Supplemental Data

Experimental Procedures

Protein Expression and Purification: A subfragment of budding yeast Las17 containing residues 529-633 was subcloned from pRL101 (1) into the pGV67 expression vector (2) to make a GST-TEV-Las17-VCA expression vector. BL21(DE3)RIL *E. coli* transformed with this vector were grown to an $O.D._{595}$ of 0.6-0.7 and induced with 0.4 mM IPTG at 22 °C overnight. GST-Las17-VCA was purified from the lysate using a glutathione sepharose affinity column, dialyzed to reduce the salt concentration to 100 mM, then loaded on a 6mL Resource Q column using a gradient of 100 to 600 mM. Peak fractions were pooled, concentrated, and diluted with a no salt buffer to give a final concentration of 20 mM Tris pH 8.0, 50 mM NaCl, and 1 mM DTT before reconcentrating and flash freezing in liquid nitrogen. Bovine and budding yeast Arp2/3 complex were purified as previously described (2,3), except that the yeast strain used was KEBY88 (Table S1). Rabbit skeletal muscle actin was purified (4) and pyrene labeled (5) as previously described. The entire coding region of budding yeast coronin was amplified from genomic DNA and cloned into p21dHT or pGV67 with BamHI and SacI restriction sites. The WD-CC (residues 1 to 410 and 594 to 651), WDU (residues 1-603), and UCC (401-651) were created by amplifying either plasmid containing coronin gene using non-overlapping 5'-phosporylated primers to mutate or delete target sequences or insert new sequences. Crn1-foldon was created using the same method using long primers that inserted the 27 amino acid bacteriophage T4 fibritin foldon trimerization motif in place of the coiled coil domain (6). The foldon domain is inserted after residue 603 in Crn1. The primer sequences were as follows:

5'-
TGCGTAAAGATGGTGAATGGGTGCTGCTGAGTACCTTTCTGTGAGAGCTCCGTCGACAAGCT;
5'-
CATATGCTTGACCATCACGAGGCGCTTCCGGGATATAACCACTACCCTTAGGTGATGTCCTT
GCCGC

Full-length, WD-CC, foldon, ALEE and AKK mutant proteins were expressed and purified as His-tagged proteins. For expression, Bl21(DE3)RIL *E. coli* transformed with a p21dHT expression vector was grown to an $O.D._{595}$ of 0.6-0.7, induced with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside, and grown overnight at 22 °C. Cells were lysed by sonication, clarified by centrifugation, and the soluble fraction was loaded on a Ni-Sepharose 6 Fast Flow column. Protein was eluted with a buffer containing 250 mM imidazole and dialyzed overnight to decrease salt concentration, loaded onto a 6mL Resource Q column at pH 8.0 and eluted with a gradient of 50 mM to 500 mM NaCl. Protein was then concentrated in an Amicon-Ultra concentration device before loading on a Superdex200 gel filtration column. The UCC, WD and WDU constructs were expressed as GST-fusions. Expression conditions were identical to the

His-tagged constructs. Protein was purified on a glutathione sepharose column eluted with 20 mM Tris pH 8.0, 140 mM NaCl and 50 mM glutathione. Peak fractions were pooled and a 25:1 ratio (by mass) of TEV protease to recombinant proteins was added. The reaction mix was dialyzed overnight against 20 mM Tris pH 8.0, 50 mM NaCl and 1mM DTT before running ResourceQ and Superdex200 gel filtration column as described for the His-tagged constructs. Wsp1-VCA and N-terminally labeled Wsp1-Rh-VCA were purified as described (2). Cysteine point mutants of N-WASp-VCA were made by amplifying N-WASp-VCA in pGV67 (7) using non-overlapping 5'-phosporylated primers and ligating the blunt ended products. Purification of N-WASp-VCA mutants was carried out as described (7).

Pyrene actin polymerization assays: A solution of 2 µL of 0.5 mM $MgCl_2$ and 2 mM EGTA was added to 20 µL of a 5x mix of 15% pyrene actin monomers in G-buffer. A mix (78 µL) containing Arp2/3 complex, coronin, GST-Las17-VCA, buffers and salts was added 2 min later to bring the final concentrations to 10 mM imadazole pH 7.0, 50 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$ and 1 mM DTT. Fluorescence measurements were made at 10 second intervals on a Tecan Safire2 plate reader or a Perkin Elmer LS-55 fluorescence spectrometer using an excitation wavelength of 365 nm and an emission wavelength of 407 nm. The maximum rate of polymer formation was determined by plotting the slope of each polymerization curve at each time point and converting RFU/s to nM actin/s assuming that the total amount of polymer at equilibrium is equal to the total concentration of actin minus 0.1 µM, the critical concentration. For high concentrations of coronin, maximum elongation rates were taken during the initial 400 s of the reaction to avoid noise due to filament bundling.

