

Supplementary Materials and Methods

Antibodies and constructs

Antibodies and DNA constructs are listed in Supplementary Tables 3 and 4 respectively.

Alignment and phylogenetic tree

Protein sequences were obtained from Uniprot <http://www.uniprot.org/> and aligned using MacVector software. The phylogenetic tree was constructed based on the major eukaryotic superclasses as previously defined¹⁴ and based on previous identification of Arp2/3 complex and Scar/WAVE complex sequences^{15,54}. BLAST homology search on the NCBI website <https://blast.ncbi.nlm.nih.gov/Blast.cgi>. *Dictyostelium*, human or a close relative were searched against the complete translated genome of open reading frames from these organisms.

HMM logo was generated by feeding the Pfam database of the DUF1394 domain into Skylign⁵⁵.

CYRI-B structure prediction

The predicted protein structure of CYRI-B₃₁₋₂₉₂ was generated by the protein homology/analogy recognition engine (Phyre)⁵⁶ using the cytoplasmic fmr1-interacting protein 1 (PDB 3P8C) as a template with 100% homology confidence and 18% sequence identity.

Mammalian cell lines and growth conditions

CHL-1, HEK293T, COS-7 cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS and 2 mM L-Glutamine (DMEM).

ROSA26:CreER^{t2+}; *Ink4*^{-/-}*Cyri-B*^{wt/wt}; *Rac1*^{fl/fl} mouse tail skin fibroblasts and ROSA26:CreER^{t2+}*Ink4*^{-/-}; *Cyri-B*^{fl/fl} mouse embryonic fibroblasts were maintained in DMEM complemented with 1 mg/mL of primocin.

COS-7 cells transfected with the doxycycline-inducible system were grown in 10% tetracyclin-free FBS (ClonTech) and treated with 5 µg/mL doxycycline for 48h.

MDCK cells were maintained in 5% FBS and 2 mM L-Glutamine supplemented minimum essential medium, high glucose, high sodium bicarbonate.

WM852 cells were grown in RPMI supplemented with 10% FBS and 2 mM L-Glutamine.

All mammalian cell lines used in this study were maintained in 10 cm plastic dishes at 37 °C and 5% CO₂.

Cell lines were regularly tested for mycoplasma contamination (MycoAlert - Lonza).

CLICK Chemistry of Mammalian CYRI-B

HEK293T cells plated on 24-well plate were transfected with 1 µg of pEGFPN1 or CYRI-B-EGFP (wild-type or G2A mutant) using Lipofectamine 2000 and were processed the next day. C14:0-azide was synthesised as previously described⁵⁷. Transfected HEK293T cells were incubated with 100 µM of C14:0-azide (in DMEM with 1 mg/mL defatted BSA) for 4 h at 37 °C. Cells were washed twice in PBS and lysed on ice for 10 min in 100 µL lysis buffer (150 mM NaCl, 1 % Triton X-100, 50 mM Tris-

HCl, pH 8.0, containing protease inhibitors). Cell lysates were centrifuged at 10000 x g for 10 min at 4 °C to remove cell debris. Alkyne IR-800 Dye to C14:0 azide was conjugated for 1 h at room temperature (RT) with end-over-end rotation by adding an equal volume of freshly mixed click chemistry reaction mixture (10 μM 800 CW alkyne infrared dye, 4 mM CuSO₄, 400 μM Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine, and 8 mM ascorbic acid in dH₂O) to the supernatants. GFP-tagged proteins were isolated using the μMACS GFP isolation kit following manufacturer's protocol and resolved by SDS-PAGE as described below. Protein acylation was quantified by expressing the intensity of the CLICK signal relative to the protein signal.

Yeast Two-Hybrid screen

Screening was performed at Hybrigenics services *as per* their standard protocols. Briefly, the coding sequence for the constitutively active full-length Rac1 (NM_006908.4 ; mutations G12V, C189S) was PCR-amplified and cloned into pB27 as a C-terminal fusion to LexA (LexA-Rac1). All libraries use the prey vector pP6. pB27 and pP6 are derived from the original pBTM116⁵⁸ and pGADGH⁵⁹ plasmids, respectively.

