ONLINE METHODS

Protein purification and crystallization. Expression constructs for crystallization were cloned into pET29b using standard PCR based methods, and mutants were generated using Quickchange site-directed mutagenesis. BCAR3 residues 502–825 (Uniprot: O75815) was expressed with a C-terminal histidine-tag in E. coli BL21(DE3), and purified by Ni^{2+} affinity chromatography followed by anion-exchange (Source Q 10/10), resulting in typical final protein concentrations of 10–12 mg.mL^{-1} BCAR3 in 10 mM HEPES (pH 7.9), 100 mM NaCl and 2 mM DTT. Initial crystals of wild-type protein grew by vapor diffusion from 15–20 % PEG3350, 0.04 M citric acid and 0.06 M Bis-Tris propane (pH 6.4) at room temperature. A contaminant lower molecular weight species was avoided by creating a BCAR3 (502–825) M536L construct, and grew crystals for data collection after mixing 3 µL of BCAR3 with 1.5 µL of the original mother liquor incorporating 4 % polypropylene glycol 400 additive.

The complex between NSP3 (Uniprot: Q8N5H7 isoform 2) and p130Cas (Uniprot: P56945) was prepared by mixing cell lysates following expression of C-terminally his-tagged NSP3 382–703 and untagged p130Cas 645–870. The complex was purified by both affinity and anion-exchange chromatography in a final buffer consisting of 10 mM Tris (pH 8.0), approximately 150 mM NaCl and 2 mM DTT. Initial crystals were obtained using wild type NSP3–p130Cas proteins, while a construct incorporating C497S and C598S mutations in NSP3 (referred to as NSP3) crystallized in an identical manner and used for data collection. Final crystals were prepared by mixing complex with mother liquor containing PEG3350 and sodium citrate (pH 7.8) in a 2:1 ratio. Diffraction was markedly improved by exchange of sodium citrate for sodium acetate in the mother liquor following crystal growth. Both BCAR3 and NSP3–p130Cas crystals were prepared for data collection by introducing glycerol up to 20 % and 15 % respectively prior to flash freezing.

Structure determination and refinement. Diffraction data from BCAR3 crystals was collected using a Rigaku Superbright rotating-anode source. Data was integrated and scaled XDS^40 and SCALA^41 and the structure of BCAR3 was solved by molecular replacement in Phaser^42 using the structure of NSP3 solved below as search model. The model was refined using Phenix^43 and
rebuilt using COOT\textsuperscript{44}. Phases of the NSP3–p130Cas complex were obtained from crystals containing selenomethionine derivatized NSP3 as well as by soaking native complex crystals in 10 mM thiomerosal for 1 hour prior to flash-freezing. Derivative data were collected wavelengths corresponding to the peak of anomalous dispersion for selenium and mercury at NSLS beamline X29. Data were integrated, reduced and scaled with HKL2000\textsuperscript{45}, or XDS and SCALA. Phasing by MIRAS and density modification were performed using AutoSharp\textsuperscript{46}, and partially built using Buccaneer\textsuperscript{41}. The model was completed in COOT and refined against native diffraction data using Phenix. Data-collection and final refinement statistics of both structures are displayed in Table 1. Molecular models were created using FFAS\textsuperscript{47} and Modeller\textsuperscript{48}, and all figures were created using Pymol (http://pymol.org/).

**Protein Biochemistry.** Expression constructs for target GTPases Rap1a (1–167, Uniprot P62834)\textsuperscript{49}, rRas (27–196, Uniprot P10301) (PDBid:2FN4), Rap2 (1–167, Uniprot P10114)\textsuperscript{50} and C3G (830–1077, Uniprot Q13905)\textsuperscript{26} were created by ligation independent cloning into a modified pET vector incorporating an N-terminal histidine-tag. Proteins were purified by affinity chromatography and size-exclusion chromatography on a Superdex 200 column.

Isothermal titration calorimetry was carried out using a MicroCal iTC\textsubscript{200} calorimeter, and proteins at stated concentrations were prepared in a matched buffer containing 10 mM Tris, 100 mM NaCl and 0.5 mM TCEP. Analytical ultracentrifugation sedimentation equilibrium (SE) experiments were performed in ProteomeLab XL-I (BeckmanCoulter) analytical ultracentrifugewith protein in 20mM Tris (pH 8.0), 150mM NaCl and 1mM DTT. SE data were analyzed using HeteroAnalysis software (by J.L. Cole and J.W. Lary, University of Connecticut).

**Nucleotide Exchange Assays.** For GDP exchange assays GTPases were loaded with mant-GDP using standard protocols\textsuperscript{51}, and exchange of mantGDP in an excess if unlabelled GDP was measured by tracking fluorescence at 460 nm following excitation at 355 nm in a Molecular Devices fMax fluorescent plate reader at 25 °C. Exchange assays were carried out with ~0.3 μM GTPase in a buffer containing 10 mM HEPES (pH 7.8), 100 mM NaCl, 0.5 mM TCEP and 2 mM MgCl\textsubscript{2}, and ~0.1 mg.mL\textsuperscript{-1} BSA was included to stabilize GTPases in solution.
**Antibodies.** A rabbit polyclonal antibody towards the 11 C-terminal residues of NSP3 was used for immunoprecipitations (10 μg) and a Shep1 SH2 antibody\(^2\) for immunoblotting (0.5 μg.mL\(^{-1}\)). The following primary antibodies were also used: p130Cas (BD Transduction laboratories; 1:600 dilution of a 0.25 mg.ml\(^{-1}\) stock for immunoblotting), BCAR3 (Santa Cruz, 1 μg.mL\(^{-1}\) for immunoblotting and 2 μg for immunoprecipitation), GFP (GeneTex; 1 μL serum for immunoprecipitation).

**Transwell migration assays.** Cells co-transfected with NSP3 constructs in pcDNA3 and the peGFP-N3 plasmid (Clontech) were seeded on Transwell filters coated on both sides with 10 mg.mL\(^{-1}\) fibronectin (Millipore). EGF (20 ng.mL\(^{-1}\)) was added in the lower chamber as a chemoattractant, in comparison to no EGF as a control. The cells were allowed to migrate for four hours, and the mean eGFP intensity from transfected cells on the lower side of the filters was measured from microscope images. Comparisons between wells with EGF were made by one-way ANOVA and Dunnett’s post-hoc test. Similar results were obtained in four different experiments.

**Probing NSP–Cas interactions in cells.** COS cells were cultured in DMEM supplemented with 10 % fetal bovine serum (FBS) and transiently transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were lysed in RIPA buffer and centrifuged at 16,000 g for 15 min at 4°C. For immunoprecipitations, cells lysates were incubated at 4°C with antibody for 2 hours, followed by incubation with Gamma-bind beads (GE Health Care Health Sciences) for 1 hour. Beads were washed with RIPA buffer and immunocomplexes were eluted by boiling for 5 min in SDS sample buffer. Extracts were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore). Following primary antibody incubation, membranes were probed with HRP-conjugated anti-mouse or anti-rabbit antibodies (Millipore; 1:5,000 dilution).
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