Supporting Information

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SI Materials and Methods

Cloning, Expression, and Purification of GST and GFP Fusion Proteins. A vector coding for full-length coronin with GFP at the C terminus under the control of actin15 promoter was described previously (1). This plasmid was referred to as GFP–cor WT. The GFP–coronin MUT1 and MUT2 constructs were created by site-directed mutagenesis using appropriate primers and the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and the sequence verified. Cloning of GFP–p21-activated kinase a (PAKa) and generation of a dominant-negative PAKa (GFP–PAKa–c) was described earlier (2). The plasmids were transformed into AX2 and *corA* knockout cells by electroporation. Transformants expressing the respective GFP-tagged proteins were selected by using G418 (Geneticin, Life Technologies). The expression levels were determined by Western blots. For the expression of different coronin deletion proteins as GST fusion proteins, appropriate coding sequences were PCR amplified and cloned into the expression vector pGEX 4T-2 (GE Healthcare) using BamHI and XmaI sites. GST fusion proteins were expressed in *Escherichia coli* strain XL1 blue and purified from the soluble fraction using Glutathione Sepharose affinity columns (GE Healthcare). A 200 bp fragment encoding the Cdc42- and Rac-interactive binding (CRIB) domain of coronin was cloned into the expression vector pBsrN2 (3) and introduced into AX2 cells. The transformants were selected with blasticidine 1.5 μg/mL (MP Biomedicals). Rac proteins were expressed as GST- and GFP-tagged fusion proteins.

GFP–myosin heavy chain kinases (MHCKs) expression plasmids were procured from the DictyStock center (www.dictybase.org) and expressed in wild-type and coronin mutant cells. The expression levels were analyzed by Western blotting with anti-GFP mAb K3-184-2.

Loading of Rac GTPases with GDP or GTPγS. For direct interaction assays, GST, GST–Rac1a, and RacC bound to Glutathione Sepharose columns were loaded with GDP or GTPγS in nucleotide exchange buffer (25 mM Hepes, pH 7.4, 100 mM NaCl, 10 mM EDTA, and 1 mM DTT) for 1 h at 4 °C. Equivalent amounts of thrombin cleaved fragment (NT CRIB) were added to the preloaded columns and incubated for1 h at 4 °C. After repeated washing, the bound proteins were separated by SDS/PAGE [15% (vol/vol) polyacrylamide] and visualized with Coomassie Blue. For interaction of full-length coronin with Rac GTPases, AX2 cells (5×10^7) were lysed by sonication in lysis buffer [25 mM Tris·HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 2 mM EGTA, 2 mM DTT, and 1% Nonidet P-40 with EDTA-free protease inhibitor mixture (Roche)], and equivalent amounts of cell lysates were added to columns containing GST, GST–Rac1b, and GST–RacB preloaded with either GDP or GTPγS. After 2 h of incubation at 4 °C, beads were washed with wash buffer (lysis buffer without protease inhibitor), and pull-down eluates were analyzed in Western blots with anticoronin mAb 176-3-6 (4). Interaction of Dictyostelium GTPases activating protein 1 (DGAP1) with Rac1a was analyzed as described. Probing was with mAb 216–394-1 (5).

Immunoprecipitation and Pull-Down Experiments. For coronin self-association studies, equivalent amounts of AX2 cells (5×10^7) expressing GFP–cor WT and GFP–MUT were lysed by pipetting several times in 500 μL lysis buffer (10 mM Tris·HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40 with protease inhibitor mixture) and incubated on ice for 20 min. We then added 500 μL of dilution buffer (10 mM Tris·HCl, pH 7.5, 100 mM NaCl, and 1 mM EDTA) to the clarified lysate and incubated it with 20 μL of GFP–TRAP beads (ChromoTek) for 2 h at 4 °C. The beads were washed and the immunoprecipitates were analyzed by Western blots with anti-GFP mAb K3-184-2 (3) and anticoronin mAb 176-3-6 (4). Interaction of Rac1a GTPase with coronin fragments was investigated by pull-down assays. AX2 cells expressing GFP–Rac1a (5×10^7 cells) were lysed (25 mM Tris·HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM DTT, and 5% glycerol) supplemented with protease inhibitors (Sigma) and incubated with equivalent amounts of GST and coronin GST fusion proteins bound to Glutathione Sepharose beads for 2 h at 4 °C. The pull-down eluates were immunoblotted with GFP-specific mAb K3-184-2. Interaction of full-length coronin with Rac1a GTPase was investigated by coimmunoprecipitation experiments as described above.

