Supplementary Materials

Supplementary Materials and Methods

Creation of Knockout and Knock-in Stains

To make the RacB knockout and knock-in strains, the upstream region of the *racB* gene (~6 kb) was cloned from the *Dictyostelium* genome as an *Eco*RI fragment using standard methods. This region contains a unique *Bgl*II site ~1.6 kb upstream and *Kpn*I site ~300 bp upstream of the RacB translation start ATG. The DNA was digested with *Bgl*II and *Eco*RI, and the fragment containing a *Kpn*I site was subcloned into the pBS-SK vector digested with BamHI and EcoRI (pBS-RacB-up).

For the *rac*B knockout construct, a 5' fragment was amplified from the pBS-RacB-up by using the primers T3 and ATCAGGATCCCAACAGCACCATCACCTAC and subsequently digested with enzymes *Not*I and *Bam*HI. A 3' fragment was amplified with the primers AATTGGATCCCAACAGAATACGTTCCAACTG and T7, and subsequently digested with *Bam*HI and *Xho*I.

For the *rac*B knock-in constructs, a 5' fragment was amplified from pBS-RacB-up by using the primers T3 and GATTTCTAGATTATGTAAACTTTTTAACCTG and subsequently digested with enzymes *Not*I and *Xba*I. Using the primers TTTTTCTAGATGGAACAAAAATTAATTTCAGAAGAAGATTTAAATCAATCAATTAAATTGGTGG and ATTTGGATCCGTTTTTATAAAATTGAACATTTTGAATTC, a fragment encoding a myc-tagged RacB was amplified from a *rac*B cDNA and digested with *Xba*I and *Bam*HI. The 3' fragment used for the knock-in construct is the same one used to create the knockout construct.

The *rac*B knockout and knock-in constructs were made by inserting the blasticidin resistance (Bsr) cassette or hygromycin resistant gene into the *Bam*HI site. Supplementary figure S1 shows maps of the RacB

knockout and knock-in constructs and well as a Southern blot of the parental KAx-3 and *racB* null strains. Description of the maps and the digestion for the Southern blot analysis of the racB null strain is found in the Legend to Figure S1. Three independent knockout clones were obtained. All exhibited the same chemotaxis, developmental and F-actin activation phenotypes.

The *racgef1* knockout construct was made by inserting the Bsr cassette into the *Spe*I site of the RacGEF1 cDNA in the pSP72 vector (see supplementary figure S2). The vector was digested with *Eco*RV, and the DNA was transformed into *Dictyostelium* cells. Supplementary figure S2 shows maps of the RacGEF1 knockout construct and well as a Southern blot of the parental KAx-3 and *racgef1* null strains.

Knockout clones were determined by Southern blot analysis after digestion of genomic DNA with EcoRV. Description of the maps and the digestion for the Southern blot analysis of the *racgef1* null strain is found in the Legend to Figure S1. Three independent knockout clones were obtained. All exhibited the same phenotypes.

Plasmids

All cDNAs of Rac and RacGEF were cloned by PCR from a 12-16 h developmental λZAP library (Schnitzler, G.R., Fischer, W.H. and Firtel, R.A. 1994, Cloning and characterization of the G-box binding factor, an essential component of the developmental switch between early and late development in Dictyostelium. Genes Devel., 8, 502-514.). Rac cDNAs and the DNA of the CRIB domain of PAKa, and WASP were subcloned into the pGEX vector for GST fusion, the DNAs of RacGEFs' DH domain into pET21b for His-tagging, and the DNA of the CRIB domain of PAKa, and PAKc into pMAL-c2X for MBP fusion. RacGEF1 and its fragments were fused at the NH2 terminus with GFP and subcloned into the DIP-J expression vector.