The bait was screened against the different libraries using a mating approach with YHGX13 (Y187 *ade2-101::loxP-kanMX-loxP*, *mat alpha*) and L40deltaGal4 (*mat-a*) yeast strains as previously described⁶⁰. Positive colonies were selected on a medium lacking tryptophan, leucine and histidine, and supplemented with 3-aminotriazole. The prey fragments of the positive clones were amplified by PCR and sequenced at their 5' and 3' junctions. Interacting proteins were identified in the GenBank database (NCBI).

GST Pull-down of Mammalian CYRI-B and GTPases

DH5alpha *E. coli* cells were grown at OD_{600nm} 0.4 and induced for 4h with 0.2 mM IPTG. Pellet was resuspended in ice-cold buffer A (50 mM NaCl, 50 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 3 mM DTT) and sonicated, followed by a 30 min spin at 20000 rpm to yield lysate. GST tagged proteins were immobilized on pre-washed glutathione-sepharose beads for 30 min at 4°C with gentle agitation and unbound proteins were washed out 3 times in buffer A.

Cells transfected with GFP constructs were collected in ice-cold lysis buffer (100 mM NaCl, 25 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 1X protease and phosphatase inhibitors, 0.5% NP-40). 1.5-2 mg of proteins were mixed with pre-equilibrated beads with gentle agitation during 2h at 4°C. Beads were then washed 3 times in washing buffer (100 mM NaCl, 25 mM Tris-HCl pH 7.5, 5 mM MgCl₂), resuspended in sample buffer containing DTT and resolved by SDS-PAGE as described below.

MBP Pull-down

Recombinant proteins were purified as mentioned above and immobilized on MBP-trap beads. Beads were mixed with similar amount of recombinant GST-tagged proteins in ice-cold buffer A (see above) containing 0.05% Triton X100. Binding was allowed for 2h at 4°C and beads were then thoroughly washed in ice-cold buffer A. Proteins were eluted by adding boiling sample buffer directly to the beads and prepared for SDS-PAGE.

Mutagenesis of Mammalian CYRI-B

Point mutation was inserted using the Q5-site directed kit (New England Biolabs) and following the manufacturer's instructions. Primers were designed using NEBaseChanger - see Supplementary Table 5.

Protein purification for SPR analysis

E. Coli BL21 CodonPlus (DE3)-RIL (Agilent Tech.) and *E. Coli* BL21 (DE3) pLysS (Promega) were used for GST-tagged and His-Tagged proteins respectively.

Pre-culture was grown overnight in L-Broth (LB) containing appropriate antibiotics. Once reaching OD_{600nm} 0.4, protein expression was induced using 0.2 mM IPTG and culture was kept overnight at 20°C under agitation (200 rpm). Cells were lysed in Buffer 1 (200 mM NaCl, 30 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 3 mM β-mercaptoethanol) containing protease inhibitors and passed through a 20,000 psi-pressurised microfluidizer. The soluble fraction was collected by centrifugation (30 min, 20000 rpm) and loaded onto an equilibrated GSTrap HP or HisTrap HP column using an AKTA machine (GE Healthcare). Proteins were either directly eluted using Buffer 1 containing either 20 mM GSH for GST-tagged proteins or 300 mM Imidazole pH 7.5 for His-tagged proteins. Cleavage on the column was performed overnight with the appropriate protease, flowing at 0.1 ml/min in a loop connected to the AKTA machine. Proteins were gel purified (HiLoad 16/600 Superdrex 75pg or HiLoad 16/600 Superdrex 200pg) in Buffer 2 (150 mM NaCl, 25 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 2 mM β-mercaptoethanol), snap-frozen and stored at -80°C.

Surface Plasmon Resonance (SPR) protein binding assay

SPR analysis was performed using Biacore T200 (GE Healthcare) equilibrated with buffer 2 (see above) supplemented with 0.5% of surfactant P20. GST-tagged proteins were immobilised at 22°C onto CM5 sensor chip functionalized with anti-GST and reached ~320 RU. Same procedure was used for His-tagged protein onto NTA sensor chip and reached 650 RU. All immobilisation steps were done at a flow rate of 10 μL/min. Serial dilution of each analyte was injected across a reference flow cell and the flow cell containing the ligand at a flow rate of 30 μL/min. Data were solvent corrected, reference subtracted, quality controlled and evaluated using the Biacore T200 evaluation software. Affinity was determined by curve fitting a 1:1 binding model.

