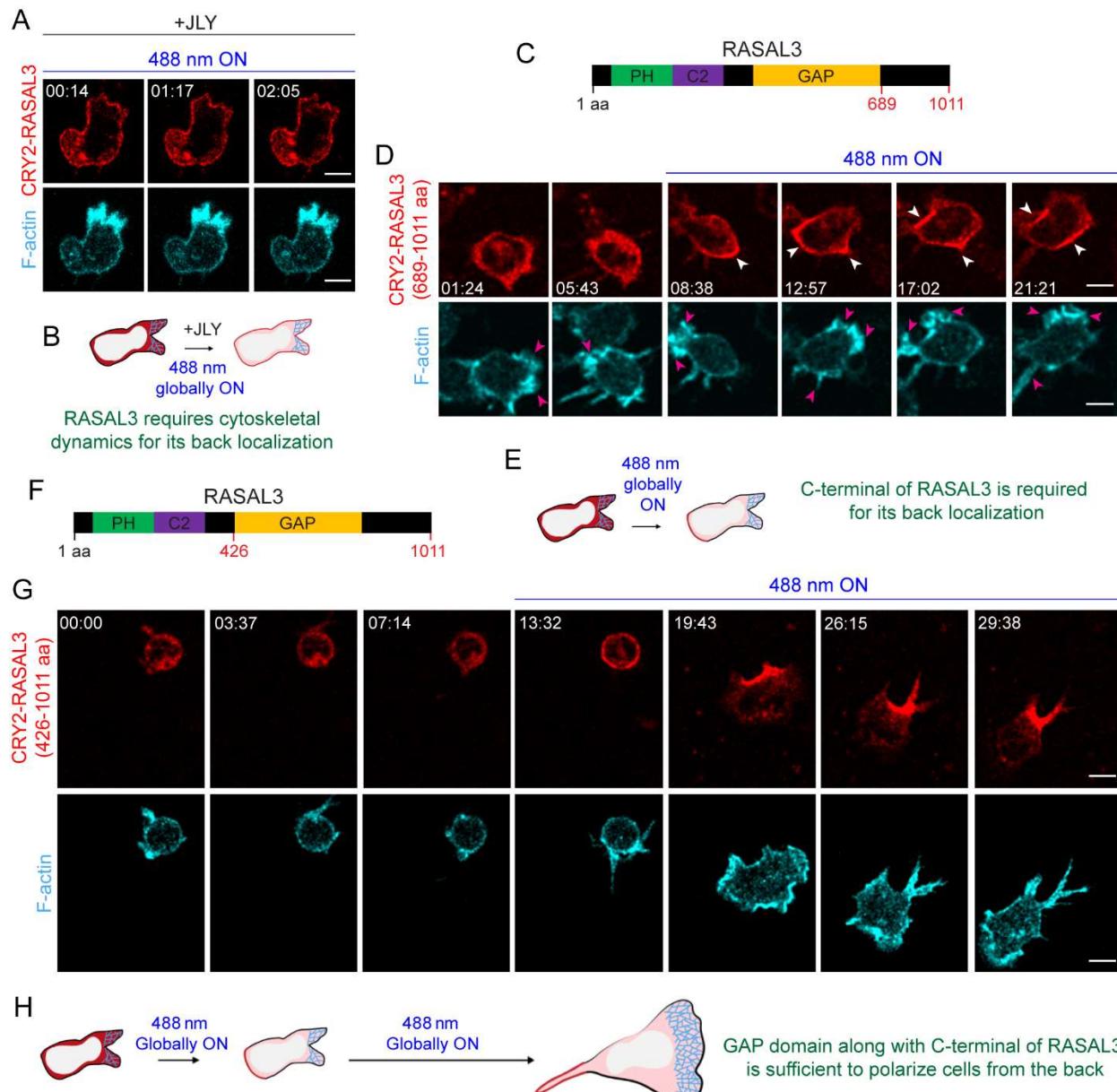
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1251 **Figure S3. RASAL3 back localization is dependent on cytoskeletal dynamics and its C-**
1252 **terminal tail whereas polarizing function of RASAL3 is due to its GAP domain along with**
1253 **C-terminal tail. (A)** Time-lapse confocal images of JLY cocktail-treated HL-60 neutrophil
1254 expressing CRY2PHR-mCherry-RASAL3 (red; upper panel) and LifeAct-miRFP703 (cyan; lower
1255 panel), after 488 nm laser was turned on globally. Time in min:sec format. Scale bars represent 5
1256 μm. **(B)** Cartoon shows that JLY treatment caused RASAL3 to recruit uniformly instead of
1257 localizing to the back. **(C, F)** Schematics showing RASAL3 protein sequence (1-1011 amino acid
1258 long). Two truncation mutants were generated in this study: **(C)** RASAL3₆₈₉₋₁₀₁₁ which consists of
1259 only the C-terminal tail (689-1011 amino acids) and **(F)** RASAL3₄₂₆₋₁₀₁₁ consisting of the GAP
1260 domain along with the C-terminal tail (426-1011 amino acids). **(D, G)** Time-lapse confocal images
1261 of differentiated HL-60 neutrophil expressing **(D)** CRY2PHR-mCherry-RASAL3 (689-1011 aa)
1262 or **(G)** CRY2PHR-mCherry-RASAL3 (426-1011 aa) (red; upper panel) and LifeAct-miRFP703
1263 (cyan; lower panel), before or after 488 nm laser was turned on globally. Time in min:sec format.
1264 Scale bars represent 5 μm. **(E, H)** Cartoons demonstrate phenomenon observed with recruiting
1265 CRY2PHR-mCherry-RASAL3 (689-1011 aa) or CRY2PHR-mCherry-RASAL3 (426-1011 aa) in
1266 differentiated neutrophils.

