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Supplemental Data

Coronin 1B Coordinates

Arp2/3 Complex and Cofilin Activities

at the Leading Edge

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Supplemental Experimental Procedures

Antibodies

Antibodies were obtained from Cell Signaling Technologies (pSer^{PKC}, pLIMK1/2, LIMK1), Upstate Biotechnology (p34Arc, Cortactin), Cytoskeleton (Cofilin), Chemicon (actin), Biosource (pPaxillinSer126, pErk1/2), Roche (GFP), Sigma (Myc) and Jackson ImmunoResearch (Cy2, Rhodamine Red-X, Cy5, and HRP conjugated secondary antibodies). AlexaFluor phalloidins were from Molecular Probes. Cells were obtained from ATCC. Inhibitors Ro32-0432 and okadaic acid were from Calbiochem. Antibodies to Coronin 1B (4245.Exp) were affinity purified as described (Cai et al., 2005).

Cell culture and viral transduction

Cell culture, immunoprecipitation and immunoblotting were performed as described (Cai et al., 2005). Transient transfections were performed using FuGENE 6 (Roche) for HEK293 cells, PolyFect (Qiagen) for Rat2 cells and Lipofectamine 2000 (Invitrogen) for NIH3T3 cells. Retroviral packaging, infections, and fluorescence-activated cell sorting

were as described (Bear et al., 2000). Lentivirus production and infection were as described (Rubinson et al., 2003).

Molecular cloning

SSH1L-GFP was constructed by amplifying the coding sequence of human Slingshot-1L (IMAGE clone 6376684) and inserting it into a pMSCV-based retroviral vector. The CS version of SSH1L-GFP was generated by overlapping PCR mutagenesis and confirmed by sequencing. SSH1L-Myc was generated by subcloning the SSH1L coding sequence into a pMSCV vector containing a C-terminal Myc tag. Wild type or Serine 3 mutant Cofilin constructs were cloned in the pMSCV-based retroviral vector by PCR. Cofilin S3A and S3D mutants were generated by encoding the mutation in the PCR primers. The short hairpin RNA (shRNA) construct used to knockdown Coronin 1B (KD-1B) was generated according to the instructions found at http://www.unc.edu/~cail/biotool/2shRNA.html. The control shRNA (NS; GATCGACTTACGACGTTAT) has no exact match in the human, mouse or rat genome. The shRNA vector pLL-5.0 is a modification of the original pLentiLox 3.7 vector (Rubinson et al., 2003), in which the CMV promoter driving GFP expression was replaced with the 5' LTR promoter from pMSCV and a short multi-cloning site to facilitate lower expression levels of GFP fusion proteins for rescue or imaging. A schematic example of a construct in pLL-5.0 is shown in Fig.7A.

Imaging and lamellipodial co-localization analysis

Immunofluorescent staining and imaging were performed as previously described (Cai et al., 2005). Except where indicated, cells were imaged using spinning disk confocal microscopy. To stimulate uniform lamellipodial protrusion around the periphery of the cell, Rat2 cells were serum starved overnight and simulated with 2 µg/ml EGF for 15 minutes before fixation. To perform the lamellipodial co-localization analysis, confocal stacks were combined by wavelet-based extended depth of focus projection (Forster et al., 2004) and analyzed using a custom ImageJ macro. Briefly, an ImageJ plugin (http://www.epfl.ch/demo/edf/) was used to combine multiple images from a multichannel z-stack (4 slices for each channel with 200 nm interval) into a single in-focus RGB file, and further processed using a custom macro (http://www.unc.edu/~cail/code/channel.txt). All three immunofluorescence images were collapsed into a single image that was then converted to a binary mask by thresholding (Fig. S1F). Dilation or erosion operations were iteratively performed to create a series of contour lines both inside and outside the perimeter of the cell using a custom ImageJ macro (http://www.unc.edu/~cail/code/edge2.txt) (Fig S1D, E). Pixel intensities for each labeled antibody or GFP along the contour lines were extracted from each channel and the average intensity is plotted as a function of distance from the edge of the cell (Fig. S1G). Since each point on the graph represents the average intensity of approximately 2000 pixels, variation of signal due to fixation, staining or imaging artifacts is minimized. For cells that did not show continuous protrusion around the entire periphery of the cell, as determined by the anti-Cortactin labeling (Fig. S1), a hand drawn polygon mask was used to exclude those regions lacking peripheral Cortactin staining. Extracted pixel intensities were exported to Excel for analysis and Prism (Graph-Pad) for graphing.