RacB Activation Assay

The RacB activation assay is a modification of the protocol initially established for the activation of Rac in mammalian cells (Benard et al., 1999). The levels of RacB-GTP were measured by affinity precipitation using MBP- (maltose-binding protein) or GST-CRIB (Cdc42 and Rac interactive binding region) of DdPAKa, DdPAKc or DdWASP. Log phase vegetative cells were washed and resuspended at a density of 8-9 x 10^6 cells/ml in Na/K phosphate buffer for 5 h with 30 nM cAMP added every 6 min. The cells were brought to a basal stimulatory state by adding caffeine (1 mM final concentration) to inhibit adenylyl cyclase to the pulsed cells and incubating them for 30 min with continued shaking. The cells were collected by centrifugation and resuspended at a density of 4 x 10^7 cells/ml in Na/K phosphate buffer containing 1 mM caffeine. The 1 ml samples were stimulated with 1 µM cAMP (final concentration) in a 5 ml syringe attached to a filter holder. At the indicated times after stimulation, the cells were disrupted by rapidly filtering through a 3 micron pore size Nucleopore filter into 250 µl of 5X reaction buffer (50 mM HEPES, pH 7.5, on ice, 500 mM NaCl, 100 mM $MgCl_2$, 1 mM DTT, 2.5% Triton X-100) containing 5 µg aprotinin, 5 µg leupeptin, and 20 µg GST-PAK CRIB protein. 35 µl of a 1:1 slurry of glutathione-Sepharose beads were added to the supernatant and incubated under gentle agitation for 30 min at 4°C. The beads were collected by centrifugation at 3,000 rpm for 1 min and then washed three times by sequentially gently resuspending the beads in reaction buffer and then centrifugation at 3,000 rpm for 1 min and then suspended in sample buffer and subjected to 13% SDS-PAGE and Western blot analysis with an anti-myc monoclonal antibody. The amount of RacB (RacB-GTP) bound to beads was quantified by densitometry of the developed Western blot film. The wild-type level at 0 sec was set at 1.0. The levels of RacB-GTP in a specific mutant strains were divided by relative level of total myc-RacB protein in that strain compared to the level of myc-RacB protein in wild-type cells (a ratio of the total myc-RacB protein levels as determined by Western blotting). In all cases, RacB activation and total RacB protein were co-analyzed with each mutant strain.

In vitro RacB binding assay

The RacB-GTP binding assay was performed by affinity precipitation using the MBP- (maltose-binding protein) or GST-CRIB (Cdc42 and Rac interactive binding region) of DdPAKa, DdPAKc, or DdWASP.

For the assay with the MBP-CRIB of DdPAKa or DdPAKc, 5 μg GST-RacB was incubated in 0.5 ml of reaction buffer [10 mM HEPES buffer (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and 50 μg/ml BSA] containing 1 μM His-GEF1(DH), and 100 μM GTP-γ-S or GDP at 23°C for 30 min. 20 μg MBP-CRIB and 35 μl of a 1:1 slurry of amylose-Sepharose beads were added to the reaction mixture and incubated under gentle agitation for 1 h at 4°C. The beads were collected by centrifugation at 3,000 rpm for 1 min and washed with 1 ml of the reaction buffer. After being washed three times, the beads were suspended in sample buffer and subjected to 13% SDS-PAGE gel and Western blot analysis with an anti-GST polyclonal antibody.

To perform the assay with the GST-CRIB of DdWASP, log-phase vegetative (KAx3/mycRacB knock-in) cells (5 x 10^6 cells) were harvested and lysed in 1 ml of lysis buffer [10 mM HEPES buffer (pH 7.5), 1% Triton X-100, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT] containing 5 μg aprotinin and 5 μg leupeptin at 4°C. After centrifugation at 15,000 rpm for 10 min, the supernatant was combined with 100 μM GTP-γ-S or GDP, and incubated for 15 min at 4°C. 20 μg MBP-CRIB protein and a 35 μl of a 1:1 slurry of glutathione-Sepharose beads were added to the supernatant and incubated under agitation for 1 h at 4°C. The beads were collected by centrifugation at 3,000 rpm for 1 min and washed with 1 ml of the lysis buffer. After being washed three times, the beads were suspended in sample buffer and subjected to 13% SDS-PAGE gel and Western blot analysis with an anti-myc monoclonal antibody.