Proximity ligation assay

COS-7 cells expressing CYRI-B-HA and MYC-Rac1 constructs were plated on laminin-coated coverslips and used for DuoLink *in situ* proximity ligation assay (Sigma - mouse and rabbit - Red detection) using the manufacturer's protocol. Mouse anti-HA (Covance) and Rabbit anti-MYC-tag (CST) were used at 1:400 and 1:200 respectively. Incubation with either antibody was performed as a negative control.

Enforced mitochondrial localisation

The Rac1A cDNA (gift of A. Kortholt, University of Groningen) was mutagenised to P29S/Q61L the stop codon removed. It was cloned N-terminal to mCherry-

mitochondrial anchor, or N-terminal to mCherry to give a cytosolic version. Likewise, PakB-CRIB was cloned N-terminal to either GFP alone (to give a soluble CRIB fusion) or GFP-mito (to give a mitochondrial-targeted version). CYRI was similarly used in its WT or R155D R156D double mutant. The mitochondrial anchor consists of the C-terminal tail (aa 602-658) of *gema*, the *Dictyostelium* mitochondrial-anchored Rho1/2 GTPases.

Live cell images were acquired at separate times using single-channel hardware setups to ensure zero channel bleed-through or dual excitation of fluorophores. To note, the cells move between image captures. Dual images were captured using a double band-pass filter that allows both red and green signals to pass simultaneously. The same cells are shown in the red, green and dual images.

Transfection, siRNA Treatment and Knockout Mammalian Cells.

Oligos used are listed in Supplementary Table 5.

Cells were plated a day before transfection at 70% of confluence and later transfected using Lipofectamine 2000 according to the manufacturer's instructions. 2-5 μg of DNA was used per reaction based on a 6-well plate format.

siRNA oligonucleotides targeting CYRI-B (Qiagen): Mouse tail fibroblasts and COS-7 cells were respectively treated with 75 nM of *Mus musculus* CYRI-B siRNA and 25 nM of *Homo sapiens* CYRI-B siRNA (recognised *Cercopithecus aethiops*) or matched concentration of control siRNA (AllStars Negative siRNA – Qiagen) were transfected using Lullaby transfection reagent according to manufacturer's instructions. The same step was repeated 48h later and cells were analysed after 24h.

For CrispR/Cas9 mediated knock out, sgRNA were selected using the MIT CrispR designing tool (<http://crispr.mit.edu/>). Annealed oligonucleotides were cloned into pLentiCrispRv2-Puro. Briefly, HEK293T cells were seeded at 1.5×10^6 cells/10cm dish. Cells were transfected with 10 μg of the selected plasmid (Vector^{Ctrl} or containing a gRNA against CYRI-B) 7.5 μg of pSPAX2 (Addgene 8454) and 4 μg of pVSVG (Addgene 12260) in a final volume of 440 μL of sterile water, and complemented with 500 μL 2X HBS and 120 mM CaCl_2 . Solution was incubated 30 min at 37°C before adding to HEK293T cells. Medium was removed after 24h and replaced by 6 mL of 20 % FBS DMEM. Meanwhile, recipient cells were plated at 1×10^6 cells/10cm dish. The day after, supernatants were filtered through a 0.45 μm pore membrane and mixed with 25 μg of hexadimethrine bromide (4.2 $\mu\text{g}/\text{mL}$ final) before infecting recipient cells. Infection was repeated the next day and stably transfected cells were selected with 1 $\mu\text{g}/\text{mL}$ of puromycin.

Same procedure was used for lentiviral infection of the MDCK cells and cells were selected with 5 $\mu\text{g}/\text{mL}$ of puromycin.

For CrispR COS-7 *cyri-b* knockout cells, human gRNAs against *CYRI-B* (CrispR#1 or #2 - See Table 5) were cloned into a pSpCas9(BB)-2A-GFP vector (Addgene plasmid #48138) using the restriction enzyme *BbsI* as described in⁶¹ COS-7 cells were seeded onto 6 cm dishes and transfected the day after using Lipofectamine 2000 with 5 μg of pSpCas9(BB)-2A-GFP (empty vector or *CYRI-B* targeting CrispR gRNA)

following the manufacturers guidelines. Cells were grown for approximately 24 h before FACS sorting. The transfected cells were trypsinised, resuspended in serum free DMEM with DAPI (1 µg/ml) and filtered through a 0.45 µm pore membrane for FACS. For FACS, gates were drawn to sort by cell size, live/dead and GFP positive cells. GFP positive sorted cells were incubated with DMEM complete and left to grow at normal culturing conditions. Knockouts for *CYRI-B* were analysed by western blotting.

