Supplemental Information includes 7 Supplemental Figures, Supplemental Experimental Procedures and 6 Movies.

Figure S1 Related to Figure 1

Figure S2 Related to Figure 2

Figure S3 Related to Figure 2

Figure S4 Related to Figure 2

Figure S5 Related to Figure 3

Figure S6 Related to Figure 4

Figure S7 Related to Figure 7 and Experimental Procedures

Movies S1-S6 Related to Figure 3

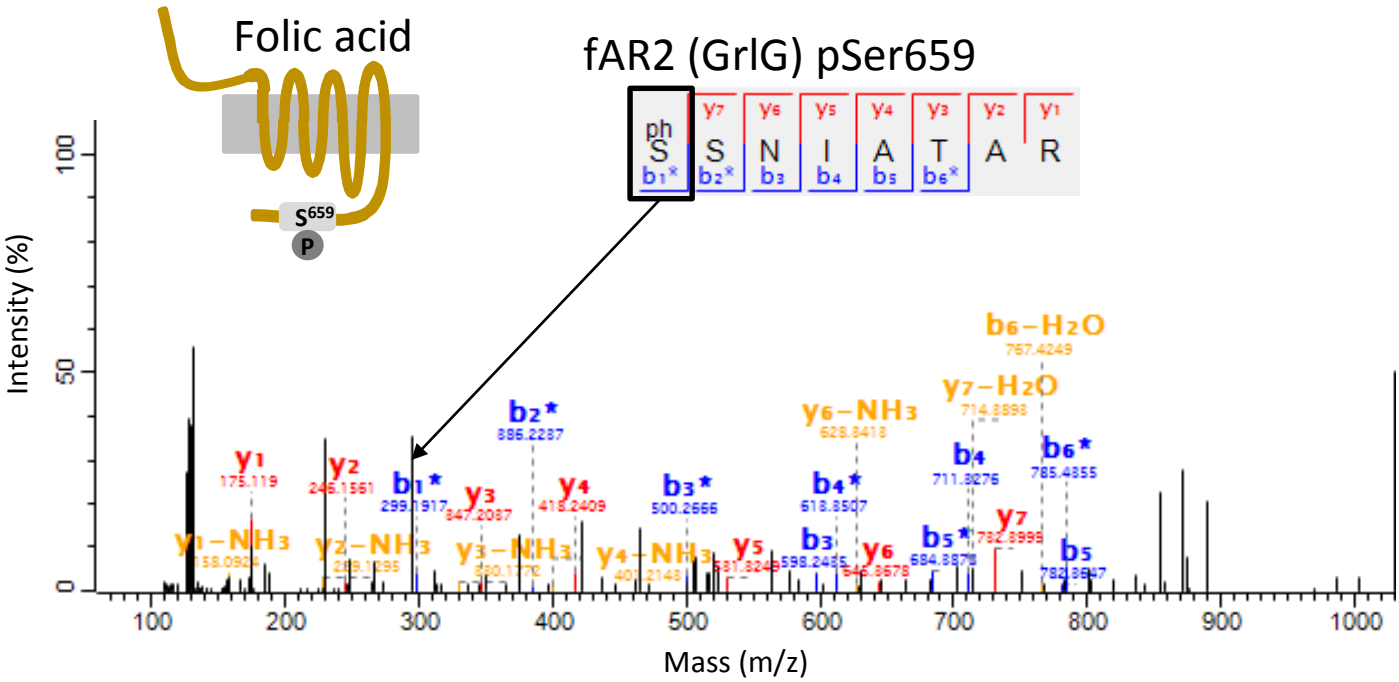


Figure S1. MS/MS sequencing spectrum of the phosphorylated Ser659 peptide in fAR2 (GrlG).