F-actin polymerization and myosin II assembly

F-actin polymerization and myosin II assembly were assayed as previously described (Steimle et al., 2001). Log-phase vegetative cells were washed twice and resuspended to 10 ml at a density of 5 x 10^6 cells/ml with 12 mM Na/K phosphate buffer pH 6.2 and pulsed with 30 nM cAMP for 5 h at 6 min intervals. After

pulsing, the cells were harvested and resuspended to 10 ml with 12 mM Na/K phosphate buffer containing caffeine (1 mM final concentration) and incubated with shaking for 30 min. Subsequently, the cells were stimulated with cAMP (10 μM final concentration), and, at the indicated times after stimulation, 0.5 ml aliquots of cells were lysed by addition of an equal volume of 100 mM MES (pH 6.8) buffer containing 1% Triton X-100, 5 mM EGTA, 10 mM $MgCl_2$, 5 μg aprotinin, and 5 μg leupeptin. Triton-resistant cytoskeletal pellets were collected by centrifugation at 15,000 rpm for 15 min, suspended in 2X sample buffer, and subjected to 11% SDS-PAGE gel. The amounts of actin and myosin II were determined by densitometric analysis of the scanned Coomassie-stained gels. The level of myosin II and actin in wild-type cells at 0 sec (unstimulated cells) was set at 1.0. The levels of actin or myosin II in the mutant strains were divided by the relative level of total lysate protein in a given strain compared to the level of total lysate protein in wild-type cells.

Chemotaxis Assay

Chemotaxis analysis was performed as described previously (Funamoto et al., 2001). Briefly, log-phase vegetative cells were washed twice and resuspended at a density of 5 x 10^6 cells/ml with 12 mM Na/K phosphate buffer and pulsed with 30 nM cAMP for 5 h at 6 min intervals as described above. Approximately 50 μl of cells was added to a 35 mm plastic Petri dish containing 3 ml of 12 mM Na/K phosphate buffer. The Petri dish has a hole covered by a glass cover slip. A region of the cover slip containing a reasonable density of cells (cells should not be touch and the density needs to be low enough so that cells will not bump into each other during the chemotaxis assay but high enough to have ~10-15 cells in the field during the assay). A glass capillary needle (Eppendorf Femptotip) filled with 150 μM cAMP solution was positioned to stimulate cells by using an Eppendorf Patchman micromanupulator and the response of the cells was recorded by using a time-lapse video recorder and NIH Image software. Computer analysis was performed using DIAS software (Soll and Voss, 1998; Wessels et al., 1998).

PAKc and Akt/PKB Kinase Activity Assay

The Akt/PKB kinase activity was assayed as previously described (Meili et al, 1999). The PAKc kinase activity assay is a modification of that used for Akt/PKB. Cells were lysed with an equal volume of 2X NP40 lysis buffer (2X PBS, 100 mM NaF, 2% NP40, 4 mM EDTA, 2 mM pyrophosphate, 2 mM Na_3VO_4, and protease inhibitors leupeptin and aprotinin) on ice for 10 min. The lysate was pre-cleared by centrifugation for 10 min. To immunoprecipitate myc-PAKc, 1 µl of anti-myc monoclonal-antibody from Invitrogen (1 mg/ml) and 30 µl of 50% slurry of protein G-Sepharose were added to the supernatant. The beads were washed twice with lysis buffer and twice with kinase buffer (25 mM MOPS, pH 7.4, 25 mM β-glycerophosphate, 20 mM $MgCl_2$, 1 mM DTT). The beads are resuspended in and incubated with 75 µl of kinase buffer containing 5 µM cold ATP, 10 µCi [γ-^{32}P]ATP, and 5 µg H2B as substrate at room temperature for 15 min. The reaction was stopped by the addition of 25 µl of 5X sample buffer (250 mM Tris, 500 mM DTT, 10% SDS, 50% glycerol, and 0.5% bromophenol blue) and boiled for 2 min. Samples were separated by 10% SDS-PAGE, blotted onto a PVDF (polyvinylidene difluoride) membrane, and exposed to film.