Generation of knockout mouse embryonic fibroblast and mouse tail skin fibroblast cell lines were obtained by adding 1 µM of hydroxytamoxifen in the growth medium every 3 days over 7 days.

FRET imaging of Mammalian Cells

The Rac1-Raichu-mTq2-sREACH probe is described in²⁹. Cells were transfected with the probe, plated the day after on laminin and imaged. FRET images were acquired with the Nikon FLIM/TIRFsystem Z6014 microscope equipped with a Plan Achromat 63x/1.45 oil objective and a 465 nm LED. Dishes were placed in a 37°C heated chamber perfused with 5% CO₂. FRET efficiency was calculated by standardizing the probe lifetime to the average lifetime of the donor alone as follows:

$$FRET\ efficiency\ (\%) = \frac{Average\ lifetime\ donor - Lifetime\ probe}{Average\ lifetime\ donor} \times 100$$

Active Rac1 pulldown

COS-7 cells were plated on laminin-coated dishes for 1h, washed twice with ice-cold PBS and lysed using 50 mM Tris-HCl pH 7.4, 500 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 10 mM MgCl₂, 1X protease and phosphatase inhibitors. Cleared lysates were incubated with recombinant GST or GST-CRIB-PBD obtained from DH5alpha cells as described above. 1-1.5 mg of lysate were incubated for 2 h at 4°C with a similar amount of GST-construct immobilised on glutathion-sepharose beads. Beads were washed 3 times with 50 mM Tris-HCl pH 7.4, 500 mM NaCl, 10 mM MgCl₂ and prepared for SDS-PAGE analysis as described below.

SDS-PAGE and Western Blotting of Mammalian Cells

Lysates were collected on ice by scraping cells in RIPA Buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1% Triton X100, 0.1% SDS, 1X protease and phosphatase inhibitors) and centrifuged 10 min at 15000 rpm and 4°C. Protein concentration was measured at OD_{600nm} using Precision Red.

20-40 µg of protein were resolved on a NuPAGE Novex 4-12% Bis-Tris gels and transferred onto a nitrocellulose membrane using the BioRad system. Membranes were blocked in 5% non-fat milk in TBS-T (10 mM Tris pH 8.0, 150 mM NaCl, 0.5% Tween 20) during 30 min before overnight incubation with primary antibodies at 4°C. Membranes were washed 3x 5 min in TBS-T and incubated 1h with Alexa-Fluor conjugated secondary antibodies. Blots were then washed 3x 5 min and imaged using the LiCor Odyssey CLx.

All images were then analysed using Image StudioLite v.5.2.5.

Immunofluorescence of Mammalian Cells

Cells were collected and plated onto sterile 13mm glass coverslips coated overnight at 4°C with 10 µg/mL of rat-tail collagen I, 10 µg/mL fibronectin or 10 µg/mL laminin diluted in PBS. Coverslip were washed 3x in PBS before seeding cells. Cells were fixed with 4% paraformaldehyde for 10 min, permeabilised (20 mM Glycine, 0.05% Triton X100) for 10 min and blocked with 5% BSA-PBS for 30 min. Primary and secondary antibodies were diluted in blocking buffer and incubated 1h in a dark and humidified chamber. Coverslips were washed twice in PBS and once in water before being mounted on glass slides using ProLong Gold antifade reagent. Images were taken using an inverted Olympus FV1000 confocal microscope using a Plan Apochromat N 63x/1.40 oil SC or an Uplan FL N 40x/1.30 oil objective.

Images were processed and analysed using Fiji software (ImageJ v1.48t)¹⁶.