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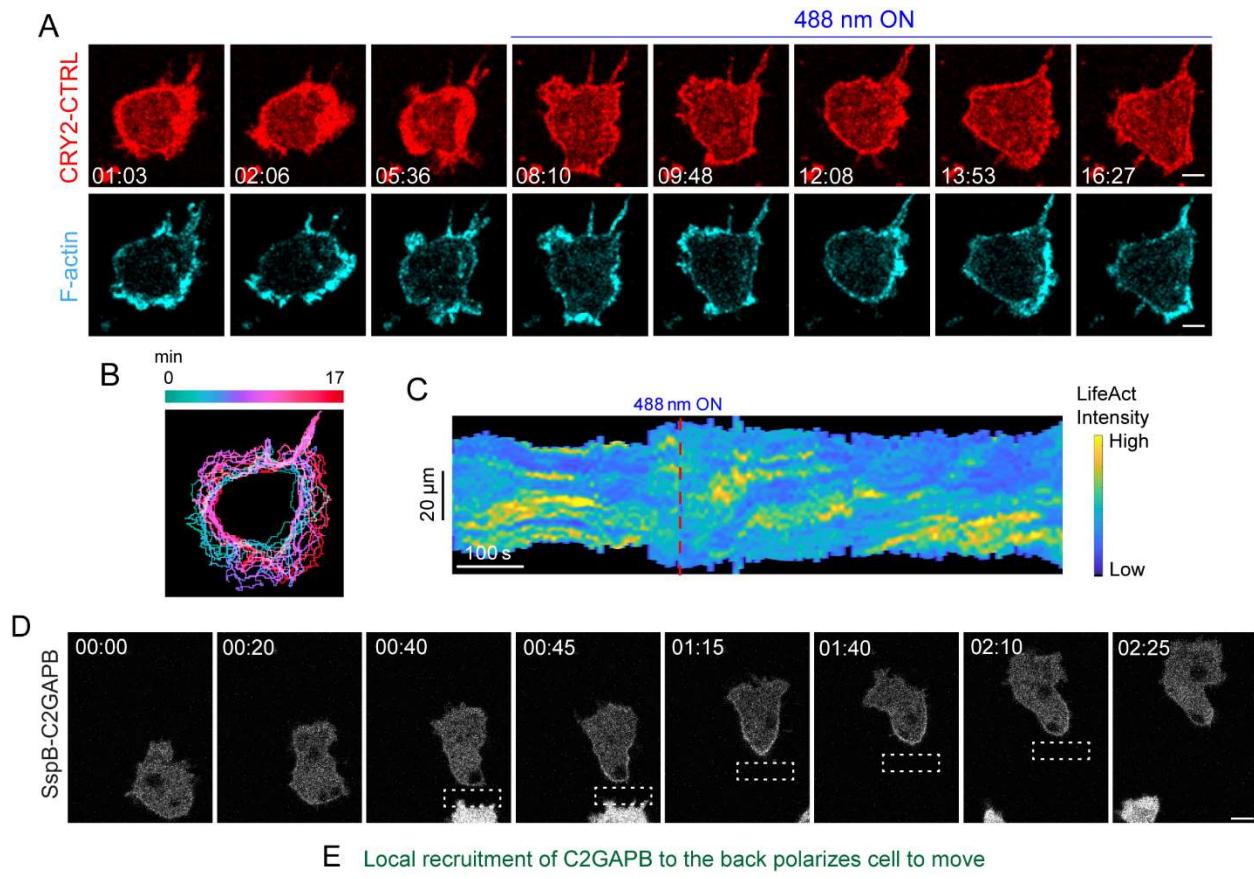
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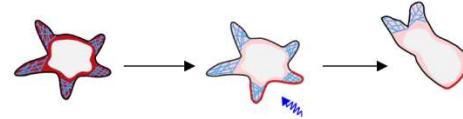
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E Local recruitment of C2GAPB to the back polarizes cell to move