RacGEF1 Membrane Localization Assay

Log phase vegetative cells (racgef1$^-$/GFP-RacGEF1Total) were washed and resuspended at a density of 5 x 10^6 cells/ml in Na/K phosphate buffer and pulsed with 30 nM cAMP for 5 h at 6 min intervals. After pulsing, the cells were collected by centrifugation at 400 x g for 3 min and resuspended at a density of 1 x 10^7 cells/ml in Na/K phosphate buffer containing 1 mM caffeine. The cells were treated with 60 µM LY294002 (diluted from 5 mM stock solution dissolved in DMSO) or DMSO (control) and incubated for 25 min by shaking. Cells were stimulated with cAMP (10 µM cAMP final concentration). At the indicated times after stimulation, 0.5 ml of the cells were lysed by adding to an equal volume of 2X Triton lysis buffer (2X PBS, 100 mM NaF, 1% Triton X-100, 4 mM EDTA, 2 mM pyrophosphate, and 1 mM DTT) containing protease inhibitors (5 µg leupeptin and 5 µg aprotinin) on ice for 10 min. Triton-resistant cytoskeletal pellets

were collected by centrifugation at 15,000 rpm for 15 min, suspended in 2X sample buffer, and subjected to 6% SDS-PAGE gel. The amount of GFP-RacGEF1 protein was determined by Western blotting using anti-GFP polyclonal-antibody from Santa Cruz Biotech. The level of GFP-RacGEF1 protein was determined by densitometric analysis of the developed film. The level of RacGEF1 in the cytoskeletal fraction in DMSO-treated (control) cells at 0 sec (unstimulated) was set at 1.0.

In Vitro GEF1 Membrane Localization Assay

Log phase vegetative cells were washed and resuspended at a density of 5 x 10^6 cells/ml in Na/K phosphate buffer for 5 h with 30 nM cAMP added every 6 min. The cells were collected by centrifugation and resuspended at a density of 1 x 10^7 cells/ml in Na/K phosphate buffer containing 2 μM caffeine. The cells were treated with 60 μM LY294002 or DMSO (control) and incubated for 25 min by shaking. At the indicated times after stimulation with final 10 μM cAMP, the cells were lysed with an equal volume of 2X Triton lysis buffer (2X PBS, 100 mM NaF, 1% Triton X-100, 4 mM EDTA, 2 mM pyrophosphate, 1 mM DTT, and protease inhibitors leupeptin and aprotinin) on ice for 10 min. Triton-resistant cytoskeletal pellets were collected by centrifugation, suspended in 2X sample buffer, and subjected to SDS-PAGE. The amount of GFP-GEF1 protein was determined by Western blotting using anti-GFP polyclonal-antibody from Santa Cruz Biotech.

Legends for Supplementary Figures

Figure S1. Production of RacB knockout and knock-in strains. A. The upper section is a map of the RacB locus created from data made available in the *Dictyostelium* genomic sequence database (Fey P, Gaudet P, Just EM, Merchant SN, Pilcher KE, Dyck PA, Kibbe WA, Chisholm RL "dictyBase" http://www.dictybase.org/). A map of the RacB cDNA is shown. The RacB cDNA was obtained by amplifying inserts in the λZAP cDNA library by PCR as described in the Supplementary Materials and Methods. The cDNA clone was sequenced and the sequence agreed with the predicted open reading frame (ORF) from the sequence of the RacB genomic locus.

A map of the RacB knockout construct is shown and is described in Supplementary Materials and Methods, as is the digestion used to create the knockout strains. The Bsr cassette encoding blasticidin resistance inserted as shown and as described in the Supplementary Materials and Methods. For the knock-in construct, a myc-tagged RacB ORF construct using the RacB cDNA (Supplementary Materials and Methods) was inserted.