For determining the levels of activated Rac in *Dictyostelium discoideum* cells, a pull-down with the GTPase binding domain (GBD) from rat PAK1 kinase (GST–PAK–GBD), which specifically interacts with the GTP-bound form of Rac1, was carried out followed by Western blot analysis with polyclonal antibodies against Rac (6). The amount of activated Rac from AX2 wild-type and coronin mutant cells were calculated by ImageJ, and the data are represented in a bar chart.

Kinase Assay. Kinase assays were performed as described previously (7). Equivalent amounts of cells ($2–3 \times 10^7$) were lysed (25 mM Tris·HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, and 1 mM DTT) supplemented with protease inhibitors (Sigma) and phosphatase inhibitors (PhosphoSTOP, Roche). The clarified lysate was incubated with 20 μL of GFP–TRAP beads (ChromoTek) for 2 h at 4 °C. The beads were washed four times in lysis buffer without protease inhibitors and once in kinase buffer (25 mM Mops, pH 7.4, 20 mM MgCl2, and 30 mM KCl supplemented with phosphatase inhibitors). The purified GFP–MHCK proteins were further incubated with Histone 2B substrate and ATP mix (ATP and ^{32}p-γ-ATP) for 10 min at room temperature, and the kinase activity was measured by autoradiography.

Miscellaneous Methods. Isolation and analysis of cytoskeletal fractions were done as described earlier (4). Briefly, equivalent amount of cells (2.5×10^7) were collected from Petri dishes in 300 μL of Soerensen phosphate buffer (17 mM Na^+/K^+ phosphate, pH 6.0) and lysed in 2× cytoskeletal buffer (25 mM Tris·HCl, pH 7.5, 30 mM KCl, 5 mM EGTA, 1% Nonidet P-40) supplemented with protease inhibitors (Sigma). After a 10 min incubation on ice, the tubes were transferred to room temperature and incubated for another 10 min. Detergent insoluble fractions were collected by centrifugation at $10,000 \times g$ for 4 min, and equivalent amounts of pellet and the supernatant fractions were loaded in a 10% polyacrylamide gel. The gels were stained with Commassie Blue, and myosin II was quantified using ImageJ. To quantify nuclei number, growth phase cells were collected from Petri dishes and fixed with methanol. Nuclei were stained with DAPI, and nuclei in more than 500 cells of each strain were quantified. To analyze CRIB sequences of coronin, CRIB motifs of *Dictyostelium discoideum* coronin (P27133), *Homo sapiens* p-21 activated kinase (HsPAK1) (Q13153), *Homo sapiens* Wiskott–Aldrich syndrome protein (HsWASP) (P42758), *Dictyostelium discoideum* Wiskott–Aldrich syndrome protein (DdWASP) (Q7KWP7), and *Dictyostelium discoideum*

p21-activated protein kinase b (DdPAKb) (Q869N2) were retrieved and aligned using the clustalW2 online program (8). The aligned sequence was processed using ESPript 2.2 (9) for representation.