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488 nm OFF

Local 488 nm ON

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1295 **Figure S4. Recruitment of RasGAP, and not CTRL, moves cell rapidly. (A)** Time-lapse
1296 confocal images of differentiated HL-60 neutrophil expressing CRY2PHR-mCherry-CTRL
1297 (control without RASAL3, red; upper panel) and LifeAct-miRFP703 (cyan; lower panel), before
1298 or after 488 nm laser was globally applied. Time in min:sec format. Scale bars represent 5 μ m. **(B)**
1299 Color-coded (at 1 min intervals) outlines of the cell shown in (A). **(C)** Representative kymograph
1300 of cortical LifeAct intensity in CTRL-expressing neutrophil before or after 488 nm laser was
1301 switched on. A linear color map shows that blue is the lowest LifeAct intensity whereas yellow is
1302 the highest. Duration of the kymograph is 17 mins. The cartoon summarizes membrane
1303 recruitment, F-actin polymerization or cell shape status corresponding to the kymograph. **(D)**
1304 Time-lapse confocal images of vegetative *Dictyostelium* cells expressing mRFPmars-SspB R73Q-
1305 C2GAPB which is recruited to the back of the migrating cell by applying 488 nm laser near it, as
1306 shown by the dashed white box. Time in min:sec format. Scale bars represent 5 μ m. **(E)** Cartoon
1307 demonstrates C2GAPB-mediated phenomenon shown in (D).

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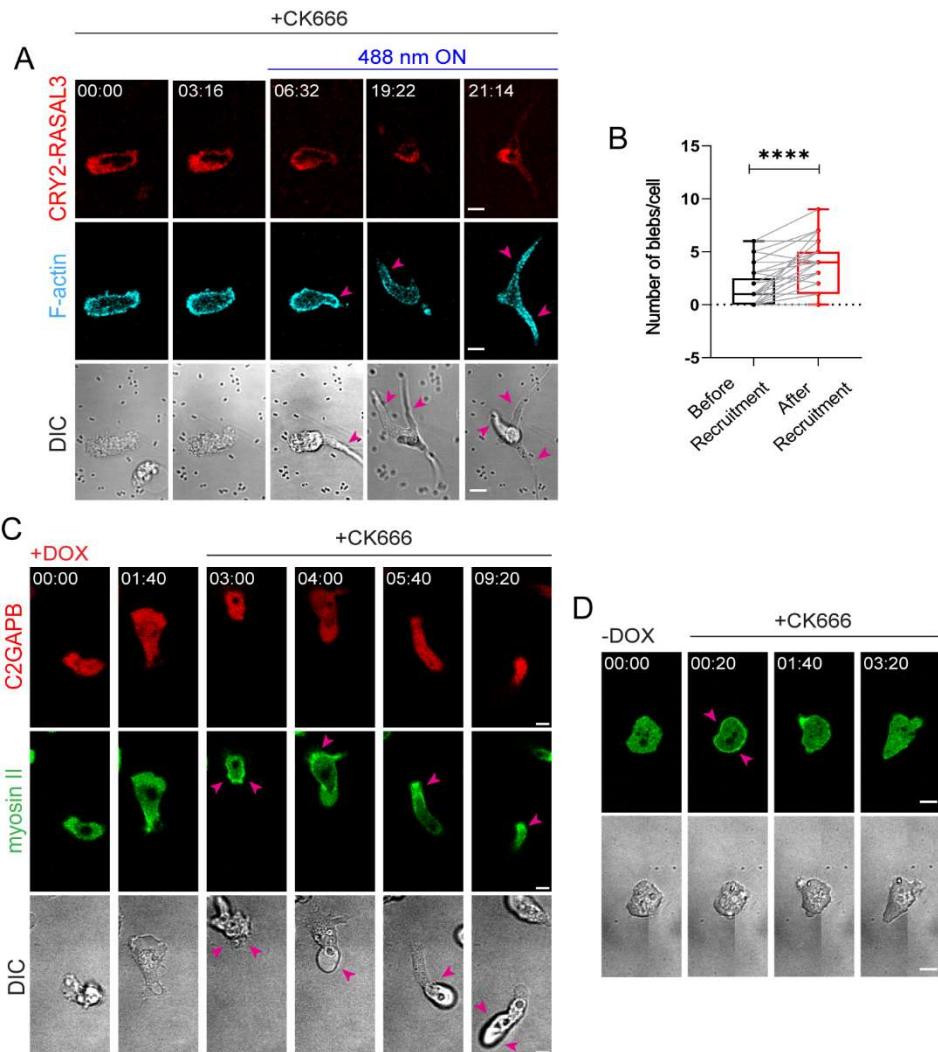
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1340 **Figure S5. RasGAPs increase cellular contraction.** **(A)** Time-lapse confocal images of CK666-
1341 treated differentiated HL-60 neutrophil expressing CRY2PHR-mCherry-RASAL3 (red; upper
1342 panel) and LifeAct-miRFP703 (cyan; lower panel), before or after 488 nm laser was globally
1343 applied. Pink arrows denote long blebs which appear after recruited RASAL3 localized to the back
1344 of CK666-treated cell. Time in min:sec format. Scale bars represent 5 μ m. **(B)** Box-and-whisker
1345 plot of number of blebs per cell within a minute, before (black) or after (red) RASAL3 recruitment.
1346 $n_c=37$ from atleast 3 independent experiments; asterisks indicate significant difference, ***P \leq
1347 0.0001 (Wilcoxon-Mann-Whitney rank sum test). Time-lapse confocal images of vegetative
1348 *Dictyostelium* cells expressing mRFPmars-C2GAPB (red; top panel) and myosin II-GFP (green;
1349 middle panel) before **(C)** or after **(D)** overnight doxycycline treatment. CK666 was added during
1350 imaging. Since there is no C2GAPB expression without doxycycline treatment, C2GAPB (red
1351 panel) is not shown in **(D)**. Pink arrows denote appearance of long blebs (DIC channel) in
1352 C2GAPB-expressing cells after CK666 was added. Time in min:sec format. Scale bars represent
1353 5 μ m.