Analysis of free actin filament barbed ends *in vivo*

Free actin filament barbed ends were detected in fibroblasts as described previously with slight modifications (Bryce et al., 2005). Rat2 cells were infected with lentivirus expressing shRNA specific for Coronin 1B or control shRNA (co-expressing GFP-actin as a marker of infection). Cells were plated onto glass coverslips coated with 10 µg/ml fibronectin for 4 hours. Cells were washed quickly with pre-warmed PBS, and then permeabilized and labeled with 0.4 µM AlexaFluor 568 in permeabilization buffer (20 mM HEPES, 138 mM KCl, 4 mM $MgCl_2$, 3 mM EGTA, 0.2mg/mL saponin, 1% BSA, 1 mM ATP, 3 µM phalloidin) for 30 seconds. After the labeling, the cells were fixed with 4% paraformaldehyde in Kreb's S Buffer (145 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 1.2 mM $NaH2PO_2$, 10 mM glucose, 20 mM HEPES pH 7.4 and 0.4 M sucrose) for 10 min and counterstained with AlexaFluor 647 phalloidin. AlexaFluor568-actin labeling was quantified from the pixel intensity profile around the cell periphery using the method described for Fig. S1. The upper 50% of pixel intensities were used to determine the width of the zone of free barbed ends (Fig. S2). The ratio of the barbed end intensity to phalloidin intensity along the edge was normalized and plotted as a function of distance from the cell margin (Fig S2).

Recombinant Coronin 1B protein production

Coronin 1B recombinant protein was expressed and purified either from the *Drosophila* S2 expression system (Invitrogen) or a mammalian expression system. Purified protein was quantified from absorbance at 280 nm and the predicted extinction coefficient of 65890 $M^{-1}cm^{-1}$. To produce the protein from insect cells, Coronin 1B coding sequence

was subcloned into the EcoRI and EcoRV sites in the pMT/V5-HisA vector (Invitrogen). This construct was co-transfected with pBS-Puro (Benting et al., 2000) into S2 cells by Cellfectin (Invitrogen). Cells were selected and expanded according to the manufacturer's protocol to generate a stable cell line. To purify Coronin 1B protein from S2 cells, a 100 mL culture containing $1x10^7$ cells/mL was grown in a spinner flask, and induced with 1 mM $CuSO_4$ for 24 hours. Cells were harvested and washed with phosphate-buffered saline (PBS) prior to purification. The cell pellet was resuspended in 14 mL His-Base Buffer (20 mM Tris-HCl pH 8.0, 0.5% Triton-X100, 0.5 M NaCl, 5% Glycerol, 5 mM 2-mercaptoethanol, 1 mM PMSF, 10 µg/ml 1,10-phenanthroline, 10 µg/ml aprotinin and 10 µg/ml leupeptin), followed by a brief sonication on ice for 10 sec and rotation at 4°C for 30 min. Lysates were cleared by centrifugation (SS-34 rotor, 14,000 rpm, 15 min) and the supernatant was added to His-Base buffer washed Ni-NTA Beads (Qiagen) supplemented with 20 mM imidazole. After binding at 4°C for 1 hour, the beads were washed with His-Base Buffer containing 20 mM imidazole. Protein was eluted with 220 mM imidazole and dialyzed against MKEI-50 Buffer (10 mM imidazole pH 7.0, 1 mM EGTA, 2 mM $MgCl_2$, 50 mM KCl, 0.2 mM ATP and 0.1 mM DTT). For mammalian expression, the Coronin 1B coding sequence was inserted into the multi-cloning sites of the pTT5SH8Q2 vector that contains the 8xHis and StrepTagII affinity tags (Durocher et al., 2002; Shi et al., 2005). HEK293 cells growing in Dulbecco's modified Eagle's medium (high glucose) with 5% calf serum were transfected with the construct using linear polyethylenimine (M.W. 25,000) as described previously (Durocher et al., 2002). Three days after the transfection, cells were lysed in NewHope buffer (350 mM NaCl , 5% Glycerol , 5 mM imidazole , 50 mM Tris-HCl pH 8.0, 0.5%

Triton X-100, 1 mM PMSF, 10 µg/ml 1,10-phenanthroline, 10 µg/ml aprotinin and 10 µg/ml leupeptin). Cell lysate was cleared by centrifugation and His-tagged protein was purified by Talon Beads (Clontech Laboratories). Protein was eluted with NewHope buffer containing 250 mM imidazole. Elution fractions containing Coronin 1B were pooled and further purified by StrepTactin Beads (Qiagen). The beads were washed with NewHope Buffer and MKEI-50 Buffer. Protein was eluted with MKEI-50 Buffer with 2.5 mM D-desthiobiotin, and dialyzed against MKEI-50 Buffer. To produce phosphorylated Coronin 1B, 50 nM phorbol 12-myristate 13-acetate (PMA) was used to stimulate HEK293 cells for 30 minutes prior to harvesting. Activated sodium orthovanadate (4 mM) was added to all buffers used during the purification of phospho-Coronin 1B. The phosphorylation status of this protein was further verified by both $pSer^{PKC}$ blotting and immunoprecipitation, as well as mass spectrometry analysis.