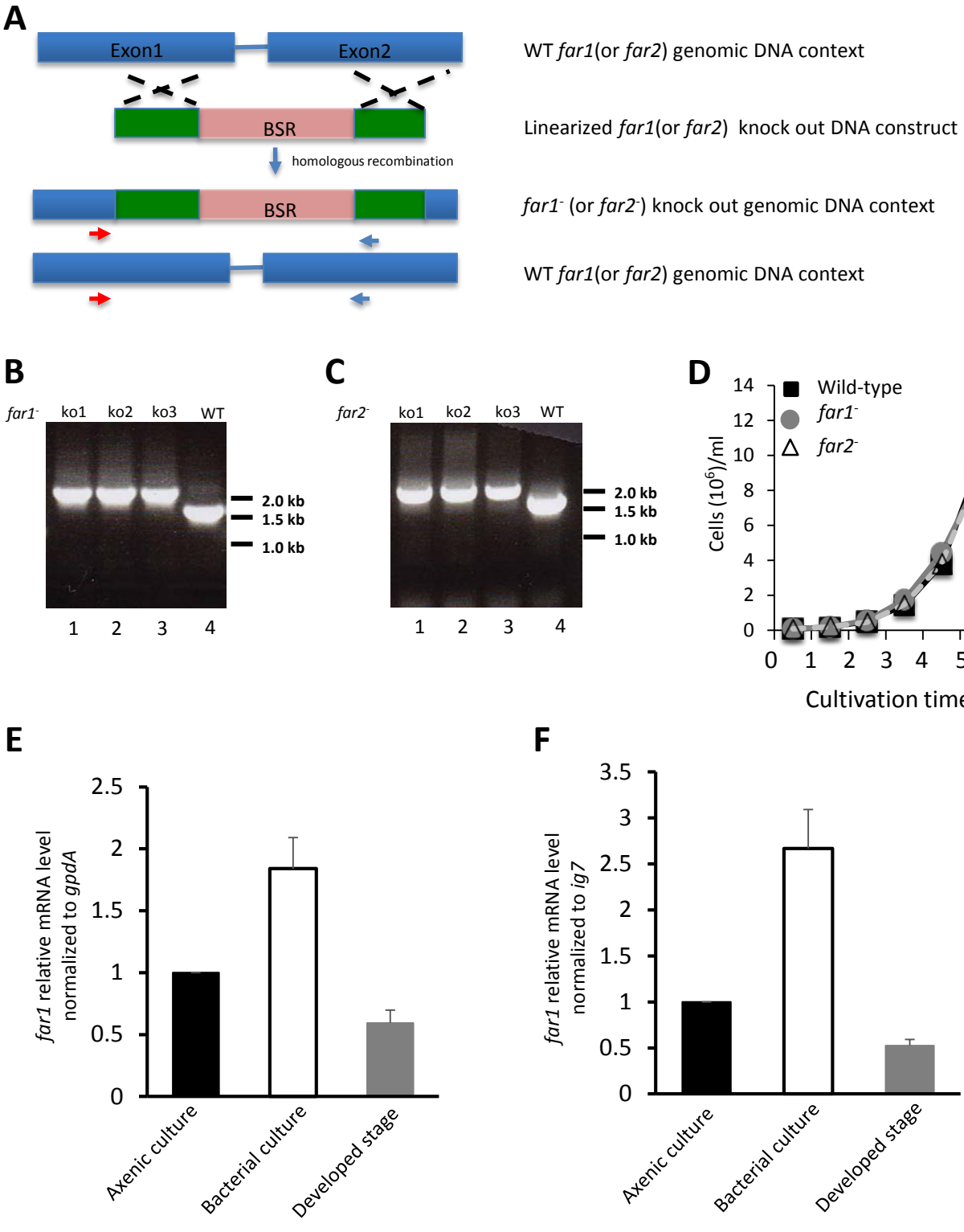


Figure S2. Disruption of *far1 (grlL)* and *far2 (grlG)* genes using homologous recombination.

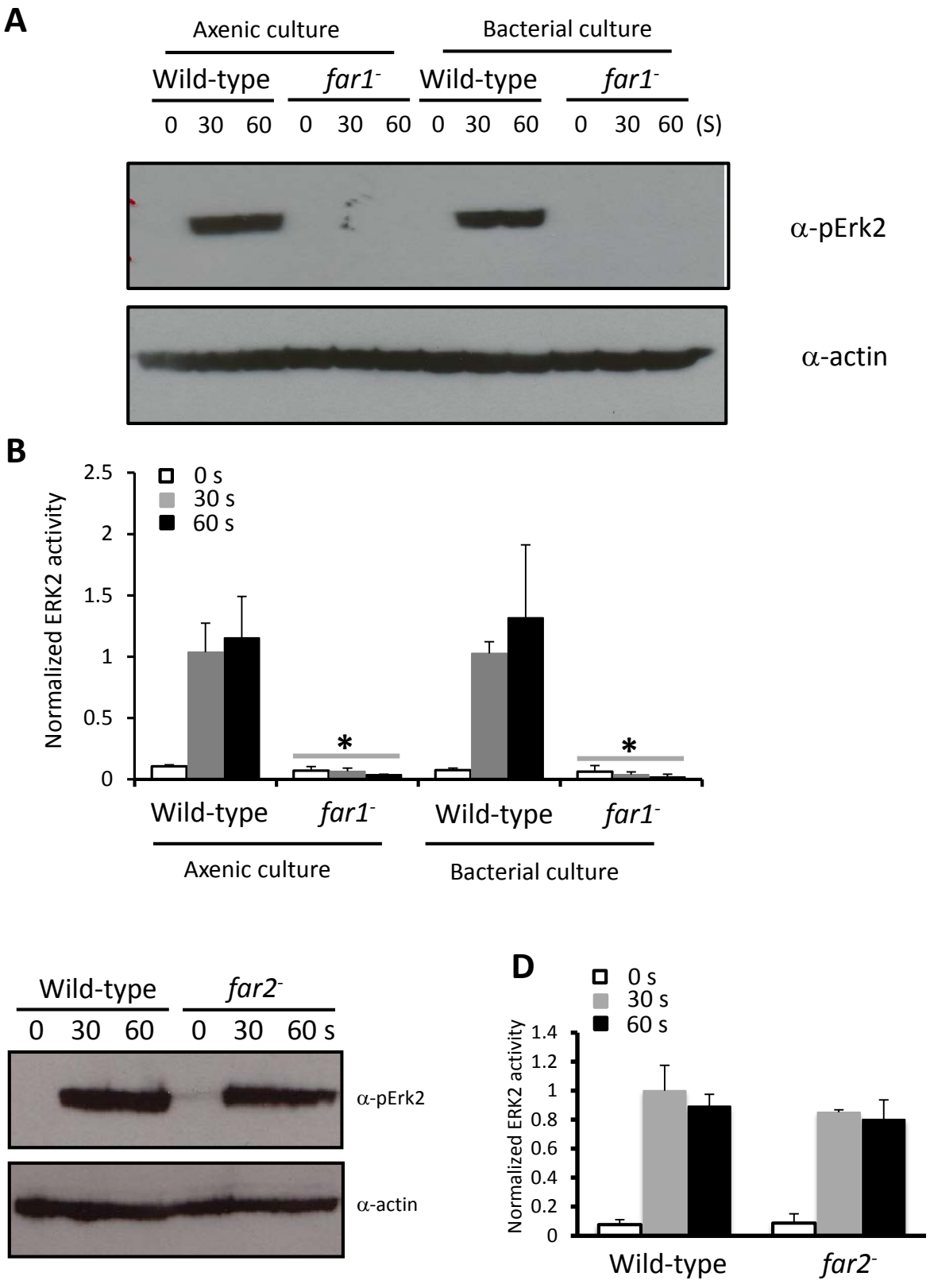


Figure S3. ERK2 signaling is impaired in *far1*- cells but not in *far2*- cells.