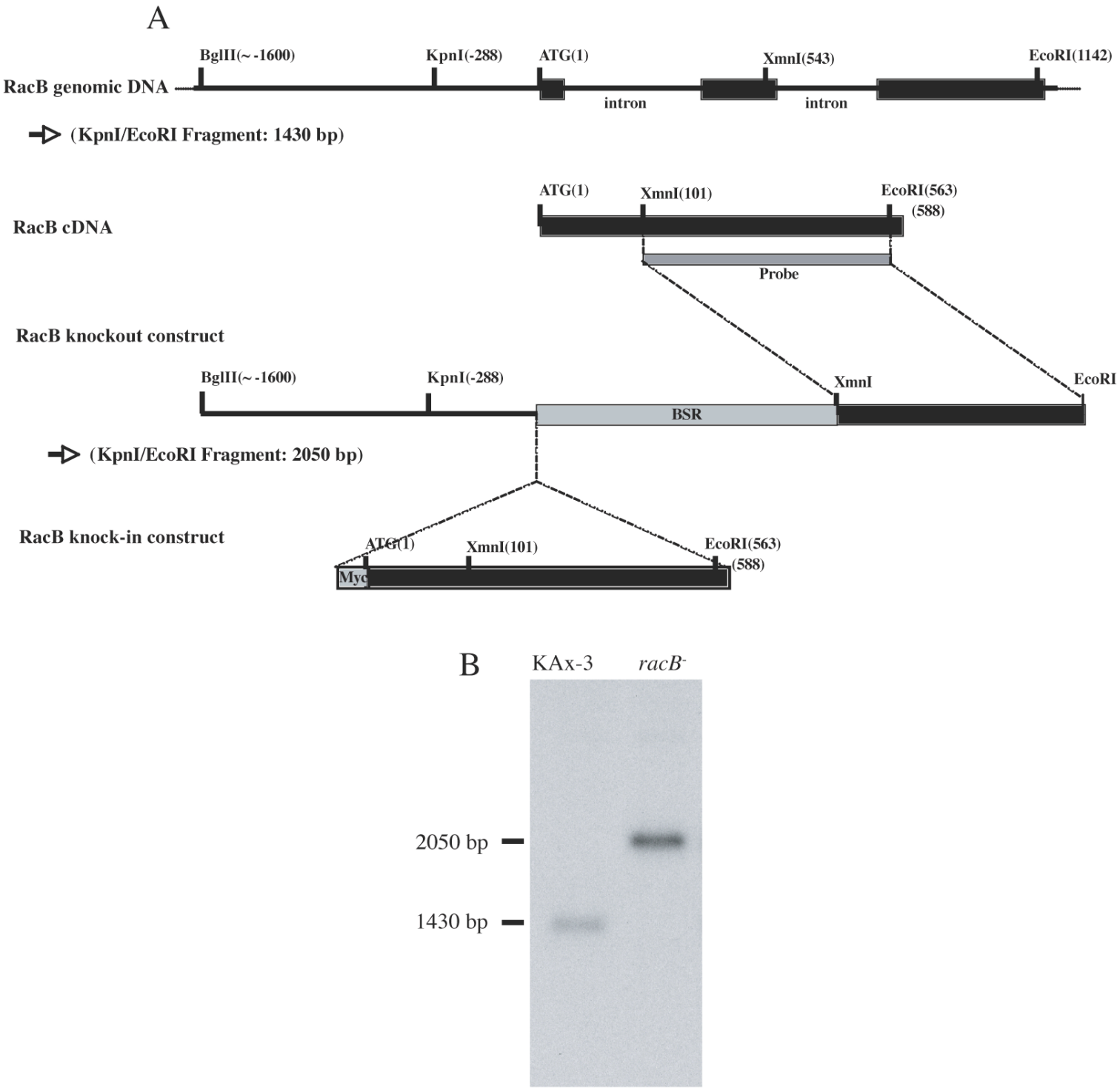
B. Southern map of the *racB* knockout strain. DNA from KAx-3 wild-type cells and a *racB* null strain were digested with *Kpn*I and *Eco*RI and probed using the *Xmn*I/*Eco*RI fragment from the cDNA clone shown in part A. The endogenous band (1430 bp) is missing in the knockout strain and a new band of the expected size of 2050 bp is present. In addition, the *racB* null strain does not express a RacB transcript (see Fig. 3A).