Membrane dynamics analysis

CHL-1 cells were transfected with GFP-LifeAct (5 µg AMAXA kit-T, program T-020) and incubated overnight in complete DMEM. Cells were then plated onto a glass bottom dish coated with laminin for 3 h before imaging within a contained unit at 37°C and 5% CO₂. Time-lapse images were taken using a Nikon microscope with a CoolLED GFP filter set (470 nm LED) and a Nikon Plan Apo VC 100x/1.4 NA oil immersion objective and captured using a Photometrics PRIME camera. GFP images were taken at 1 frame per second for a total of 3 min. For each frame, a binary mask was made of the cell based on the intensity of its LifeAct signal, and the intensity of an associated edge image made by Canny edge detection. Differences between binary images from one frame to the next were used to find areas of extension or retraction, with extended areas positively valued and retracted areas negatively valued. Co-ordinates for an outline of the binary image of each frame were extracted from the ROI class in ImageJ, and were used to measure the mean intensity of the corresponding difference image in a 5x5 px area. These values were then written for each cell to a new 2D image that we refer to as an "unwrapped kymograph", with each two rows representing one frame and each column representing one outline coordinate point for that frame. After smoothing this unwrapped kymograph, areas of protrusion were identified by thresholding, with their extension in the y direction (time) measured. This gave us an estimate of the active lifetime of each protrusion, and a mean protrusion lifetime for each cell. Images were processed using Metamorph and Fiji softwares.

Plugin used for creating kymograph will be provided upon reasonable request.

Rac1 photo activation

Transfection protocol: MEFs were transiently transfected by electroporation (Amaya kit T) with 5 µg of photoactivatable Rac1 plasmid³¹ (pTriEx-LOV2-Ja-Rac1-mCherry). The transfected cells were suspended in complete DMEM media, and plated onto laminin-coated glass-bottom 35 mm dishes. After several hours, the media was replaced with serum-free DMEM and incubated overnight in darkness.

Imaging: Imaging was performed on a Zeiss 880 confocal microscope with a stage incubator perfused with CO₂. Time-lapse imaging of moderate mCherry expressing

cells was done for 150 frames at 2 second intervals between frames. Two images were collected for each frame at 568 nm with bi-directional scanning averaged over two frames to image the mCherry tag, and a transmitted light detector to show a bright field image of the cell morphology, both at 1024x1024 resolution. An initial 29 frames (1 minute) was collected with 568 nm excitation to document baseline protrusive activity. Photoactivation of Rac1 was started at frame 30, and continued for each frame to 150, with a pulse of 458 nm excitation in a 100 pixel diameter region of interest. The 568 nm excitation was at 7.5% laser power, with gain of 600-800 depending on the brightness of the cell, and the pinhole set at 300 to maximize collection of light levels and depth of field to capture ruffles. The 458 nm excitation used laser power of 10% and scan speed set for a pixel dwell time of 8 μ sec. Movies were processed using the Plugin found in Supplementary Note 1

Chemotaxis assay

Chemotaxis assays with WM852 human melanoma cells were performed as described in³⁵. Briefly 8×10^4 cells were seeded onto fibronectin coated coverslips and left overnight in serum-free RPMI. Coverslips were mounted onto Insall chambers with RPMI containing 10% fetal bovine serum as the chemoattractant, and images were taken every 15 min for 48 h with a Nikon TE2000-E time-lapse microscope using Metamorph software. Cells were manually tracked using MTrackJ plugin in Fiji. All cells that moved independently of other cells were chosen for tracking. Approximately 120 cells were tracked for each condition from 3 independent repeats per condition (see also legend Figure 6).

Random Migration Assay for Mammalian Cells

6-well glass bottom plates were coated overnight as described above. 1×10^5 cells were plated and imaged every 10 min for 17 h using a Nikon TE2000 microscope, PlanFluor 10x/0.30 objective and equipped with a heated CO₂ chamber. Images were analysed using Fiji software⁶³ (ImageJ v1.48t). Individual cells were tracked using the mTrackJ plugin, and spider plots were generated using the chemotaxis and migration tool plugin (v.1.01).

***Dictyostelium discoideum* Cells**

Axenic *D. discoideum* strains Ax3 was used as wildtype. *cyri* knockout cells were generated in Ax3 genetic backgrounds. Ax3-derived *napA* KO cells are described previously⁶⁴. Cells were grown in HL5 medium (Formedium) with 100 U/ml penicillin and 100 μ g/ml streptomycin in 10 cm plastic Petri dishes and incubated at 21°C.