In vitro Slingshot phosphatase assay

The *in vitro* Slingshot phosphatase assay is a modification of a previously published protocol (Nagata-Ohashi et al., 2004; Niwa et al., 2002). Briefly, SSH1L-myc was immunoprecipitated from transiently transfected HEK293 cells using myc antibodies (9E10). Purified SSH1L-myc on beads was washed extensively with SSH Buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 2 mM EGTA, 2 mM $MgCl_2$, 1% NP-40, 1% Triton X-100, 10% glycerol, 1 mM PMSF, 10 µg/ml 1,10-phenanthroline, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 10 mM sodium fluoride) before use. Purified Coronin 1B was phosphorylated *in vitro* using purified PKCα as described previously (Cai et al., 2005). Coronin 1B was phosphorylated *in vivo* by 100 nM PMA stimulation for 30 minutes prior

to lysis. Cells were lysed in 1 ml SSH Buffer containing 1 µM Ro32-0432. Cleared lysate was divided into five aliquots and varying amounts of SSH1L-Myc on beads was added to each aliquot. Dephosphorylation reactions were performed at 30°C for 30 minutes. The reaction was stopped by adding 700 µl chilled RIPA containing 4 mM activated sodium orthovanadate. The SSH1L-Myc beads were spun out of the lysate and Coronin 1B was immunoprecipitated from the supernatant. Following immunoprecipitation, the unbound fraction was concentrated by chloroform/methanol precipitation. The concentrated unbound fractions, the SSH1L-Myc beads and Coronin 1B immunoprecipitates were analyzed by immunoblotting as indicated in Fig. 4D.

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Supplemental Figures

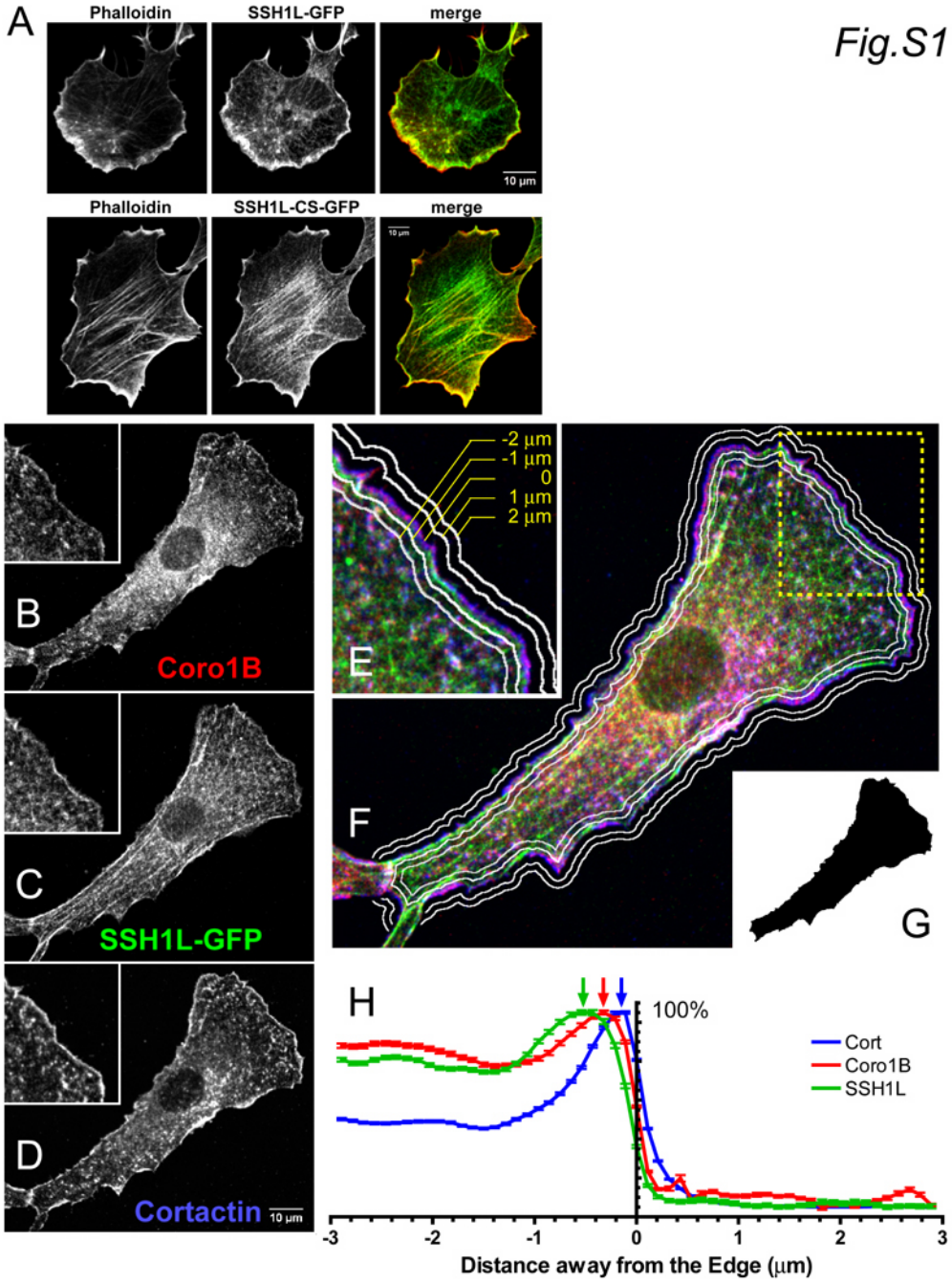


Fig.S1

Figure S1: SSH1L localizes to the back of lamellipodia
A- Rat2 fibroblasts expressing either WT SSH1L-GFP or CS SSH1L-GFP (green) were imaged for F-actin with Alexa Fluor 568-phalloidin (red).