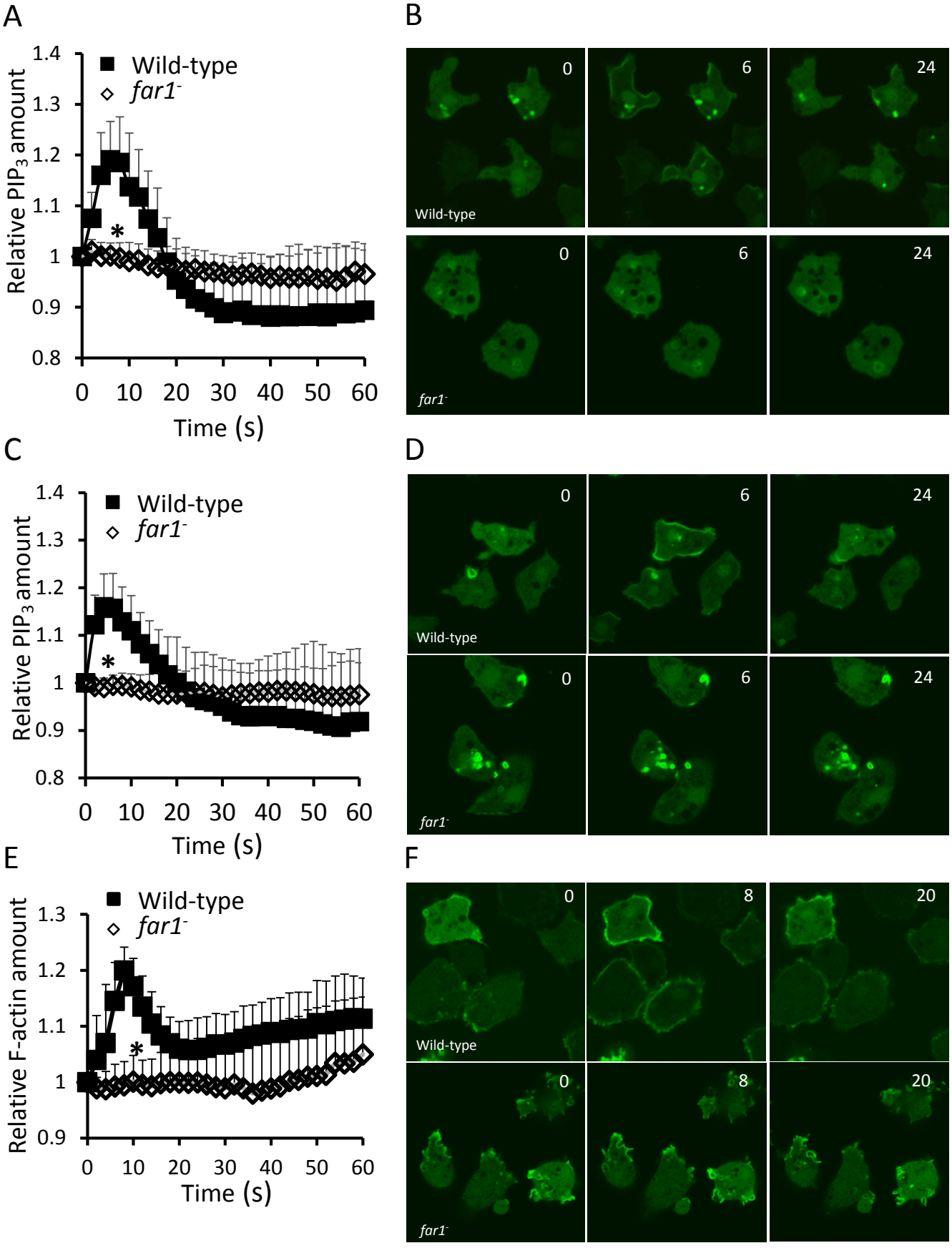
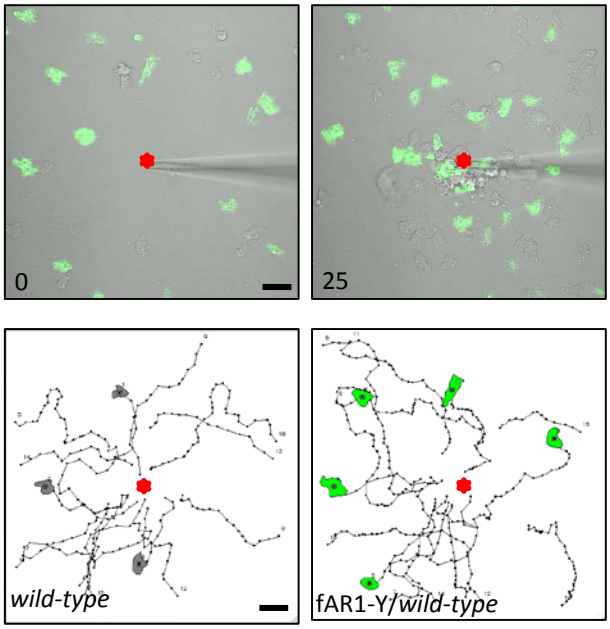


Figure S4. Folic acid induced PH_{CRAC}-GFP and LimEΔcoil-GFP translocation is abolished in *far1*$^{-}$ cells.

A



B

	wild-type (transparent)	fAR1-Y/wild-type (green)
Net path length (μm)	87.2±19.8	89.2±17.5
Directionality	0.77±0.14	0.71±0.15
Speed (μm/min)	14.68±3.70	10.63±2.46

Figure S5. Chemotaxis analysis of wild-type and fAR1-Y/wild-type cells migrating to a micropipette filled with 100 μM folic acid.