***Dictyostelium discoideum* GFP-Trap with Formaldehyde Crosslinking**

Cells were collected in PBS and lysed by adding ice-cold 3x lysis/crosslinking buffer (1x buffer: 20 mM HEPES pH 7.4, 2 mM MgCl₂, 3% formaldehyde, 0.2% Triton X-100). After 5 min with gentle agitation at 4 °C, formaldehyde was quenched for 10min on ice using 1.75 M Tris pH 8.0. Samples were centrifuged at 22000g for 4 min at 4 °C. Pellet was successively washed and resuspended with 1 mL of ice cold quenching buffer (0.4 M Tris pH 8.0, 0.2% Triton X-100), wash buffer A (100 mM HEPES pH 7.4, 2 mM MgCl₂, 0.2% Triton X-100) and wash buffer B (100 mM HEPES pH 7.4, 2 mM

MgCl₂), with 3 min centrifugation step between washes. Final resuspension was performed using 1mL of ice-cold RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.15% SDS, 5 mM EDTA, 2 mM DTT) and incubated 1h at 4 °C with gentle agitation. Supernatants were mixed with pre-equilibrated GFP-Trap beads (Chromotek) following manufacturer's protocol. Beads were washed 3x with 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM EDTA followed by 1 wash with 10 mM Tris-HCl pH 8.0. Samples were eluted after incubation with 2x SDS loading buffer and heating 10 min at 70°C before loading on a SDS-PAGE.

***Dictyostelium discoideum* GFP-NAP1 'in gel' Proteolytic Digestion - Mass Spectrometry Analysis**

Eluates from GFP-NAP1 immunoprecipitation were separated by SDS-PAGE and stained with Coomassie blue. Each gel lane was divided in 6 slices and digested⁶⁵. Tryptic peptides from in gel digestions were separated by nanoscale C₁₈ reverse-phase liquid chromatography using an EASY-nLC II (Thermo Fisher Scientific) coupled online to a Linear Trap Quadrupole - Orbitrap Velos mass spectrometer (Thermo Scientific) and desalted using a pre-column C₁₈ NS-MP-10 100µm i.d. x 0.2 cm of length (NanoSeparations). Elution was at a flow of 300 nl/min over a 90 min gradient, into an analytical column C₁₈ NS-AC-11 75µm i.d. x 15 cm of length (NanoSeparations). For the full scan a resolution of 30,000 at 400 Th was used. The top ten most intense ions were selected for fragmentation in the linear ion trap using Collision Induced Dissociation using a maximum injection time of 25 ms or a target value of 5000 ions. MS data were acquired using the XCalibur software (Thermo Fisher Scientific).

Raw data obtained were processed with MaxQuant version 1.5.5.1⁶⁶ and Andromeda peak list files (.apl) generated were converted to Mascot generic files (.mgf) using APL to MGF Converter [<http://www.wehi.edu.au/people/andrew-webb/1298/apl-mgf-converter>]. Generated MGF files were searched using Mascot (Matrix Science, version 2.4.1), querying dictyBase⁶⁷ (12,764 entries) plus an in-house database containing common proteomic contaminants and the sequence of GFP-NAP1. The common contaminant and reverse hits (as defined in MaxQuant output) were removed.

Mascot was searched assuming trypsin digestion allowing for two miscleavages with a fragment ion mass tolerance of 0.6 Da and a parent ion tolerance of 15 ppm. The iodoacetamide derivative of cysteine was specified in Mascot as a fixed modification, and oxidation of methionine and phosphorylation of serine, threonine and tyrosine were specified in Mascot as variable modifications. Scaffold (version 4.3.2, Proteome Software) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm, resulting in a peptide false discovery rate (FDR) of 0.63%²⁰. For label-free quantification, proteins were quantified according to the label-free quantification algorithm available in MaxQuant⁶⁸.

Significantly enriched proteins were selected using a Welch-test analysis with a 5% FDR.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD010460.

Generation and Validation of *cyri*-knockout and rescued *Dictyostelium discoideum*

Primers used are detailed in Supplementary Table 5.

Standard methods were used for construction of all *Dictyostelium* knockout and re-expression vectors⁶⁹. A linear CYRI knockout construct (2758 bp in length), which consisted of a blasticidin resistance (Bsr) cassette flanked by sequences matching 5' and 3' regions in the *CYRI* (DDB_G0272190 identifier at dictybase.org) gene locus (18pb cross-over), was made by PCR amplification using the primers set 1 (5' arm) and set 2 (3' arm). PCR-amplified arms were combined with the Bsr cassette in a using the primers set 3.