B,C,D- Rat2 fibroblasts expressing WT SSH1L-GFP (panel B, green in D and E) were serum starved overnight and stimulated with 2 µg/ml EGF for 15 minutes. The cells were immunostained with Coronin 1B (panel A, red in D and E) and Cortactin (panel C, blue in D and E). Insets are magnified regions of the leading edge. Image resolution under these conditions (60x objective NA 1.45, wavelet-based extended depth of focus projection of confocal stacks) is ~220 nm.

E,F- Merged image of A, B and C. Interior and exterior contours drawn at different distances from the perimeter of the cell were generated using a custom macro in ImageJ (see Methods) and presented as white lines. D is a magnified inset of the framed region in panel E.

G- The binary mask, which was used in the analysis for the cell in panel E, was automatically generated by threshold using a macro in ImageJ.

H- The average pixel intensity of each labeled component along the contour lines in E is plotted as a function of distance from the edge of the cell. Error bars are standard error of the mean for each contour line at each point. Cortactin is in blue; Coronin 1B is in red and SSH1L-GFP is in green. Arrows indicate the maximum signal for each component.

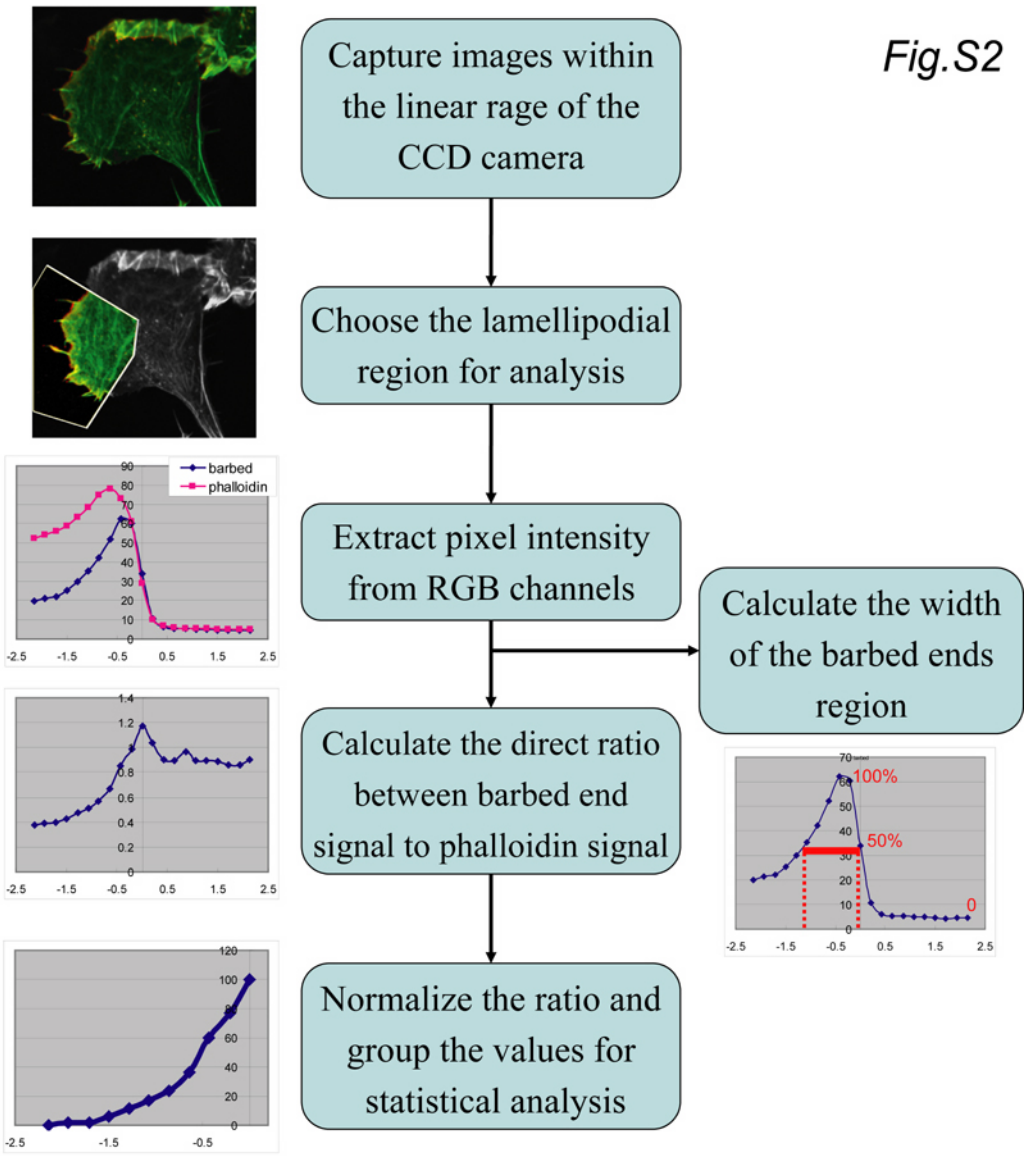


Figure S2: Barbed end assay quantification
Figure shows a flowchart of the steps involved in processing and quantifying the barbed end images used to derive the data in Figure 2D and E of the paper. Cells were plated on fibronectin coated glass coverslips for 4 hours before barbed end assay was performed (barbed ends are colored red in image). Cells were immunostained with Alexa Fluor 647-phalloidin (pseudo-colored green) to visualize F-actin distribution in the cell. Hand drawn masks (white lines) were used to limit extraction of information to lamellipodial subregions without overlap with other cells. Pixel intensities around