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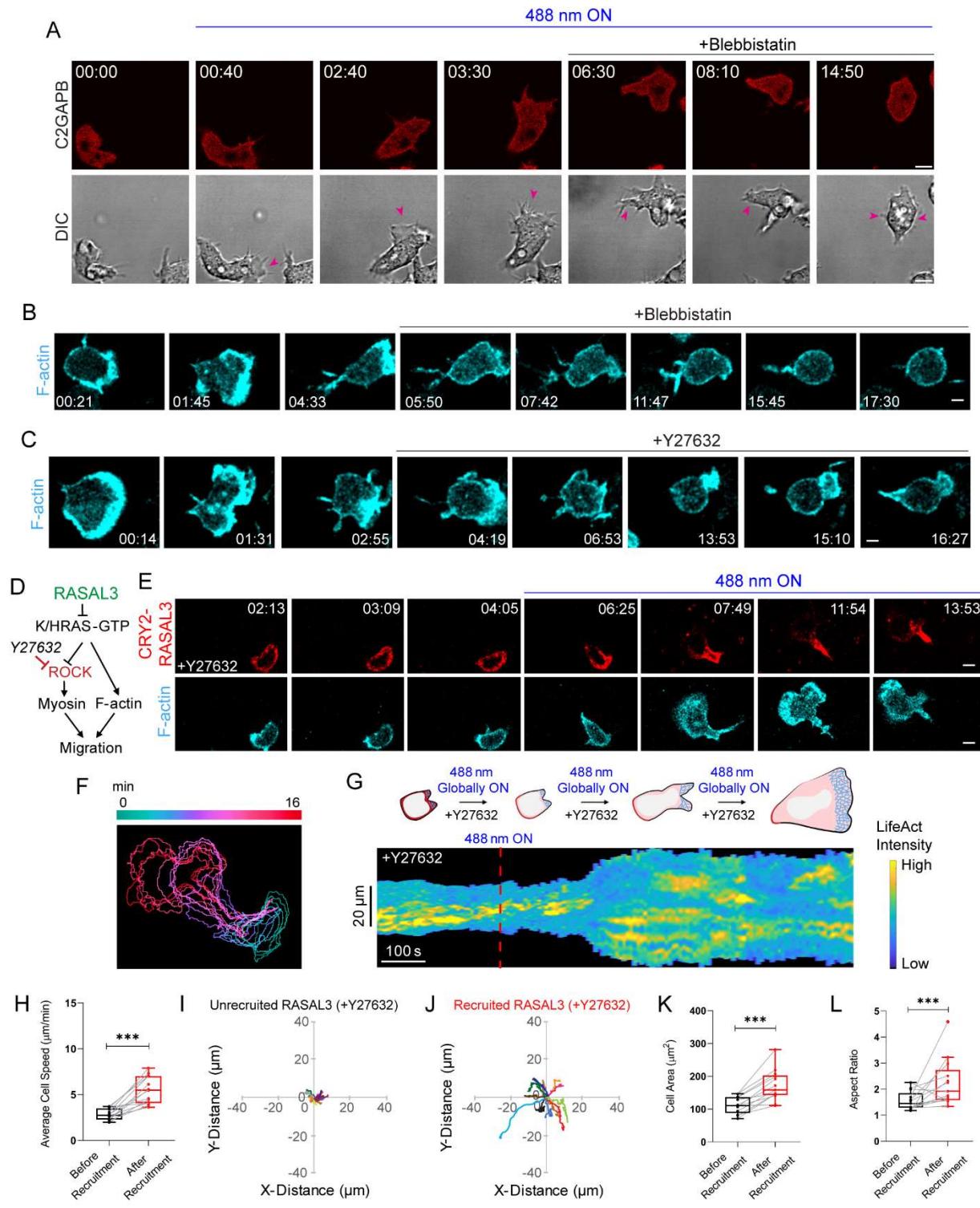
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1377 **Figure S6. Global RASAL3 recruitment overcomes ROCK inhibition whereas global**
1378 **C2GAPB recruitment does not overcome myosin inhibition.** (A) Time-lapse confocal images
1379 of vegetative *Dictyostelium* cells expressing mRFPmars-SspB R73Q-C2GAPB (red; top panel),
1380 before or after 488 nm laser was switched on globally. Blebbistatin was added during imaging.
1381 Pink arrows denote cell of interest (DIC channel). Time in min:sec format. Scale bars represent 5
1382 μm . (B, C) Time-lapse confocal images of HL-60 neutrophil expressing LifeAct-miRFP703 (cyan;
1383 lower panel), before or after (B) blebbistatin and (C) Y27632 treatment. Time in min:sec format.
1384 Scale bars represent 5 μm . (D) Strategy for testing effect of ROCK inhibitor, Y27632, on
1385 RASAL3-directed actin polymerization and migration. (E) Time-lapse confocal images of
1386 Y27632-treated HL-60 neutrophil expressing CRY2PHR-mCherry-RASAL3 (red; upper panel)
1387 and LifeAct-miRFP703 (cyan; lower panel), before or after 488 nm laser was turned on globally.
1388 Time in min:sec format. Scale bars represent 5 μm . (F) Color-coded (at 1 min intervals) outlines
1389 of the cell shown in (E). (G) Representative kymograph of cortical LifeAct intensity in Y27632-
1390 treated RASAL3-expressing neutrophil before or after 488 nm laser was turned on. A linear color
1391 map shows that blue is the lowest LifeAct intensity whereas yellow is the highest. Duration of the
1392 kymograph is 16 mins. Cartoon summarizes membrane recruitment, actin polymerization or cell
1393 shape status corresponding to the kymograph. Box-and-whisker plots of (H) cell speed, (K) cell
1394 area, and (L) aspect ratio, before (black) and after (red) RASAL3 recruitment in Y27632-treated
1395 cells. $n_c=13$ from atleast 3 independent experiments; asterisks indicate significant difference, ***P
1396 ≤ 0.001 (Wilcoxon-Mann-Whitney rank sum test). Centroid tracks of Y27632-treated neutrophils
1397 ($n_c=13$) showing random motility before (I) or after (J) RASAL3 recruitment. Each track lasts
1398 atleast 5 mins and was reset to same origin.