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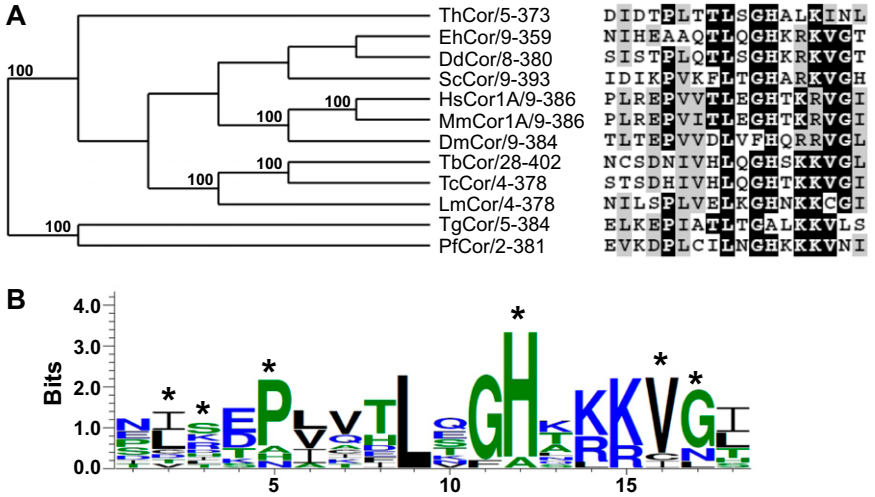

Fig. S1. Sequence and structural analysis of CRIB domain of short coronins. (*A*) Conservation of the CRIB domain in short coronins across taxa. Protein sequences of coronin from *Homo sapiens* (P31146), *Mus musculus* (O89053), *Drosophila melanogaster* (Q7JVY0), *Saccharomyces cerevisiae* (Q06440), *Entamoeba histolytica* (C4M137), *D. discoideum* (P27133), *Tetrahymena thermophila* (I7MIA8), *Trypanosoma cruzi* (Q4D4 × 6), *Trypanosoma brucei brucei* (Q57W63), *Toxoplasma gondii* (Q5Y1E7), *Leishmania major* (Q4QB38), and *Plasmodium falciparum* (O44021) were retrieved, and the core tryptophan-aspartic acid (WD) repeats were aligned using the ClustalX program (1). A bootstrap Neighbor Joining (NJ) tree was created, and the resulting cladogram is shown with supporting bootstrap values in percent. The CRIB sequence alignment of respective coronins is shown on the right side of the tree. (*B*) Sequence logo of the coronin CRIB domains. CRIB sequences of coronin proteins were retrieved, and a sequence logo was created using the WebLogo online program (2). The overall height of the stack in bits indicates the sequence conservation at that position. The CRIB consensus amino acids are highlighted with asterisks.

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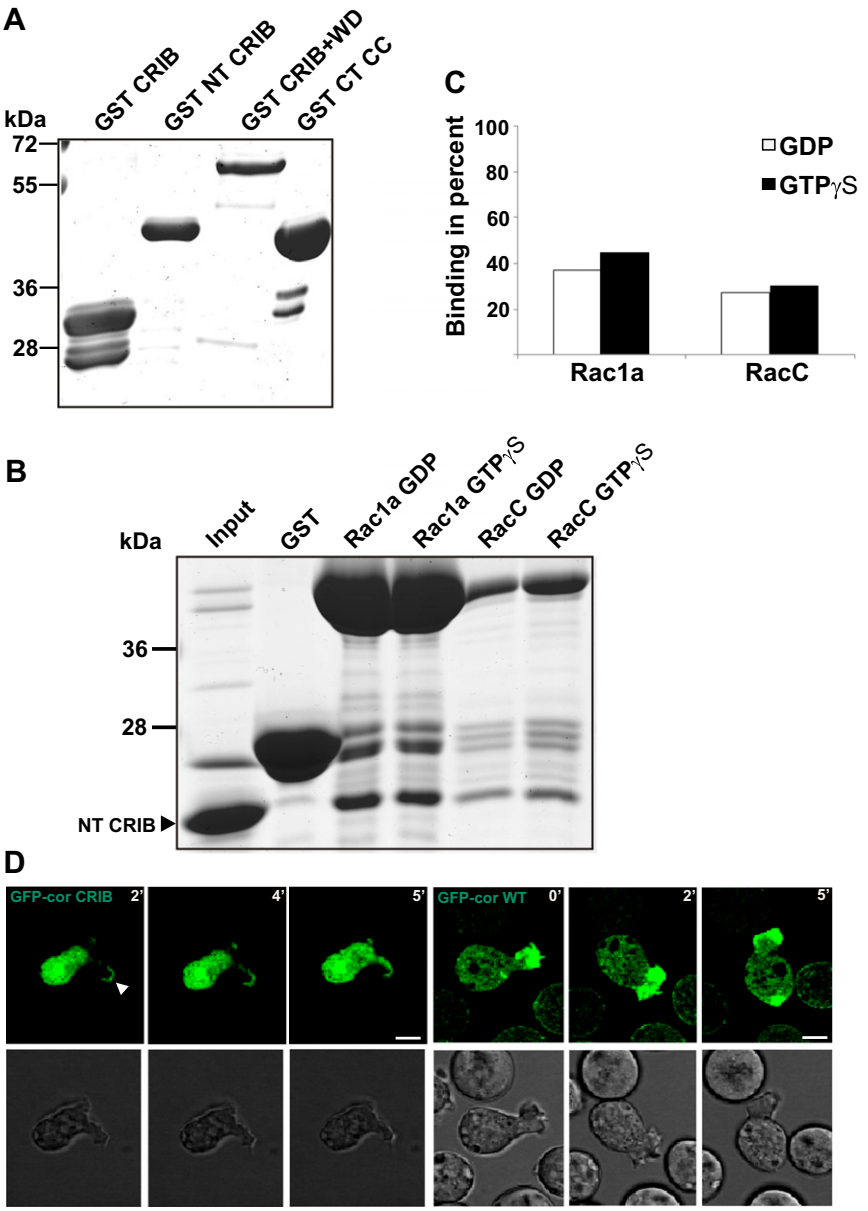