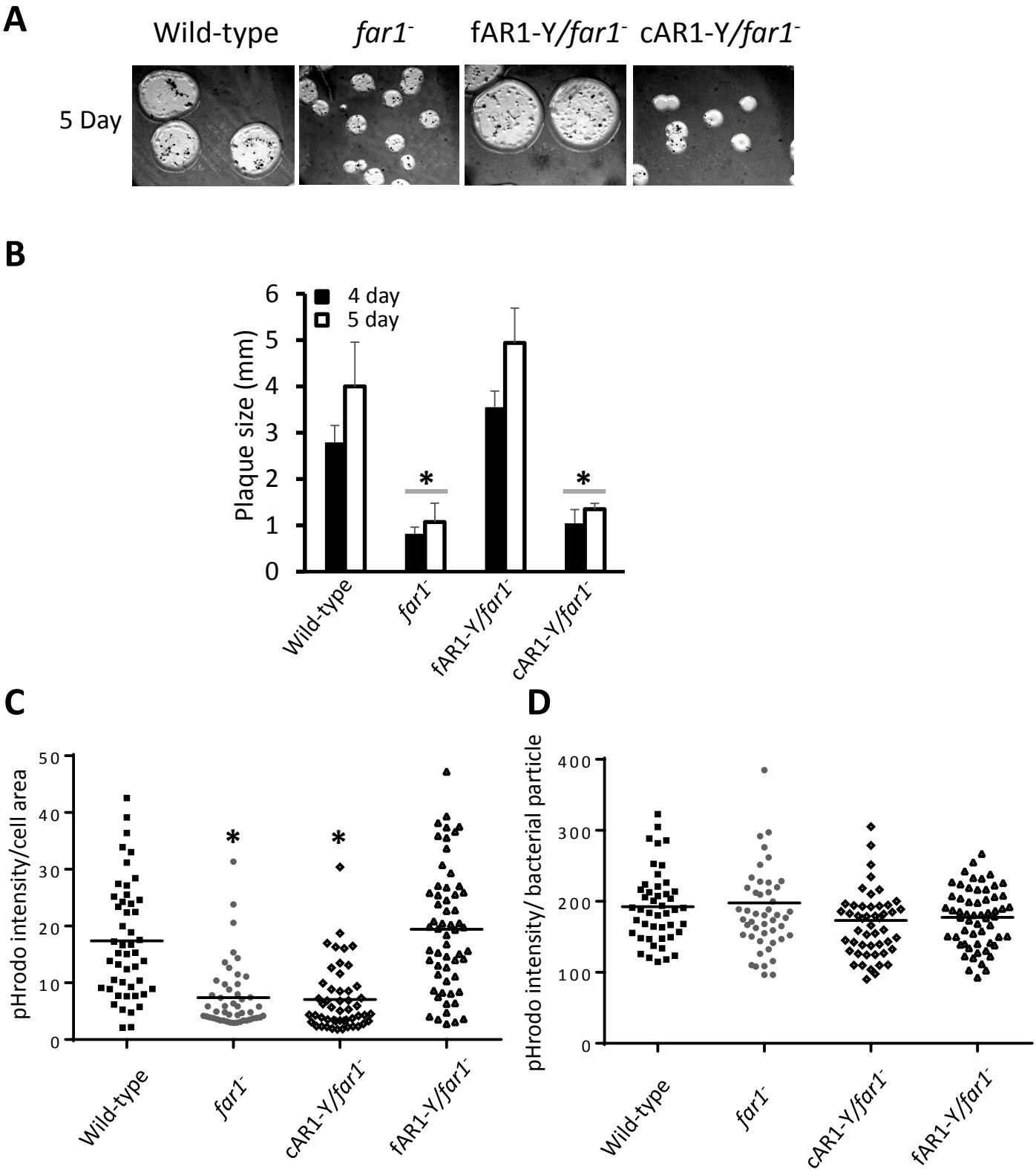


Figure S6. fAR1 is required for phagocytosis of bacteria in *D. discoideum.*

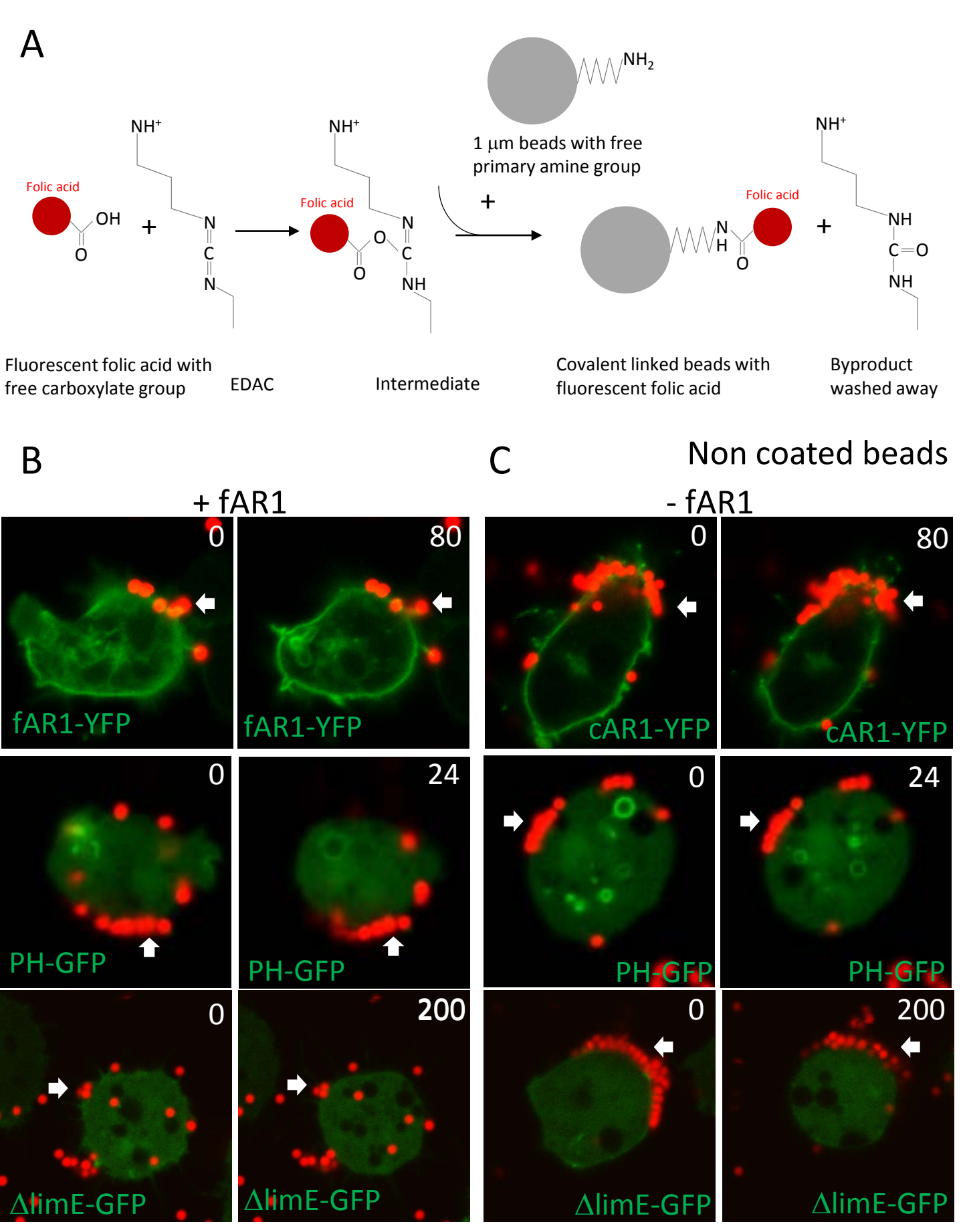


Figure S7. Non coated beads fail to trigger phagocytosis signaling.

Figure S1. MS/MS sequencing spectrum of the phosphorylated Ser659 peptide in fAR2 (GrlG), related to Figure 1.

Figure S2. Disruption of *far1 (grlL)* and *far2* (*grlG)* genes using homologous recombination, related to Figure 2.
A. A blasticidin resistance (BSR) cassette was inserted into the open reading frame of *far1* or *far2* gene in wild-type cells (WT). **B and C.** The predicted molecular weight increase of PCR products from genomic DNA of three *far1*$^-$ **(B)** and *far2*$^-$ **(C)** clones relative to WT is shown. **D.** *far1*$^-$ and *far2*$^-$ cells exhibited a similar growth rate compared with wild-type in axenic culture medium. Wild-type and different mutant *D. disocideum* cells were cultured in D3-T medium for seven days. The growth curve with mean and SD is plotted, resulting from quantification of three independent repetitions of the experiments. **E and F.** Comparison of *far1* mRNA level between vegetative wild-type cells grown in D3-T medium, bacterial culture and differentiated cells developed for 5 hours in DB buffer. *far1* mRNA levels were assessed by real-time PCR. Expression levels were normalized to *gpdA* **(E)** and *ig7* **(F)**.

Figure S3. ERK2 signaling is impaired in *far1*$^-$ cells, but not in *far2*$^-$ cells, related to Figure 2.
A. ERK2 activation in wild-type and *far1*$^-$ cells harvested from D3-T medium or bacterial culture in response to 100 μM folic acid stimulation was examined. ERK2 activation was determined by immunoblotting with anti–phospho-ERK2 antibody. Western blots for actin were used as a loading control. **B.** The mean and SD, resulting from quantification of three independent repetitions of the experiments exemplified in **A** are plotted. A student t-test indicated a statistically significant difference compared to wild-type cells (*, $P < 0.01$). **C.** ERK2 signaling is normal in *far2*$^-$ cells. ERK2 activation in vegetative wild-type and *far2*$^-$ cells in response to 100 μM folic acid stimulation was examined. ERK2 activation was determined by immunoblotting with anti–phospho-ERK2 antibody. Western blots for actin were used as loading control. **D.** The mean and SD, resulting from quantification of three independent repetitions of the experiments exemplified in **C** are plotted.

Figure S4. Folic acid induced PH_{crac}-GFP and LimEΔcoil-GFP translocation are abolished in *far1*$^-$ cells, related to Figure 2.