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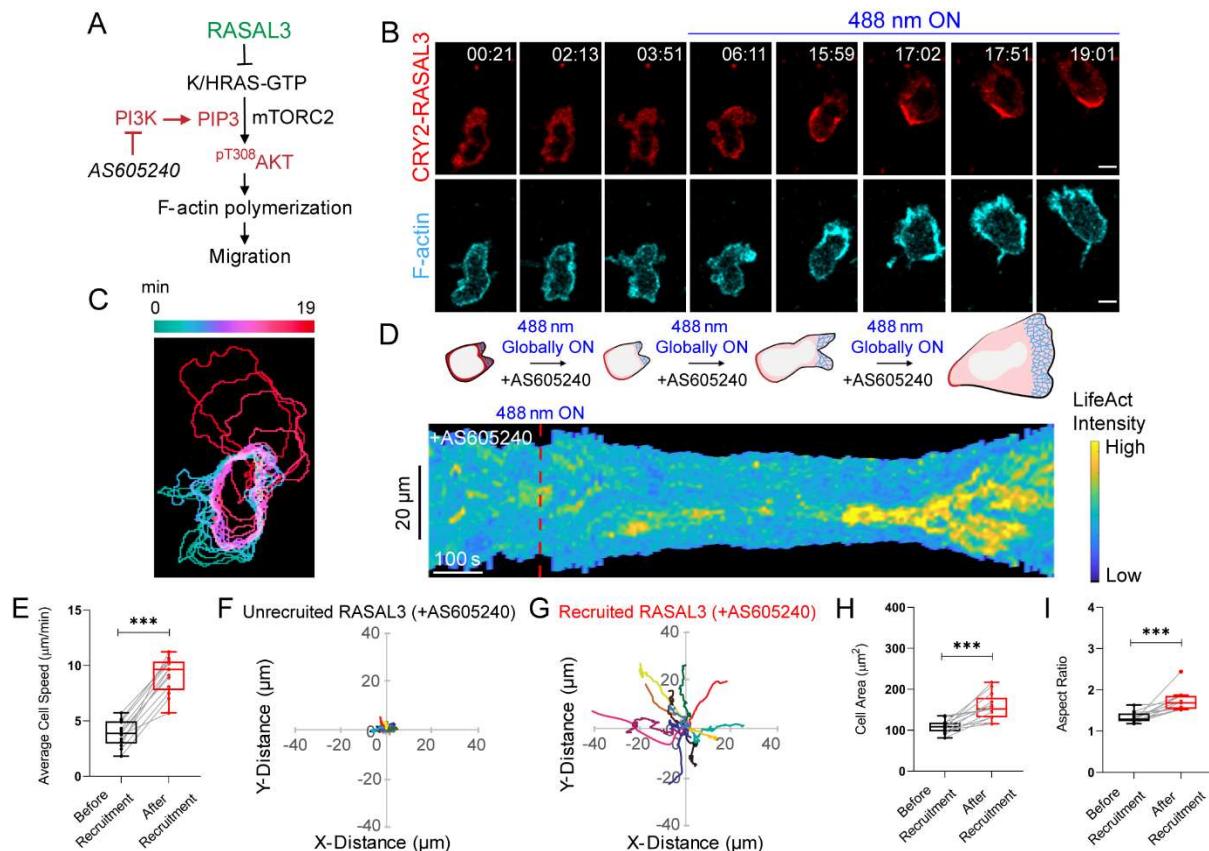
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1429 **Figure S7. Global RASAL3 recruitment overcomes PI3K γ inhibition.** **(A)** Strategy for testing
1430 effect of PI3K γ inhibitor, AS605240, on RASAL3-directed actin polymerization and motility. **(B)**
1431 Time-lapse confocal images of AS605240-treated HL-60 neutrophil expressing CRY2PHR-
1432 mCherry-RASAL3 (red; upper panel) and LifeAct-miRFP703 (cyan; lower panel), before or after
1433 488 nm laser was turned on globally. Time in min:sec format. Scale bars represent 5 μ m. **(C)**
1434 Color-coded (at 1 min intervals) outlines of the cells shown in (B). **(D)** Representative kymograph
1435 of cortical LifeAct intensity in AS605240-treated RASAL3-expressing neutrophil before or after
1436 488 nm laser was turned on. A linear color map shows that blue is the lowest LifeAct intensity
1437 whereas yellow is the highest. Duration of the kymograph is 19 mins. Cartoon summarizes
1438 membrane recruitment, actin polymerization or cell shape status corresponding to the kymograph.
1439 Box-and-whisker plots of **(E)** cell speed, **(H)** cell area, and **(I)** aspect ratio, before (black) and after
1440 (red) RASAL3 recruitment in AS605240-treated cells. $n_c=13$ from atleast 3 independent
1441 experiments; asterisks indicate significant difference, ***P \leq 0.001 (Wilcoxon-Mann-Whitney
1442 rank sum test). Centroid tracks of AS605240-treated neutrophils ($n_c=13$) showing random motility
1443 before **(F)** or after **(G)** RASAL3 recruitment. Each track lasts atleast 5 mins and was reset to same
1444 origin.