Analytical Ultracentrifugation: Sedimentation velocity experiments were carried out in a Beckman XL-I analytical ultracentrifuge. Protein samples were prepared by diluting 200-700 µM stocks into 20 mM Tris pH 8.0, 50 mM NaCl and 1 mM TCEP and loading into a two channel ultracentrifuge cell. Cells were placed in a 4-hole rotor and spun at 50,000 rpm at 20 °C and the absorbance at 280 nm continuously monitored. The resulting radial absorbance scans were fit using a non-interacting continuous c(S) distribution model (8). A confidence level of 0.95 was used to regularize the fits, and fits were considered satisfactory if the root mean square deviation was less than 0.007 and the residuals were random and less than 10% of the signal.

Fluorescence anisotropy binding assays: Arp2/3 was added to a reaction mix containing 50 nM Wsp1-Rh-VCA and 0-80 µM Crn1 in the same buffer used for the pyrenyl-actin polymerization assays. The reaction was incubated for ten minutes at room temperature before measuring the anisotropy on a Tecan

Safire2 plate reader. The excitation wavelength was 530 nm and the emission was measured at 575 nm. Data were fit to competition binding isotherms using the following equation (9) (10):

$$A(c) = A_F + \frac{(0.5)A_B}{[L_0]}\left\{\left[\left(\frac{K_D[C]}{K_C}\right) + K_D + [L_0] + [P_0]\right] - \sqrt{\left(\frac{K_D[C]}{K_C} + K_D + [L_0] + [P_0]\right)^2 - 4[L_0][P_0]}\right\}$$

Actin monomer binding assays: Actin (1.0 μM) in buffer G (2 mM Tris pH 8.0, 1 mM DTT, 0.2 mM ATP, 0.1 mM CaCl2) was incubated with 10 μM GST-Crn1 or GST-N-WASp-VCA bound to glutathione sepharose beads or with beads alone. Samples were pelleted and equal volumes of supernatant or resuspended beads were analyzed by SDS-PAGE and western blotting with an anti-actin antibody.

Actin filament binding assays: Binding of coronin to filamentous actin was measured using copelleting assays. For full-length, WD-CC, and foldon constructs, actin was added to a solution containing salts and buffers to bring the final concentrations to 4 μM actin, 10 mM imadazole pH 7.0, 50 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$ and 1 mM DTT. The reaction was allowed to proceed for one hour, then phalloidin was added from a 100 μM stock to bring the final concentration to 5 μM. The appropriate amount of this F-actin stock was added to make a 100 μL reaction mix containing the same buffer plus coronin and 1 mg/mL bovine serum albumin to reduce non-specific interactions. Reactions were incubated at 23 °C for 15 min before spinning in a TLA100 rotor at 80,000 rpm. Pellet and supernatant fractions were analyzed by SDS-PAGE followed by western blotting with an anti-his antibody (Santa Cruz Biotechnologies). For the WD(1-410) construct, essentially the same procedure was used, except that no phalloidin was added and the supernatant and pellet were analyzed by coomassie brilliant blue staining. A similar procedure was followed for the Arp2/3 recruitment assays, except that Crn1, F-actin and Arp2/3 complex were incubated for 30 min before pelleting by high speed centrifugation. Binding isotherms for the reactions without Arp2/3 complex were fit using the following equation:

$$f = \left(\frac{(K_d + [A] + [C]) - \sqrt{(K_d + [A] + [C])^2 - 4[A][C]}}{2[C]} \right)$$

Where f is the fraction bound, A and C are total actin and coronin concentrations, respectively, and K_d is the binding affinity.

Crosslinking VCA to Arp2/3 complex: Cysteine mutants of N-WASp-VCA were reduced with 5 mM tris(2-carboxyethyl)phosphine for 30 min at 22 °C. The reducing agent was then removed by applying the sample to a Biospin6 column (Biorad) and eluting in 20 mM Hepes pH 7.0, 50 mM NaCl and 1 mM

EDTA. A three-fold molar excess of benzophenone-4-maleimide (B4M) in dimethylsulfoxide was added dropwise with stirring to a 100 μM solution of VCA on ice. The reaction was stopped after 2 hours by adding dithiothreitol (DTT) to 2 mM, concentrated in a Vivaspin500 protein concentrator (Pierce) and the labeled peptide was exchanged into 20 mM Hepes pH 7.0, 50 mM NaCl and 1 mM DTT. Labeling was verified by MALDI-TOF mass spectrometry.