A-D. Wild-type and *far1*$^-$ cells expressing PH_{CRAC}-GFP were grown in either D3-T medium **(A and B)** or bacterial culture (**C and D**) and stimulated with 100 μM folic acid at 0 s. **E and F.** Wild-type and *far1*$^-$ cells expressing LimEΔcoil-GFP were grown in D3-T medium and stimulated with 100 μM folic acid at 0 s. The transient increase in fluorescence intensity was measured at the plasma membrane and graphed. The intensity of the GFP signal was normalized to the first frame of each set of cells. Mean and SD from ten cells are shown for the time course. A student t-test indicated a statistically significant difference compared with wild-type cells (*, $P < 0.01$).

Figure S5. Chemotaxis analysis of wild-type and fAR1-Y/wild-type cells migrating to a micropipette filled with 100 μM folic acid, related to Figure 3.
A. Wild-type cells are transparent and fAR1-Y/wild-type are green. Images for each strain at time 0 and 25 min are shown. Red dots show the position of the micropipette. Migration paths toward folic acid are shown below. 15 cells from each strain were used for single cell tracing. Scale bar: 20 μm. **B.** The mean and SD resulting from quantification of chemotaxis parameter from **A** are shown.

Figure S6. fAR1 is required for phagocytosis of bacteria in *D. discoideum*, related to Figure 4.
A. Cells were mixed with *K. aerogenes* and plated on SM nutrient agar plates at 22 °C. Photographs were taken after 5 days of culturing. Scale bar: 2 mm. **B.** The diameters of individual plaques in panel **A** were measured and graphed. Average plaque size and standard deviation (SD) of ten plaques from each cell line are plotted. A student t-test indicated a statistically significant difference compared with wild-type cells (*, $P < 0.01$). **C.** Relative pHrodo intensity within *D. discoideum* cells were measured and compared. A student t-test indicated a statistically significant difference compared with wild-type cells (*, $P < 0.01$). **D.** pHrodo intensity for each engulfed bacterial particles was measured and plotted for wild-type, *far1*$^-$, cAR1-Y/*far1*$^-$ and fAR1-Y/*far1*$^-$ cells.

Figure S7. Non coated beads fail to trigger phagocytosis signaling, related to Figure 7.
A. Schematic chemical reaction for making folic acid conjugated beads. **B.** Phagocytosis of 1 μm non coated latex beads (red) by wild-type expressing PH_{CRAC}-GFP, LimEΔcoil-GFP, and *far1*$^{-}$ expressing fAR1-YFP (green). **C.** Phagocytosis of 1 μm non coated latex beads by *far1*$^{-}$ cells expressing PH_{CRAC}-GFP, LimEΔcoil-GFP or cAR1-YFP (green).