the leading edge of the cells were extracted as described in Fig. S1 and two different channels were plotted separately using raw value, which was used to calculate the ratio, normalized and grouped for presentation in Fig. 2E. The width of the barbed end zone was calculated from the point at which 50% of the barbed end signal had decayed as indicated.

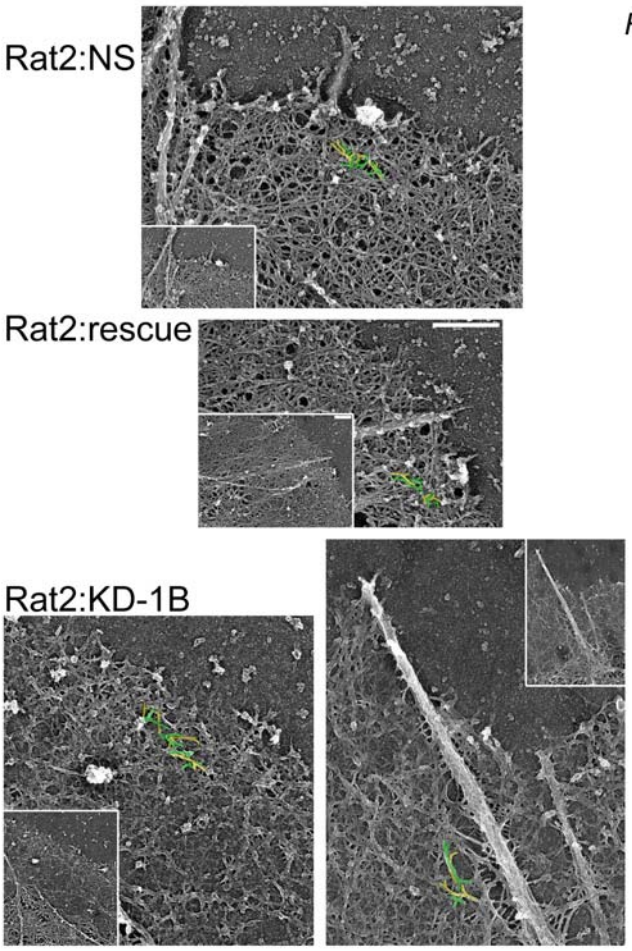
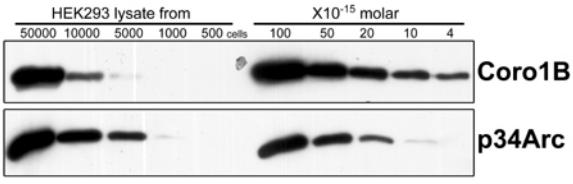


Fig.S3

Figure S3: Depletion of Coronin 1B alters actin ultra-structure at the leading edge
Platinum replica electron microscope images of lamellipodia in Rat2 fibroblasts infected with the Coronin 1B shRNA (KD-1B, without or with rescue construct expressing GFP fusion human Coronin 1B) or the control shRNA (NS). Expanded view of each cell is presented on the corner of the image. Two cells infected with Coronin 1B shRNA (KD-1B) were presented here to show the thinner architecture in the leading edge as a supplement to Fig. 2F. Some filaments are pseudo colored with yellow or green to show dendritic branch structures. White Bar = 500 nm.



Protein	Global cytoplasmic concentration (μM)	Polypeptides per cell
Coro1B	0.73	9.60E+05
p34Arc	3.5	4.60E+06

Fig.S4

Figure S4: Quantification of the concentration of Coronin 1B in HEK293 cells

Quantification of Coronin 1B and p34Arc (a subunit of the Arp2/3 complex) concentrations were determined by immunoblotting and densitometry. Only bands with signals within the linear detection range of the film were used for calculations. Dilutions of cell extract from HEK293 were blotted together with standards of purified human Coronin 1B and bovine Arp2/3. The p34Arc antibody used was raised against a peptide that is conserved between human and bovine. Average diameter of suspension HEK293 cells is 17 µm, and the average diameter of their DAPI stained nuclei is 9 µm, which were used to calculate the cytoplasm volume.