Fig. S2. Expression and analysis of coronin CRIB motif. (*A*) The different GST fusion peptides were expressed in *E. coli* XL1-blue and purified using Glutathione Sepharose 4B beads. The polypeptides were separated on a 12% polyacrylamide gel and stained with Coomassie Blue. On the left, the position of the molecular mass markers is indicated. (*B*) Direct interaction of the CRIB domain with Rac GTPases. Thrombin-cleaved NT CRIB peptide (arrowhead) was loaded onto columns containing GST–Rac1a and GST–RacC that were preloaded either with GDP or GTPγS. After incubation for 1 h, the bound proteins were separated in 15% polyacrylamide gels and stained with Coomassie Blue. (*C*) The bound CRIB peptide was quantified using ImageJ and plotted in a graph with the input set at 100%. The bars represent the average of two independent experiments. (*D*) Live cell analysis of coronin CRIB domain localization. GFP–cor CRIB and GFP–cor WT expressing cells were seeded into eight-well μ-chambers (ibidi) and imaged using a Leica laser scanning microscope (TCS SP5). Representative images are shown in the *Upper* panel, and the corresponding differential interference contrast (DIC) images are shown below. The recruitment of GFP–cor CRIB to the cell cortex is indicated by an arrowhead. The time (in minutes) is indicated. (Scale bar, 5 μm.)

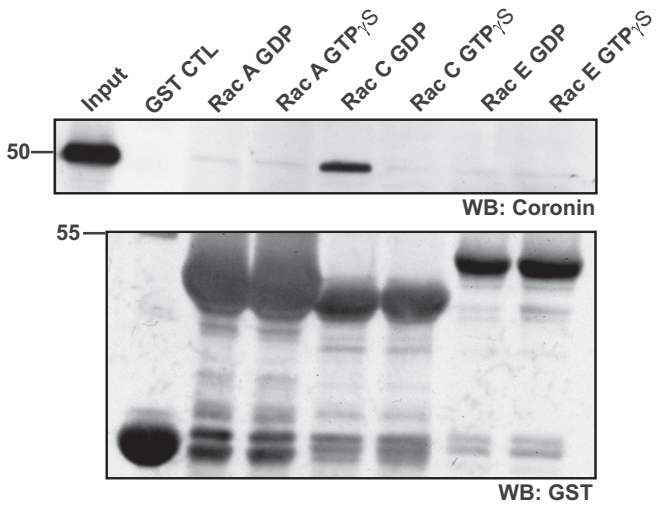

Fig. S3. Coronin–Rac interaction. GST, GST–RacA, GST–RacC, and GST–RacE bound to Glutathione Sepharose beads were loaded with GDP or GTPγS and incubated with cell lysates from AX2 cells. After repeated washing of beads, the bound proteins were immunoblotted with anticoronin antibody. The membrane was stripped and probed with DGAP1 using 216-394-1 antibody as a control.