Figure S2. RacGEF1 knockout. A. The genomic map of the RacGEF1 locus was created from data made available in the *Dictyostelium* genomic sequence database (Fey P, Gaudet P, Just EM, Merchant SN, Pilcher

KE, Dyck PA, Kibbe WA, Chisholm RL "dictyBase" http://www.dictybase.org/). A map of the RacGEF1 cDNA is shown. The RacGEF1 cDNA was obtained by amplifying inserts in the λZAP cDNA library by PCR as described in the Supplementary Materials and Methods. The cDNA clone was sequenced and the sequence agreed with the predicted ORF from the sequence of the RacGEF1 genomic locus.

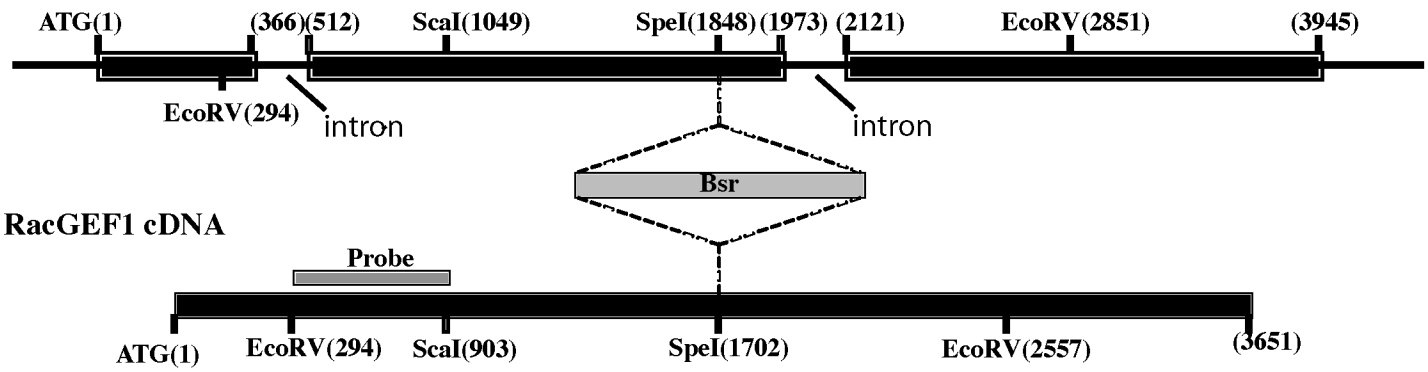
B. Southern map of the *racgef1* knockout strain. DNA from KAx-3 wild-type cells and a *racgef1* null strain were digested with *Eco*RV and probed using the *Eco*RV/*Sca*I fragment from the cDNA clone shown in part A. The endogenous band (~2500 bp) is missing in the knockout strain and a new band of the expected size of ~3711 bp is present. In addition, the *racgef1* null strain does not express a RacGEF1 transcript (see Fig. S3).

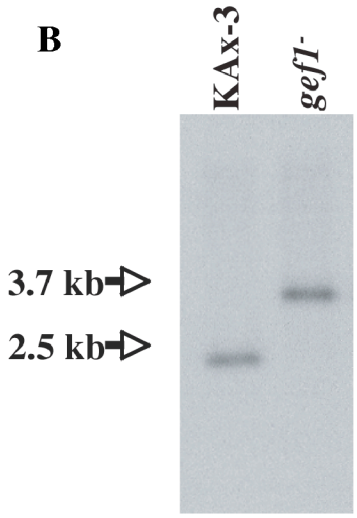
Figure S3. RacGEF1 RNA blot analysis. Total cell RNA was isolated from vegetative cells of wild-type, *racgef1* null, and several RacGEF1 wild-type and mutant overexpressing strains. All RNAs are isolated from clones used for the analysis described in the Results. After electrophoresis on formaldehyde-containing agarose gels, the RNA was blotted onto a Magnagraph 0.22 micron nylon membrane and probed with a nick-translated fragment that comprises amino acid residues 749-984. The upper and middle parts of the figure show two different exposures of the RNA blot to be able to effectively see the band corresponding to the endogenous RacGEF1 mRNA. The lower part of the figure shows ethidium bromide staining. The large and small ribosomal subunit RNAs are shown. Equal loading is shown. The positions of the endogenous RacGEF1 mRNA and the 17S and 26S rRNAs are shown.