Knockout clones were screened/validated by PCR, with primers set 4. *cyri*-knockout yield a 2450 bp PCR product, random integrants (clones with a KO construct integration elsewhere in the genome) and wild-type yield a 1983 bp PCR product.

Vector for expression of untagged CYRI was obtained by sub-cloning CYRI's genomic coding region into pDM358⁶⁹. A REMI³⁷ (non extra-chromosomal) vector was derived from this by removal of the *Dictyostelium* plasmid propagation genes and re-ligation of the vector backbone. This construct, while still having a strong promoter, is expected to be present in just one copy per cell.

Transformation of *Dictyostelium discoideum*

3.0×10^7 cells/transformation were first centrifuged (3 min, 330 x *g*, 4°C), washed with 10 ml ice-cold electroporation buffer (E-buffer; 10 mM sodium phosphate buffer pH 6.1, 50 mM sucrose), and resuspended in 400 µl ice-cold E-buffer. Cells were transferred into an ice-cold 0.2 cm electroporation cuvette and incubated 5 min with 0.5-1.0 µg of DNA on ice. Cells were electroporated (BTX-Harvard Apparatus ECM 399) at 500V, giving a time constant of 3-4ms. Cells were immediately transferred to HL5 medium in Petri dishes. Appropriate selection (50 µg/ml hygromycin or 10 µg/ml G418) was added the next day. For REMI transfections, 10 µg of linearized DNA and 50 U of restriction enzyme were used, in 0.4cm cuvettes with a Bio-Rad Gene Pulser II set at 1.2kV and 3µF.

***Dictyostelium discoideum* CYRI inclusion body purification**

BL21(DE3) pLysS cells were grown to OD_{600nm} 0.2 and induced with 0.2 mM IPTG for 4h. Cells were pelleted, frozen and resuspended with 80 mL of lysis buffer (50 mM Tris-HCl pH 8.0, 25 % sucrose (w/v), 1 mM EDTA) per 100g of cells. Cells were lysed by adding 1% lysozyme (w/v) and kept on ice for 30 min. Lysate was resuspended with 10 mM MgCl₂, 1mM MnCl₂, 10µg/mL DNase I and kept for another 30 min on ice. Finally, 200 mL of detergent buffer (0.2 M NaCl, 1% deoxycholic acid (w/v), 1% NP40, 20 mM Tris-HCl pH 7.5, 2 mM EDTA) was added to the lysate, which is then centrifuged at 5000 x *g* for 10 min. Pellet is then washed in 0.5% NP40, 1 mM EDTA and this step is repeated until a tight white pellet is obtained.

***Dictyostelium discoideum* CYRI antibody production**

Inclusion bodies were dissolved in sample buffer with DTT and loaded onto a 10% Bis-Tris acrylamide gel at 70V at 4 °C. Gel was Coomassie stained and fragments of the band corresponding to CYRI was sent to BioGenes for injection into 2 rabbits. Bleeds were collected every second week after initial immunisation/boost and tested by western blot.

(Terminal bleed from rabbit 27724 after 5th boost used at 1:100).

***Dictyostelium discoideum* Under-agarose Chemotaxis Assay**

This assay is based on a previous study⁷⁰. Surface of the 30 mm glass bottom dish (MatTek) was coated with 10 mg/ml BSA for 10 min, washed with dH₂O and dried for 5 min inside a laminar flow cabinet. 0.4% w/v SeaKem GTG agarose in SIH medium (Formedium) containing 10 μM folate was poured and set for 1h. A well was cut in the agarose and 2x10⁶ cells/mL placed in it. After 3-4h cells were imaged by Phase contrast and DIC microscopy with a Nikon Eclipse TE2000-E microscope system equipped with a QImaging RETIGA EXi FAST 1394 CCD camera and a pE-100 LED illumination system (CoolLED) at 525 nm. A 10×/ 0.45 NA Ph1 objective and a 60×/1.40 NA apochromatic DIC objective were used for phase contrast and DIC, respectively. Imaging was controlled through the μManager 1.4.9 software. All microscopy was carried out at RT and images were analysed with ImageJ/Fiji 1.49i. Pseudopod rate and split frequency was analysed from the DIC movies and manually quantified frame by frame. For analysis of cell circularity, speed and migration parameters, automated tracking plugins were developed for ImageJ (see Plugin2 in Supplementary note 2). More information will be supplied upon reasonable request.