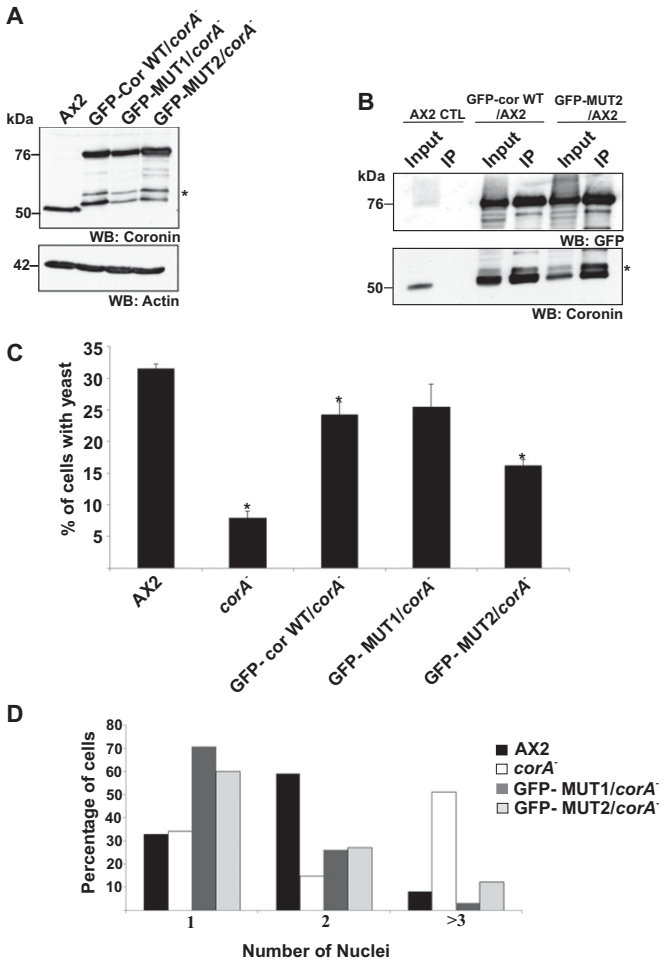

Fig. S4. Analysis of the coronin CRIB mutant proteins. (*A*) Expression level of coronin CRIB mutant. Total cell lysates prepared from equal numbers of cells (2×10^5) were separated on 12% SDS gel and Western blots performed with anticoronin antibodies. The membrane was stripped and reprobed with anti-actin antibody as a loading control. The asterisk in the blot denotes degradation products of GFP fusion proteins. (*B*) Coronin CRIB mutant protein self-associates. Equal amounts of cell lysates from AX2, AX2 expressing GFP–cor WT, and GFP-MUT2 were immunoprecipitated with anti-GFP antibodies. Lysate and precipitates were analyzed by Western blots for the presence of GFP-tagged proteins with anti-GFP antibody (WB: GFP). The membrane was stripped and probed with anticoronin antibody to detect self-association (WB: coronin). The star in the *Lower* panel indicates degradation products of the GFP fusion proteins. (*C*) AX2, *corA*$^-$, GFP–cor WT, and GFP mutant–expressing cells (rescue) were allowed to sit on coverslips and challenged with TRITC labeled yeast for 15 min before methanol fixation. Two hundred or more cells for each strain were counted. The percentage of cells that had engulfed yeast is shown in the graph (*$P < 0.05$). (*D*) Quantification of nuclei. Cells were fixed with methanol, and nuclei were stained with DAPI. Nuclei in more than 500 cells of each strain were quantified.