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1463 **Supplementary Video Legends**

1464 **Video S1**

1465 Time-lapse confocal microscopy of GFP-RBD (green; left panel) cortical waves at the substrate-
1466 attached surface in electrofused, giant *Dictyostelium* cell, before overnight doxycycline treatment
1467 ('-DOX' at top of the video). Without doxycycline, mRFPmars-C2GAPB (red; right panel) was
1468 not expressed in the cell. Top left corner shows time in min:sec format. Scale bar represents 5 μm .

1469 **Video S2**

1470 Time-lapse confocal microscopy of GFP-RBD (green; left panel) cortical waves at the substrate-
1471 attached surface in electrofused, giant *Dictyostelium* cells, after overnight doxycycline treatment
1472 ('+DOX' at top of the video). Doxycycline induced mRFPmars-C2GAPB (red; right panel)
1473 expression in the cell. Top left corner shows time in min:sec format. Scale bar represents 5 μm .

1474 **Video S3**

1475 Time-lapse confocal microscopy of PHcrac-YFP (green; left panel) cortical waves at the substrate-
1476 attached surface in electrofused, giant *Dictyostelium* cell, before overnight doxycycline treatment
1477 ('-DOX' at top of the video). Without doxycycline, mRFPmars-C2GAPB (red; right panel) was
1478 not expressed in the cell. Top left corner shows time in min:sec format. Scale bar represents 5 μm .

1479 **Video S4**

1480 Time-lapse confocal microscopy of PHcrac-YFP (green; left panel) cortical waves at the substrate-
1481 attached surface in electrofused, giant *Dictyostelium* cells, after overnight doxycycline treatment
1482 ('+DOX' at top of the video). Doxycycline induced mRFPmars-C2GAPB (red; right panel)
1483 expression in the cell. Top left corner shows the time in min:sec format. Scale bar represents 5 μm .

1484 **Video S5**

1485 Time-lapse confocal microscopy of RBD-YFP (yellow; left panel) cortical waves at the substrate-
1486 attached surface of electrofused, giant *Dictyostelium* cells, before or after global membrane
1487 recruitment of mRFPmars-SspB R73Q-C2GAPB ('Opto-C2GAPB'; red, right panel). Global
1488 recruitment was initiated when blue laser was applied globally ("488nm on" at top of the video) at
1489 '05:50' and '30:00'. Propagating RBD waves extinguished with C2GAPB recruitment, and
1490 recovered when 488nm laser was switched off again. Top left corner shows the time in min:sec
1491 format. Scale bars represent 10 μm .

1492 **Video S6**

1493 Time-lapse confocal microscopy of PHcrac-YFP (yellow; left panel) cortical waves at the
1494 substrate-attached surface of electrofused, giant *Dictyostelium* cells, before or after global
1495 membrane recruitment of mRFPmars-SspB R73Q-C2GAPB ('Opto-C2GAPB'; red, right panel).
1496 Global recruitment was initiated when blue laser was applied globally ("488nm on" at top of the
1497 video) at '16:20' or '02:00' in movie 1 or 2, respectively. Propagating PHcrac waves extinguished
1498 with C2GAPB recruitment. Top left corner shows the time in min:sec format. Scale bars represent
1499 10 μm .