***Dictyostelium discoideum* development assay**

Cells were harvested from axenic growth plates, washed twice in phosphate buffer (10 mM Na/K phosphate pH 6.5) containing 2 mM MgCl₂ and 1mM CaCl₂, and plated on 1% w/v agar prepared in the same buffer. For time-lapse imaging we used a Nikon Eclipse TE2000-E microscope fitted with a Prior ProScan II moving stage, and equipped with a QImaging RETIGA EXi FAST 1394 CCD camera and a pE-100 LED illumination system (CoolLED) at 525 nm.

cAMP needle assay

Cells were developed as described above until territories began to form, indicating production and responsiveness to cAMP waves. Cells were harvested and placed into phosphate buffer and their response to 10 μM cAMP (Eppendorf Injectman NI2 microinjector with Femtotips II) was monitored by timelapse microscopy (1 frame/5sec) using a Zeiss Axiovert A1 body with a plan/neoFluar 20x 0.5NA objective combined with a QI REtiga camera.

HSPC300-GFP analysis

Wild type or *cyri* KO Ax3 cells were transfected with HSPC300-GFP as described above and timelapse movies were obtained using a Zeiss 880 confocal microscope. Processed images were used to obtain the unwrapped kymograph. Plugin used for

this analysis will be provided upon reasonable request.

3D MDCK cysts - Culture

shRNA-expressing MDCK cells were split 1:10 the day before plating in 3D, in puromycin-free medium. Chilled 8-well chamber slides were coated by spreading 5 μ L of undiluted Matrigel over the well surface and transferred to 37°C incubator for 10 min. MDCK cells were diluted to 4×10^4 cells/mL in puromycin-free medium and thoroughly disaggregated by pipetting. Matrigel was then diluted to 4% in MEM medium and mixed with the similar volume of cells diluted at 1.5×10^4 cells/mL, bringing the final Matrigel concentration to 2%. Wells were filled with 300 μ L of the cell-Matrigel mix and cysts were grown 5 days at 37°C.

3D MDCK cysts - Immunofluorescence and Imaging

Medium was aspirated and wells were quickly washed twice with PBS. Cysts were fixed using 4% PFA for 10 min, washed, and permeabilised for 10 min at RT using 0.5% Triton X100 diluted in PBS. Cells were blocked for 30 min using PFS (0.7% (w/v) fish skin gelatin in 0.025% Saponin-PBS). Primary antibodies were diluted in PFS and incubated overnight at 4°C with gentle shaking. Cysts were washed 3x in PFS at RT. Secondary antibodies, nuclear dye and Phalloidin were diluted at 1:200 in PFS and incubated for an hour at RT before further washes. Chambers were then kept sealed in 0.02% NaN_3 -PBS at 4°C until analysis. Cysts were imaged using the Nikon A1R Z6005 confocal microscope using either a Plan Apochromat 20x/0.75 DIC N2 or a Plan Fluor 10x/0.30 DIC L/N1 objective. In order to sharpen images, factor line averaging was set up at 4. Z-stack images were acquired with a 4 μ m increment step from the bottom to the top of the cyst.

Statistics and Reproducibility

Data sets were analysed using Prism5 v5.0c and Prism7. Differences between groups were tested for normality and then analysed using the appropriate statistical test, mentioned in each figure legend. Error bars represent standard error of the mean (S.E.M) unless stated otherwise. Significance levels are given as follows: ns: $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. Cochran-Mantel-Haenszel test was generated using R software and p -values are mentioned when appropriate. All experiments were repeated independently as biological repeats at least 3x, unless stated otherwise, and always gave similar trends. Individual values are available in Supplementary Table 6.

Data and Code Availability

All data and homemade plugins from this study will be made available upon reasonable request to the corresponding authors. Complete data points from all graphs in Figures 1-7 and Supplementary Figures 2-6 are provided in Supplementary Table 6 “Statistics and Source Data”. The code used for analysis of cell protrusions in Figure 5 is available in Supplementary Note 1. The code used for tracking Dictyostelium migration under agarose is available in Supplementary Note 2.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD010460.

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