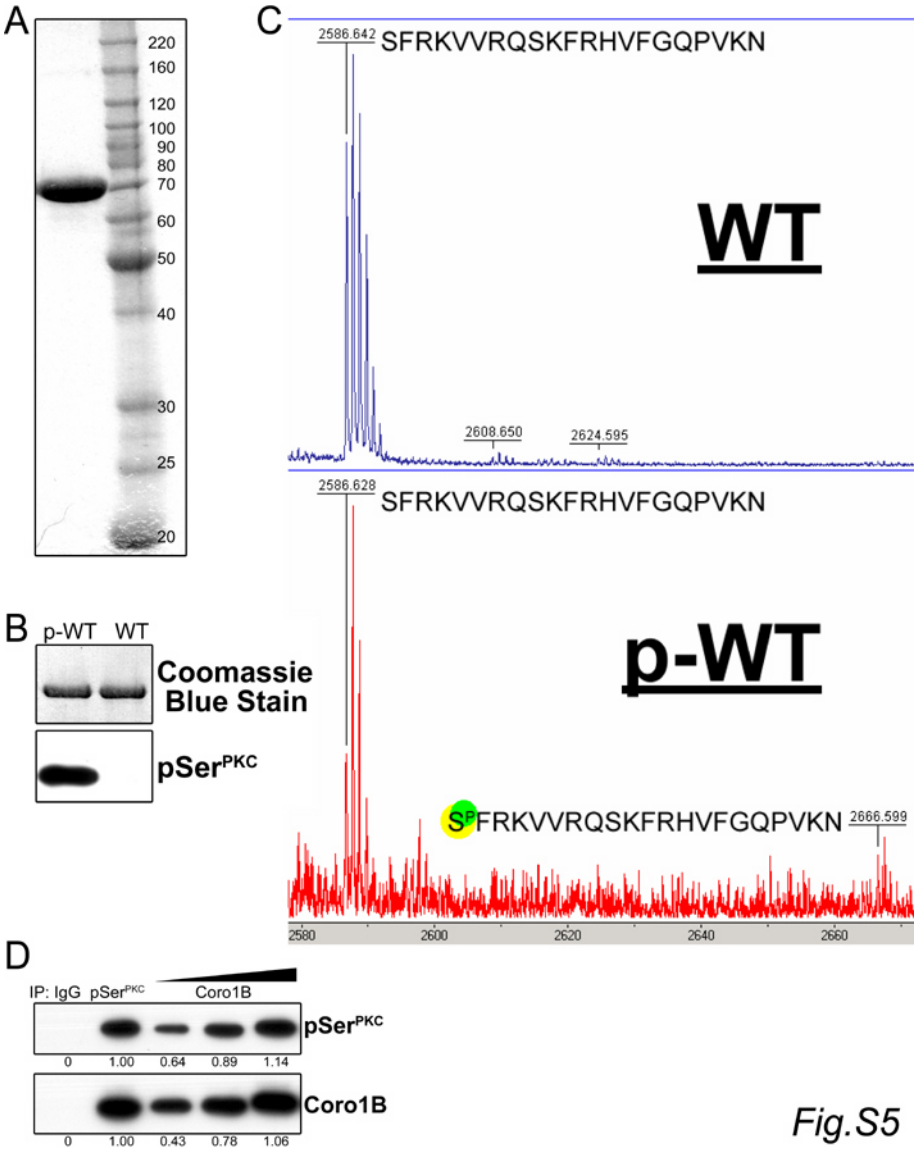


Figure S5: Protein production of wild type and phosphorylated Coronin 1B
A- 3 µg of purified Coronin 1B purifed from HEK293 cells was run on SDS-PAGE and stained with Coomassie Blue.

B- HEK293 cells over-expressing Coronin 1B were stimulated with 50 nM PMA for 30 minutes prior to protein purification. Based on our previous published work, PMA stimulation specifically promotes Serine 2 phosphorylation *in vivo* (Cai et al., 2005). Blotting with a phospho-specific antibody (pSer^{PKC}) reveals that the purified p-WT Coronin 1B protein is heavily phosphorylated.

C- 10 µg of purified wild type (WT) and phosphorylated (p-WT) Coronin 1B samples were dialyzed into water, digested with AspN overnight and subjected to mass spectrometry analysis at the UNC proteomic facility. The MS/MS spectra confirms the N terminus sequence of Coronin 1B and discovers a novel peak in p-WT sample, which matches the expected 80 Da increase for phosphorylation.

D- 1 µg of p-WT protein was immunoprecipitated with either Coronin 1B (total Coronin 1B) or pSer^{PKC} (phospho-Coronin 1B) antibodies. From pilot experiments, we determined that the Coronin 1B antibody immunoprecipitated the protein with 10-15x greater efficiency than the pSer^{PKC} antibody (not shown). Using this information, we adjusted the loads to bring the pSer^{PKC} IP'd samples into the same range as the Coro1B IP'd samples and blotted the samples with both antibodies. Using densitometry, we compared the intensity of bands to calculate the stoichiometry of phosphorylation and find that ~75% of the p-WT protein is phosphorylated.