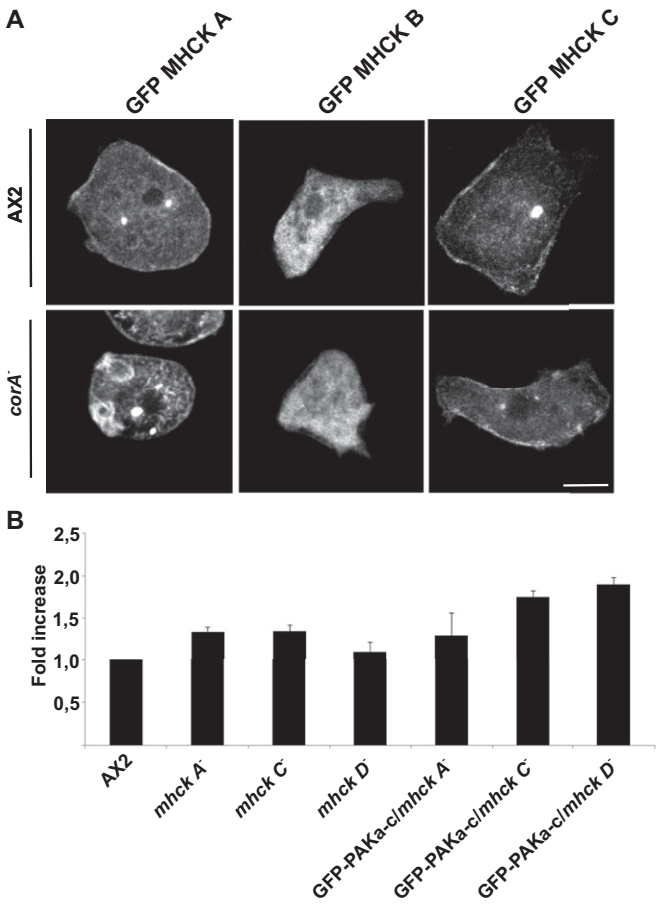

Fig. S5. Expression and analysis of GFP-tagged MHCKs and PAKa–c. (*A*) Localization of GFP–MHCKs in wild-type AX2 and *corA*$^{-}$ cells. Growth phase cells expressing various GFP–MHCKs were fixed in methanol and analyzed in microscope. (*B*) Myosin II assembly levels of MHCK knockout cells expressing GFP–PAKa–c. The bar represents the mean and SD of three independent experiments.

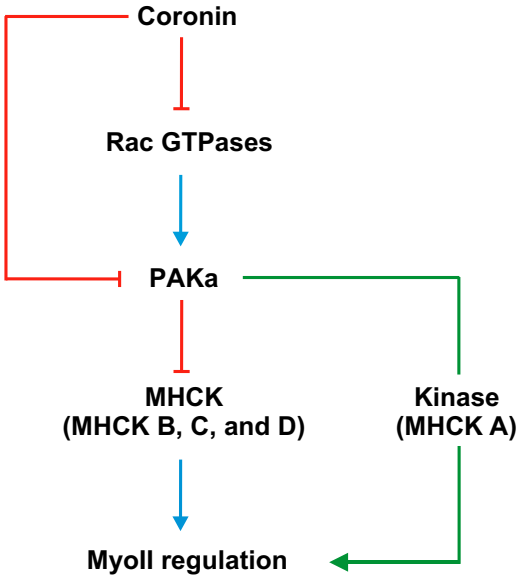

Fig. S6. Signaling cascade regulating myosin II. Signaling components of the myosin II regulation in *D. discoideum* are shown in the figure. Coronin prevents the activation of Rac GTPases by its interaction with GDP–Rac. Rac upon activation increases the kinase activity of PAKa. The activated PAKa in turn inhibits the activities of MHCKs (MHCK B, MHCK C, and MHCK D). MHCKs are responsible for the phosphorylation of myosin II heavy chain and its regulation. In addition, coronin can also regulate the activities of PAKa through direct interaction. Furthermore, PAKa can regulate myosin II assembly through a signaling cascade directly dependent on MHCK A.