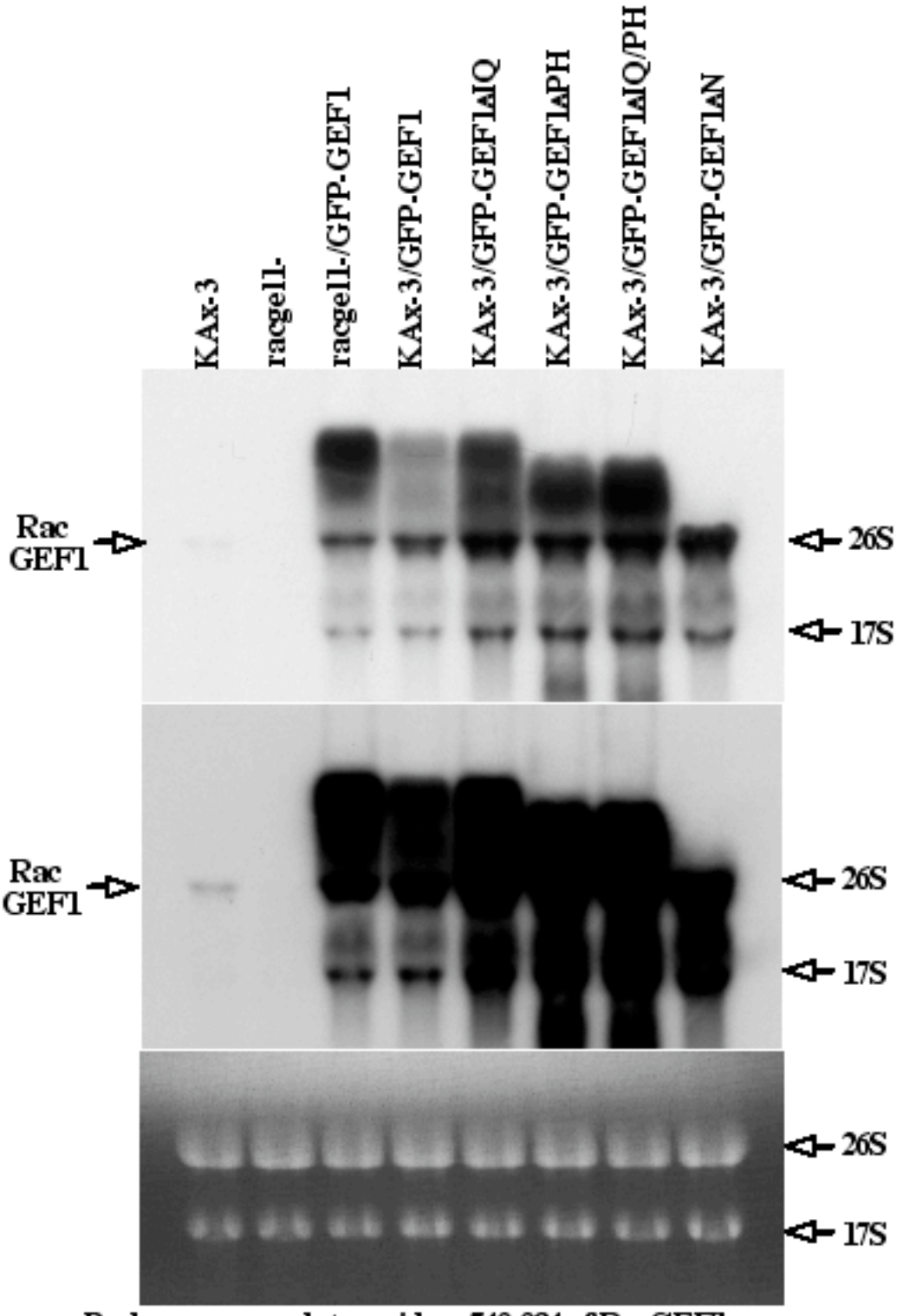
Park et al., Fig. S1

A





Park et al., Fig. S2



Probe corresponds to residues 749-984 of RacGEF1

Park et al., Fig. S3