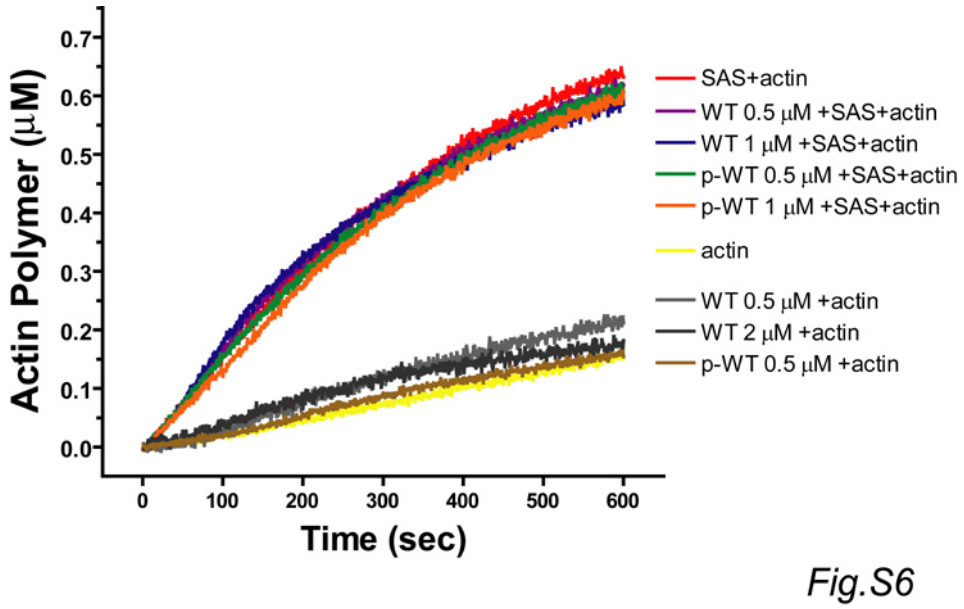


Fig.S6

Figure S6: Effects of Coronin 1B on spontaneous actin assembly and barbed end elongation induced by Spectrin-actin seeds
Plotted are actin polymer concentrations versus time in reactions containing 1 µM actin (5% pyrene labeled) with or without Spectrin-actin seeds (SAS) and Coronin 1B (wild type protein, WT; phosphorylated protein, p-WT) as indicated beside each curve: no Coronin 1B with SAS, red; 0.5 µM WT with SAS, purple; 1 µM WT with SAS, blue; 0.5 µM p-WT with SAS, green; 1 µM p-WT with SAS, orange; 0.5 µM WT, gray; 2 µM WT,

dark gray; 0.5 µM p-WT, khaki. The curve labeled actin (yellow) contained neither Coronin 1B nor SAS.

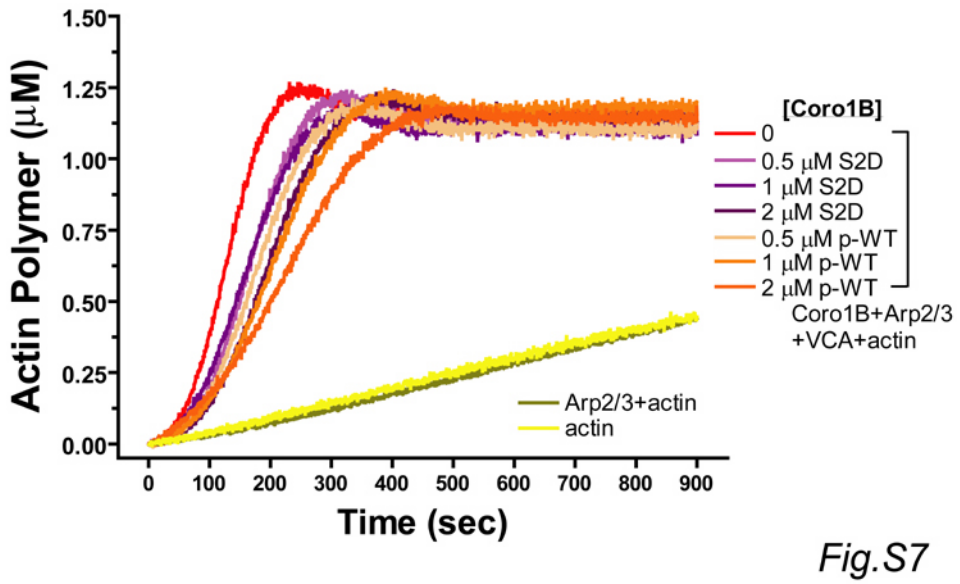


Fig.S7

Figure S7: Coronin 1B's inhibition of Arp2/3 nucleation is dependent on phosphorylation at Serine 2

These data were used to generate the dose response curve in Fig. 3B. Plotted are actin polymer concentrations versus time in reactions containing 1.5 µM actin (5% pyrene labeled), 20 nM Arp2/3, 10 nM GST-VCA, and Coronin 1B as indicated beside each curve: no Coronin 1B, red; 0.5 µM S2D, light purple; 1 µM S2D, purple; 2 µM S2D, dark purple; 0.5 µM p-WT, light orange; 1 µM p-WT, orange; 2 µM p-WT, dark orange. The curve labeled actin (yellow) and Arp2/3+actin (khaki) contained neither Coronin 1B nor GST-VCA. p-WT, wild type Coronin 1B purified from PMA stimulated cells, which has Serine 2 as the major phosphorylated site and 75% of the protein is phosphorylated; S2D, a mutant version of Coronin 1B, whose Serine 2 was mutated to Aspartic acid to mimic the phosphorylated protein.