Supplementary Experimental Procedures

Cell culture and development

For axenic culture, vegetative cells were grown in D3-T medium (KD medical) at 22°C with or without antibiotics as required. For synchronous development in shaking suspension, cells were harvested at mid-log phase, washed in development buffer (DB; 7.4 mM $NaH_2PO4{\cdot}H_2O$, 4 mM $Na_2HPO_4{\cdot}7H_2O$, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, pH 6.5) twice and then resuspended in DB to 2×10^7 cells/ml. Cells were rotated at 120 rpm on a platform shaker at 22°C for 5 hours and given exogenous 75 nM pulses of cAMP every 6 min. For growth and development on lawns of *Klebsiella aerogenes*, cells growing in D3-T medium were mixed with bacteria, and plated on SM-agar plates without antibiotics. The plates were incubated at 22°C for 3–6 days.

Quantitative phosphoproteomics analysis

Cell lysis, protein reduction/alkylation, and in-solution tryptic digestion

Wild-type cells were grown in D3-T medium and developed as described above. Vegetative cells were stimulated with 100 μM folic acid (Sigma), while developed cells were stimulated with 50 μM cAMP (Sigma). An aliquot was removed at the indicated time points. After treatment by either folic acid or cAMP, 500 μl of the treated cells were harvested at indicated time points. The cells were immediately incubated at 4 °C for 30 min in a buffer containing 50 mM Tris pH 7.5, 6 M urea, 75 mM NaCl, 50 mM NaF, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate (Sigma). The resulting cell lysates were centrifuged at 10,000 g for 10 min at 4 °C. The protein concentration in the lysate was determined by BCA protein assay (Bio-Rad). 500 μg of above cell lysates from each time point were transferred into a new tube. Each sample was added with 10 mM tris-(2-carboxyethyl) phosphine (TCEP) and incubated at 55°C for 1 hour. Then 20 mM chloroacetamide (CAA) was added to each sample and allowed 30 minutes of incubation in the dark at 22 °C. Each protein sample was mixed with six volume of cold acetone and incubated for 2 hours at -20 °C. After 10 minutes of centrifugation at 13,000 g at 4°C, the supernatant was removed completely. Finally, 10 μg of trypsin (Promega) were added into each protein samples and incubated at 37 °C for 16 hours.

TMT labeling, peptide separation and phosphopeptide enrichment

10-plex TMT reagents (Thermo Fisher Scientific) reconstituted in acetonitrile (ACN) were mixed with the corresponding protein digestion and incubated at room temperature for 2 hours. The labeling reactions were terminated by incubation with hydroxylamine for 15 minutes. The labeled peptides were lyophilized and desalted on a Waters Oasis HLB 1cc column and eluted with 60 % ACN with 0.1 % formic acid (FA). The eluted peptide mixture was lyophilized and resuspended in 100 μl high-pH Reverse Phase Liquid chromatography (RPLC) buffer A (10 mM TEAB, pH8.5) and separated on a Waters XBridge C18 column (2.1x100mm, 5μm, 130Å) with a linear 200 μl/min gradient of 0-35% buffer B (10 mM TEAB in 90% ACN) in 70 minutes on an Agilent 1200 LC device with Chemstation B.02.01 control software. Fractions were collected each minute and eventually pooled into 12 fractions. The phosphopeptides from each fraction were enriched by immobilized metal ion affinity chromatography (IMAC-FeNTA), as described in the manufacturer's protocol (Thermo Scientific) and subjected into LC/MS/MS.

LC/MS/MS Analyses and Database Search

LC was performed on a Dionex Ultimate 3000 system (Sunnyvale). After being loaded on a C18 PepMap100 precolumn (Thermo Scientific) at 6 μl/min for 5 minutes, the digested peptides were separated on a PicoFrit analytical column (250 mm long, ID 75 μm, tip ID 10μm, packed with BetaBasic 5 μm 300Å particles (New Objective) using a 72-min linear gradient of 5-35% ACN in 0.1% FA at a flow rate of 300 nl/min. Mass analysis was carried out on a LTQ Orbitrap Fusioin (Thermo Fisher Scientific) with data-dependent analysis mode, where MS1 scanned a full MS mass range from m/z 350 to 1500 at 60,000 mass resolution and 3 second of top speed mode HCD MS2 scans were sequentially carried out at resolution of 15,000 with 42% collision energy, both in the Orbitrap. Mascot Daemon version 2.3.2 (Matrix Science) was used to search peptides in the *D. discoideum* protein database. Raw MS and MS/MS data were processed and quantified using MaxQuant version 1.5.1.2 software. Reporter ion intensities for redundant peptides were summed for each TMT channel. Abundance ratios for all five time points were calculated, and each was normalized using a correction factor that was based on the ratio of the summed reporter ion

intensities for the non-enrichment samples at different time points. The final normalized and filtered GPCR phosphorylation site dynamics were analyzed and presented.

Generation of knockout cells and DNA constructs for overexpression

Both *far1*$^-$ and *far2*$^-$ cells were generated from an Ax2 background. Genes were disrupted with blasticidin-resistant (BSR) cassettes between genome nucleotides 884 and 1499 for *far1* and 982 and 1511 for *far2*, respectively, by homologous recombination. The constructs were linearized, purified, and transfected into wild-type cells by electroporation. Transformants were selected in D3-T medium containing 10 μg/ml blasticidin S. Individual colonies were picked up from independent transformations and confirmed by PCR. Two independent knock-out clones for each cell lines were obtained and exhibited the same phenotype. YFP gene was fused downstream of the indicated genes and cloned into a pDV vector containing the G418 resistance cassette. Cells were transfected, and a population was selected by growing them in D3-T medium containing 50 μg/ml G418. Constructs were confirmed by DNA sequencing. The DNA oligos used for cloning are listed in Table S1. Confirmation of expression and molecular weight of the fusion proteins was performed by Western blot with anti-GFP antibody.