1500 **Video S7**

1501 Time-lapse confocal microscopy of differentiated HL-60 neutrophil expressing CRY2PHR-
1502 mCherry-RASAL3 (red; left panel) and LifeAct-miRFP703 (cyan; right panel). RASAL3 was
1503 recruited to the membrane anchor, CIBN-CAAX (untagged), at the cell front by intermittently
1504 applying blue (488 nm) laser near it, as shown with the white box in the red channel. The
1505 disappearance of cellular protrusions at the RASAL3 recruitment site is denoted with solid white
1506 box in the blue channel. The top right corner shows time in min:sec format. Neutrophils were not
1507 exposed to chemoattractants during this experiment. Scale bar: 5 μ m.

1508 **Video S8**

1509 Time-lapse confocal microscopy of vegetative *Dictyostelium* AX2 cells before (-Dox; left panel)
1510 and after overnight doxycycline treatment (+Dox; right panel). Doxycycline induced mRFPmars-
1511 C2GAPB (red; right panel) expression in cells. The top right corner of each panel shows time in
1512 min:sec format. Cells were not exposed to chemoattractants during this experiment. Scale bar
1513 represents 20 μ m.

1514 **Video S9**

1515 Time-lapse confocal microscopy of vegetative *Dictyostelium* AX2 cell before or after global
1516 membrane recruitment of tgRFPt-SspB R73Q-Ctrl (Opto-Ctrl). Global recruitment was initiated
1517 when blue laser was applied globally (“488nm on” at top of the video) at ‘04:00’. Top left corner
1518 shows time in min:sec format. Cell was not exposed to any chemoattractant during this experiment.
1519 Scale bar represents 5 μ m.

1520 **Video S10**

1521 Time-lapse confocal microscopy of vegetative *Dictyostelium* AX2 cell before or after global
1522 membrane recruitment of mRFPmars-SspB R73Q-C2GAPB (Opto-C2GAPB). In movie 1, global
1523 recruitment was initiated when blue laser was applied globally (“488nm on” at top of the video) at
1524 ‘02:00’. In movie 2, blue laser was switched on or off multiple times during the course of the
1525 experiment, as denoted with “488nm on” or “488nm off” at the top of the movie. Top left corner
1526 shows time in min:sec format. Cells were not exposed to any chemoattractant during this
1527 experiment. Scale bar represents 5 μ m.

1528 **Video S11**

1529 Time-lapse confocal microscopy of vegetative *Dictyostelium* G β null (G β $^{-}$) cell before or after
1530 global membrane recruitment of mRFPmars-SspB R73Q-C2GAPB (Opto-C2GAPB). Global
1531 recruitment was initiated when blue laser was applied globally (“488nm on” at top of the video) at
1532 ‘08:20’. Top left corner shows time in min:sec format. Cell was not exposed to any chemoattractant
1533 during this experiment. Scale bar represents 5 μ m.

1534 **Video S12**

1535 Time-lapse confocal microscopy of LimE $_{\Delta coil}$ -YFP (yellow; left panel) cortical waves at the
1536 substrate-attached surface of electrofused, giant *Dictyostelium* cells, before or after global
1537 membrane recruitment of mRFPmars-SspB R73Q-C2GAPB (‘Opto-C2GAPB’; red, right panel).
1538 Global recruitment was initiated when blue laser was applied globally (“488nm on” at top of the

1539 video) at '15:00'. Propagating LimE waves extinguished, with C2GAPB recruitment, except for
1540 one standing wave in the middle. Top left corner shows the time in min:sec format. Scale bar
1541 represents 10 μ m.

1542 **Video S13**

1543 Time-lapse confocal microscopy of differentiated, unpolarized HL-60 neutrophil expressing
1544 CRY2PHR-mCherry-RASAL3 (red; left panel) and LifeAct-miRFP703 (cyan; right panel).
1545 RASAL3 was recruited to the membrane anchor, CIBN-CAAX (untagged), at the transient
1546 protrusions by intermittently applying blue (488 nm) laser near it, as shown with the white box in
1547 the red channel. The disappearance of cellular protrusions at the RASAL3 recruitment site is
1548 denoted with solid white box in the blue channel. 488 nm laser was switched off '03:09' onwards
1549 to visualize the effects of RASAL3 self-rearrangement on the membrane. The top right corner
1550 shows time in min:sec format. Neutrophils were not exposed to chemoattractants during this
1551 experiment. Scale bar: 5 μ m.

1552 **Video S14**

1553 Time-lapse confocal microscopy of differentiated HL-60 neutrophil expressing CRY2PHR-
1554 mCherry-RASAL3 (red; left panel) and LifeAct-miRFP703 (cyan; right panel), before or after 488
1555 nm laser was applied globally. The membrane anchor, untagged CIBN-CAAX, was expressed.
1556 Top left corner shows time in min:sec format. To start recruitment (red; left panel), the laser was
1557 switched on at '07:07' once '488 nm ON' appears at the top of the video. Cell was not exposed to
1558 chemoattractant during the experiment. Scale bar represents 5 μ m.