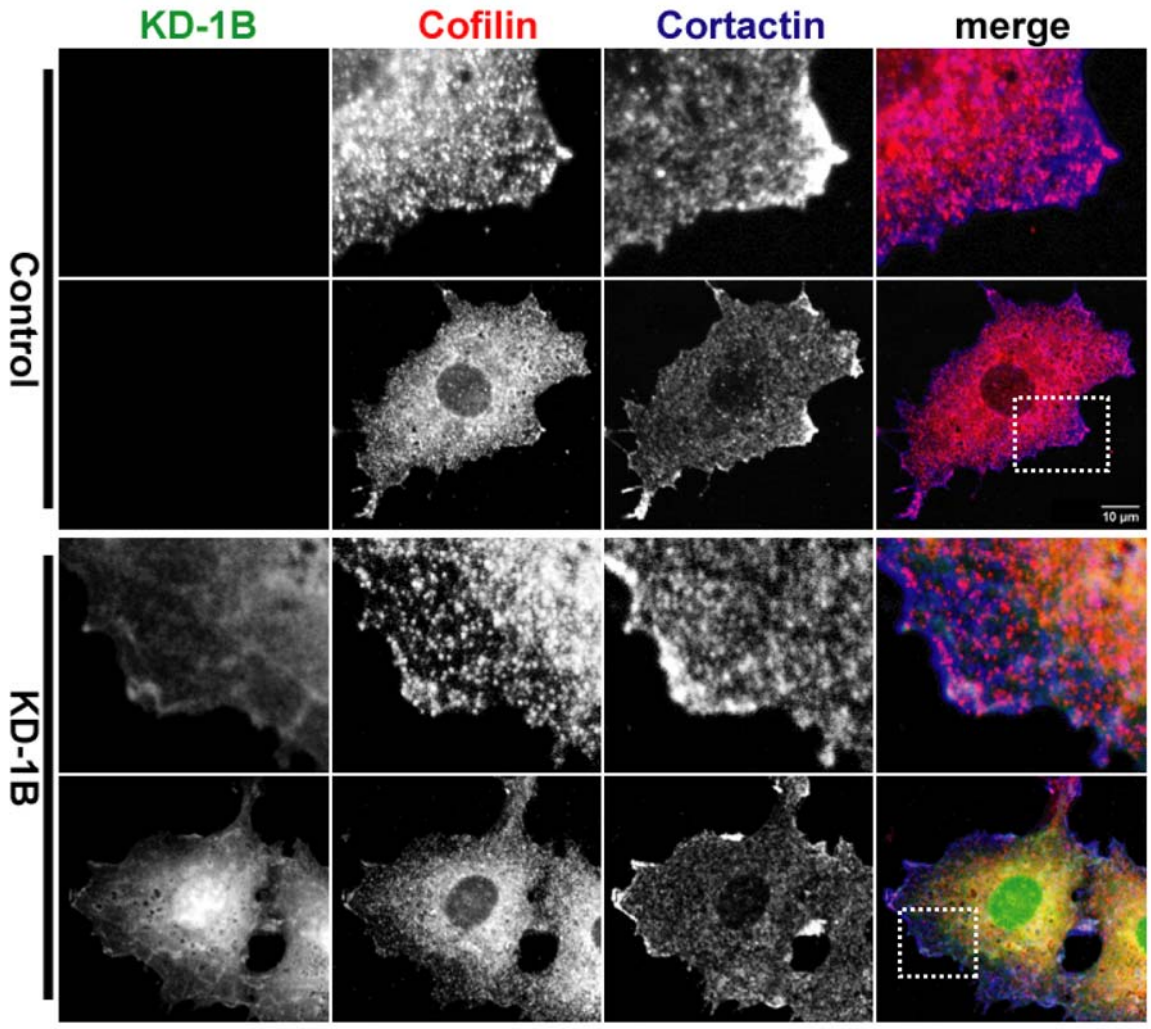


Fig.S8

Figure S8: Depletion of Coronin 1B does not alter the distribution of endogenous Cofilin and Cortactin in Rat2 fibroblasts

Rat2 fibroblasts were infected with lentivirus expressing shRNA specific for Coronin 1B co-expressing soluble GFP (labeled KD-1B in figure) and surrounding uninfected, GFP-negative cells were used as control. Cells were plated on fibronectin-coated glass coverslips for 4 hours before 4% paraformaldehyde fixation. Fixed cells were treated with methanol for 5 minutes and immunostained for Cofilin (red, polyclonal antibody from Cytoskeleton Inc.) and Cortactin (blue, 4F11 monoclonal antibody). Regions boxed by white dash lines are magnified.