ERK2 activation

Wild-type and different mutant *D. discoideum* cells were grown in D3-T medium or bacterial culture, washed twice with phosphate buffer (PB; 7.4 mM $NaH_2PO4{\cdot}H_2O$, 4 mM $Na_2HPO_4{\cdot}7H_2O$, pH 6.5) and resuspended in PB at 2×10^7 cells/ml. Cells were stimulated with 100 μM folic acid. At indicated time intervals after the stimulation, 150 μl of cell suspension was taken out and mixed with 50 μl 4 × sample buffer and then boiled for 3 min. Protein was separated by SDS–PAGE, transferred to nitrocellulose membranes, and blotted with polyclonal anti–phospho-p44/p42 MAPK (pERK2) antibody (Cell Signaling Technology) and anti-actin antibody (Santa Cruz Biotechnology).

PH-GFP translocation and LimEΔcoil-GFP translocation upon folic acid stimulation

Vegetative wild-type and different mutant *D. discoideum* cells expressing PH_{CRAC}-GFP or LimEΔcoil-GFP were prepared using the same protocol for ERK2 activation assay. Cells were plated in four well chambers (Lab-Tek) and then imaged with a Zeiss Laser Scanning Microscope 780 with a 60×, 1.3 NA Plan-Neofluar objective lens. Fluorescent frames were acquired every 2 seconds and in 30 frames total. A final concentration of 100 μM folic acid was added to the cells to induce PH_{CRAC}-GFP or LimEΔcoil-GFP translocation from cytosol to plasma membrane. The temporal-spatial intensity changes of PH_{CRAC}-GFP or LimEΔcoil-GFP in cells were directly imaged using a confocal microscope. For each cell, a region of interest (ROI) was drawn at the plasma membrane to measure the fluorescence intensity change over time. The fluorescence intensities were normalized to the first frame with the appearance of folic acid stimulation, which is defined as 1.

Folic acid binding assays

Wild-type, *far1*$^-$ and fAR1-Y/wild-type cells were cultured in D3-T medium. Cells were harvested at 1.5-2.0 × 10^6 cells/ml, washed twice with PB and resuspended in PB at $1{\times}10^8$ cells/ml. The suspension was incubated on ice for 15 min before use in the binding assay. Folate binding was measured as previously described. In brief, for 60 s, 100 μl cell suspension was mixed with 50 μl binding mix containing 500 nM FolateRSense 680 (Perkin Elmer) and 0.33 mM 8-azaguanine (Sigma) with or without 5 μM methotrexate (Sigma). Then cells from 140 μl of the mixture were separated from unbound folic acid by centrifugation at 10,000 g for 15 s through 100 μl silcon oil (AR20:AR200, 11:4, Sigma). The supernatant was carefully and completely aspirated without disturbing the cell pellet. The pellet was dissolved in 150 μl PB and the florescence intensity was measured by EnSpire Multimode Plate Reader (Perkin Elmer). Blanks values were determined in the presence of 50 μM unlabeled folate and subtracted from all data. The fluorescence intensities of folic acid bound by *far1*$^-$ and fAR1-Y/wild-type cells were normalized to that of wild-type cells, which is defined as 1.

EZ-TAXIScan Chemotaxis Assay

Wild-type and different mutant *D. discoideum* cells were harvested from SM plates and washed with PB and resuspended at 1×10^6 cells/ml. Cell migration was recorded at 30 s intervals at 22 °C for 40 min in the EZ-TAXIScan chamber (as indicated in the Fig. 4), which was assembled as described in the

manufacturer's protocol. Chips used in the chamber were pre-coated with 1 % BSA at 22 °C for 30 min. A stable gradient of folic acid or cAMP as described in Figure 4 was established for the assay. Cell migration analysis was performed with DIAS software.

Micropipette Chemotaxis Assay

Wild-type and different mutant *D. discoideum* cells were harvested from SM plates and washed with PB. Washed cells were seeded into one well chamber (Lab-Tek) and allowed to adhere for 20 min. A micropipette filled with 100 μM folic acid was connected with a microinjector to generate a chemoattractant gradient. The micropipette was placed in the same focal plane as the cells and phase contrast and fluorescent frames were acquired with an interval of 30 s for 30 min using a Zeiss Laser Scanning Microscope 510, 40 × 1.3 NA Plan-Neofluar objective lens. Cell migration analysis was performed with DIAS software.

Folic acid conjugated beads and non coated beads

To label the latex beads with folic acid, 500 μl 1 μm aliphatic amine latex beads (Thermo Fisher) were washed with PB three times and resuspended into 1 ml PB. The beads were then incubated with 24 nmol FolateRSense 680 (Perkin Elmer) in the presence of 2 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride (EDAC, Thermo Fisher) at 4 °C for 16 hours. After incubation, the coated beads were washed five times with pre-cold PB to remove excess free ligand and crosslinker. The non coated red fluorescent beads (Thermo Fisher) were washed five times with PB before used for phagocytosis assay.

Phagocytosis assay and flow cytometry

Overnight cultured *K. aerogenes* were labeled with pHrodo Red dye (Life Technology) and incubated to *D. discoideum* cells at a 50:1 ratio at 22°C as indicated in the text. After incubation, the cells were washed and resuspended in basic buffer (50 mM Tris pH 8.8 and 150 mM NaCl) to quench the fluorescence of non-phagocytized pHrodo-labelled *K. aerogenes*. The phagocytes and *K. aerogenes* were distinguished by forward and side scatter (FSC). The appearance of pHrodo in the phagocyte population was monitored as an indicator of *K. aerogenes* engulfment. The phagocyte cell population characterized by high fluorescence of pHrodo was considered as the cells which engulfed *K. aerogenes*. Data acquisition and analysis were done using FACSort flow cytometer (BD Bioscience) with Cell Quest software (v. 3.3) and analyzed using FlowJo (v. 10.0.8; Tree Star). The quantification of engulfed *K. aerogenes* among different *D. discoideum* strains was analyzed by confocal microscopy and ImageJ.