1559 **Video S15**

1560 Time-lapse confocal microscopy of differentiated HL-60 neutrophil expressing CRY2PHR-
1561 mCherry-CTRL (red; left panel) and LifeAct-miRFP703 (cyan; right panel), before or after 488
1562 nm laser was turned on globally. Membrane anchor, untagged CIBN-CAAX, was also expressed.
1563 Top left corner shows time in min:sec format. To start recruitment (red; left panel), the laser was
1564 switched on at '05:43' once '488 nm ON' appears at the top of the video. Cell was not exposed to
1565 chemoattractant during the experiment. Scale bar represents 5 μ m.

1566 **Video S16**

1567 Time-lapse confocal microscopy of differentiated, blebbistatin-treated HL-60 neutrophil
1568 expressing CRY2PHR-mCherry-RASAL3 (red; left panel) and LifeAct-miRFP703 (cyan; right
1569 panel), before or after 488 nm laser was turned on globally. Membrane anchor, untagged CIBN-
1570 CAAX, was also expressed. Cell was treated with 20 μ M blebbistatin atleast 10 mins before
1571 imaging. Pink arrow denotes the cell of interest. To start recruitment (red; left panel), the laser was
1572 switched on at '03:23' once '488 nm ON' appears at the top of the video. Cell was not exposed to
1573 chemoattractant during the experiment. Top left corner shows time in min:sec format. Scale bar
1574 represents 5 μ m.

1575 **Video S17**

1576 Time-lapse confocal microscopy of differentiated, Y27632-treated HL-60 neutrophil expressing
1577 CRY2PHR-mCherry-RASAL3 (red; left panel) and LifeAct-miRFP703 (cyan; right panel), before

1578 or after 488 nm laser was turned on globally. Membrane anchor, untagged CIBN-CAAX, was also
1579 expressed. Cell was treated with 10 μ M Y27632 atleast 10 mins before imaging. To start
1580 recruitment (red; left panel), the laser was switched on at ‘04:12’ once ‘488 nm ON’ appears at the
1581 top of the video. Neutrophil was not exposed to any chemoattractant during imaging. Top left
1582 corner shows time in min:sec format. Scale bar represents 5 μ m.

1583 **Video S18**

1584 Time-lapse confocal microscopy of differentiated, LY294002-treated HL-60 neutrophil
1585 expressing CRY2PHR-mCherry-RASAL3 (red; left panel) and LifeAct-miRFP703 (cyan; right
1586 panel), before or after 488 nm laser was applied globally. The membrane anchor, untagged CIBN-
1587 CAAX, was expressed as well. Cell was treated with 50 μ M LY294002 atleast 10 mins before
1588 imaging. To start recruitment (red; left panel), the laser was switched on at ‘04:12’ once ‘488 nm
1589 ON’ appears at the top of the video. Neutrophil was not exposed to any chemoattractant during
1590 imaging. Top left corner shows time in min:sec format. Scale bar represents 5 μ m.

1591 **Video S19**

1592 Time-lapse confocal microscopy of differentiated, AS605240-treated HL-60 neutrophil expressing
1593 CRY2PHR-mCherry-RASAL3 (red; left panel) and LifeAct-miRFP703 (cyan; right panel), before
1594 or after 488 nm laser was turned on globally. Membrane anchor, untagged CIBN-CAAX, was
1595 expressed here. Cell was treated with 20 μ M AS605240 atleast 10 mins before imaging. To start
1596 recruitment (red; left panel), the laser was turned on at ‘03:58’ once ‘488 nm ON’ appears at the
1597 top of the video. Neutrophil was not exposed to any chemoattractant during imaging. Top left
1598 corner shows time in min:sec format. Scale bar represents 5 μ m.

1599 **Video S20**

1600 Time-lapse confocal microscopy of differentiated, PP242-treated HL-60 neutrophil expressing
1601 CRY2PHR-mCherry-RASAL3 (red; left panel) and LifeAct-miRFP703 (cyan; right panel), before
1602 or after 488 nm laser was turned on globally. Membrane anchor, untagged CIBN-CAAX, was
1603 expressed. Cell was treated with 20 μ M PP242, 10 mins before imaging. To start recruitment (red;
1604 left panel), the laser was turned on at ‘06:04’ once ‘488 nm ON’ appears at the top of the video.
1605 Cell was not exposed to any chemoattractant during imaging. Top left corner shows time in min:sec
1606 format. Scale bar represents 5 μ m.

1607 **Video S21**

1608 Two-dimensional simulation results of the excitable network for varying RasGAP levels. The
1609 video shows three different simulations, with 80% WT, WT and 110%-RasGAP levels,
1610 sequentially. The area is a square with sides 40 μ m long and the time-stamp denotes seconds. The
1611 three colors correspond to Ras (red), PIP2 (green) and PKB (blue).

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