To visualize the beads engulfment by *D. discoideum* cells, vegetative wild-type and *far1*$^-$ expressing different protein markers grown in D3-T medium were harvested, washed with PB and settled in a 4 well chamber for 10 min. A 10 fold excess of beads were added to the *D. discoideum* cells and the engulfment process was recorded with a Zeiss Laser Scanning Microscope 780 with a 60×, 1.3 NA Plan-Neofluar objective lens.

Bacteria Binding

Wild-type and *far1*$^-$ cells were mixed with pHrodo-labelled *K. aerogenes* at a 1:50 ratio on ice. After 15 min, the cells mounted on a slide in the presence of 2 mM NaN_3 buffer were analyzed by confocal microscopy. For flow cytometry, cells were suspended in PB and analyzed for the percentage of pHrodo positive cells, which represents the cells that bind *K. aerogenes*.

Real time PCR

Vegetative wild-type cells cultured in D3-T medium or bacterial lawn and differentiated cells developed by cAMP pulse for 5 hours were harvested and washed with PB buffer. Total RNA were isolated using RNAqueous-4PCR Total RNA Isolation Kit (Thermo Fisher Scientific). 1 μg DNase treated RNA was converted to cDNA using qScript™ cDNA Synthesis Kits (Quanta Biosciences). A 5 % volume of the cDNA reaction was used as template for real-time PCR using a CFX Connect Real Time System (Bio-Rad) and PCR products were detected with SYBR Green I (Roche). Real-time PCR conditions set as 95°C, 120 s, 95°C, 15 s; 60°C, 60 s, 45 cycles, followed by a melting curve analysis (65 to 95°C, rising by 0.5°C each step of 3 s). Expression levels were normalized to *gpdA* and *ig7*. The sequence of primers used for real time PCR are listed in the Table below.

DNA oligos used in this study

Oligo name	Sequence	Constructs
Miao331	atctGGTACCgccaaaatgtatgaaggcagtctaacg	*far2* gene deletion
Miao332	atctAAGCTTagtgtcttgtggaacatcaaaaccatagg	*far2* gene deletion
Miao333	atctGGATCCgtaccattaatgatctccccattgg	*far2* gene deletion
Miao334	AgctACTAGTcttgattcttttcatctggttctatttg	*far2* gene deletion
Miao335	atcgGTCGACatgatgccaactcatatgattcaac	*far1* gene deletion
Miao336	atcgAAGCTTccatagatgtttgattgtgggaaacc	*far1* gene deletion
Miao337	atctCTGCAGgattcactcggtttggatggtattggc	*far1* gene deletion
Miao338	atctGGATCCgtgcagcagctcttgaacttgcttc	*far1* gene deletion
Miao339	cctatggttttgatgttccacaagacact	*far2*$^-$ screen
Miao340	ttccaatggggagatcattaatggtac	*far2*$^-$ screen
Miao341	ggtttcccacaatcaaacatctatgg	*far1*$^-$ screen
Miao342	gccaataccatccaaaccgagtgaatcg	*far1*$^-$ screen
Miao348	agcGGATCCatgaaaaaaataatatttttagtattatttttaatatttatttttaaag	fAR2-YFP
Miao351	agcTCTAGAatcttgattcttttcatctggttctatttgttg	fAR2-YFP
Miao352	agcGGATCCatgaaattaataataaaaaatttaatacttttattagtatcatgtttatac	fAR1-YFP
Miao355	agcTCTAGAattatcattttgtgcagcagctcttgaacttgcttcaattgg	fAR1-YFP
Miao375	tttCattgcaggtggtatggg	*far1* gene RT PCR
Miao376	tctaccagcaccagttgcagtatc	*far1* gene RT PCR
Miao385	GGTTGTCCCAATTGGTATTAATGG	*gpda gene* RT PCR
Miao386	CCGTGGGTTGAATCATATTTGAAC	*gpda gene* RT PCR
Miao389	TTACATTTATTAGACCCGAAACCAAGCG	*ig7* gene RT PCR
Miao390	TTCCCTTTAGACCTATGGACCTTAGCG	*ig7* gene RT PCR

Supplemental Movies

Movie S1
EZ-TAXIScan chemotaxis assay toward a linear folic acid gradient by vegetative wild-type (WT, left), *far1*$^-$ (*grlL*$^-$, middle) and fAR1-Y/*far1*$^-$ (GrlL-Y/*grlL*$^-$, right) cells. Images were captured at 30 sec intervals. Corresponds to Figure 3A-3C.

Movie S2
Chemotaxis analysis of *far1*$^-$ (transparent) and fAR-Y/*far1*$^-$ (green) cells migrating to a micropipette filled with 100 μM folic acid. Images were captured at 30 sec intervals. Corresponds to Figure 3D and 3E.

Movie S3
Tracing movie for *far1*$^-$ (left) and fAR1-Y/*far1*$^-$ (right) cells shown in Movie S2. Corresponds to Figure 3D and 3E.

Movie S4
Chemotaxis analysis of wild-type (transparent) and fAR1-Y/wild-type (green) cells migrating to a micropipette filled with 100 μM folic acid. Images were captured at 30 sec intervals. Corresponds to Figure 3 and S6.

Movie S5
EZ-TAXIScan chemotaxis assay toward a linear folic acid or cAMP gradient by differentiated *far1*$^-$ and fAR1-Y/*far1*$^-$ cells. Upper left, *far1*$^-$ in a folic acid gradient; upper right, fAR1-Y/*far1*$^-$ in a folic acid gradient; lower left, *far1*$^-$ in a cAMP gradient; lower right, fAR1-Y/*far1*$^-$ in a cAMP gradient. Images were captured at 30 sec intervals. Corresponds to Figure 3F and 3G.

Movie S6
Tracing movie for *far1*$^-$ cells and fAR1-Y/*far1*$^-$ cells in a folic acid or cAMP gradient shown in Movie S6. Upper left, *far1*$^-$ in a folic acid gradient; upper right, fAR1-Y/*far1*$^-$ in a folic acid gradient; lower left, *far1*$^-$ in a cAMP gradient; lower right, fAR1-Y/*far1*$^-$ in a cAMP gradient. Corresponds to Figure 3F and 3G.