Crosslinking reactions were carried out by mixing bovine Arp2/3 complex and VCA-B4M adducts with or without Crn1 in 10 mM imadazole pH 7.0, 50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA and 1 mM DTT in 1.5 mL plastic reaction tubes and exposing on a transilluminator equipped with 350 nm bulbs for 10 min on ice. Reactions were separated by SDS-PAGE and stained with coomassie brilliant blue or transferred to PVDF membranes and blotted with anti-Arp2 or anti-Arp3 antibodies (Santa Cruz Biotechnologies). ImageJ was used to quantify gel bands.

Preparing *S. cerevisiae* strains: *CRN1* plus flanking 5' and 3' sequences were knocked of the KEBY88 strain (a gift from Tom Stevens) with the KanMX6 marker using PCR-based homologous recombination (11). CRN1 plus 154 upstream and 211 downstream nucleotides was amplified from genomic DNA and cloned into pRS405. The resulting vector was digested with AflII and integrated into the *leu2-3,112* locus in the knockout strain. Abp1 was C-terminally GFP-tagged by homologous recombination using a HIS3 marker cassette. For each strain, we used diagnostic PCR to confirm the integrations had occurred and that a single CRN sequence had been inserted in the *leu2-3,112* locus. The actin patch dynamics of each mutant were compared to the control strain ("WT" in table S1) in which CRN1 is knocked out and reintegrated into the LEU locus.

Confocal microscopy and image analysis: Cells were grown for at least 24 hours at $O.D._{595}$ below 1.5 in YEPD medium at 30 °C, harvested by centrifugation at 350 g and washed twice in SD broth. The resuspended pellet was transferred to 25 % gelatin, 0.02 mg/mL NPG pads made with SD, covered with a No. 1 coverslip, and sealed with VALAP (equal parts Vaseline, lanolin, and paraffin). Movies of Abp1-GFP fluorescence in live *S. cerevisiae* cells were made using a Nikon Eclipse TE2000-U microscope equipped with an argon laser, a Yokogawa CSU10 spinning-disc head (Wallac), and a charge coupled device (CCD) camera (ORCA-AG, Hamamatsu). Movies were collected at 5 frames/s for 45 s (225 frames total) at 23 °C, using the open source software package Micromanager (http://www.micro-manager.org). Images were adjusted for optimal contrast and identification of patches was automated using previously described software (12). Tracks of individual patches were selected and characterized using a plugin for ImageJ (National Institute of Health). Patches that did not meet the following criteria were discarded: (1) The patch originated at the cortex (2) The patch moved away from the cortex (3) The

patch was observed for at least 6 s (30 frames) (4) Birth and death of the patch was observed within the time course of the movie (5) The patch could be readily distinguished from other patches. The lifetimes of each phase of patch motion were compared for each genotype. The patch start and end times were determined as the first and last frames, respectively, in which the patch could be distinguished from the background. To determine the length of the assembly and mobile phases, we plotted mean squared displacement from the patch origin as a function of time for each patch, as previously described. We then defined the inflection point in this plot as the last frame at which no patch motion was observed (13). The assembly phase lifetime was set as the time difference between the inflection point and the start frame plus the duration of a single frame. The moving phase lifetime was set as the time difference between the end frame and the inflection frame. The total patch lifetime was set as the time difference between the end frame and the first frame plus the duration of a single frame.

References

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Figure Legends

Fig. S1. Low concentrations of Crn1 decrease the actin polymerization rate in the absence of Arp2/3 complex. Plot shows a time course of 2 μM 15% pyrene actin polymerization with 0-100 nM Crn1. Conditions: 10 mM imidazole, pH 7.0, 50 mM KCl, 1 mM MgCl2, 0.2 mM ATP, 1 mM DTT.

Fig. S2. Crn1 is synergistic with GST-Las17-VCA. Plot of maximum polymerization rate versus activator concentration for reactions containing 25 nM Arp2/3 complex, 2 μM 15 % pyrene actin, and either ratios of GST-Las17-VCA and Crn1 totaling 60 nM or GST-Las17-VCA alone.

Fig. S3. ScCrn1-WDU and Crn1-UCC do not activate but weakly inhibit Arp2/3 complex. Plots shows time courses of 2 μM 15% pyrene actin polymerization with 20 nM Arp2/3 complex, and 40 nM GST-Las17-VCA and either 0-3 μM Crn1-WDU (A) or 0-50 μM Crn1-UCC (B). We note that the inhibition at extremely high Crn1-UCC concentrations may be due to competition with VCA for binding to Arp2/3 complex.

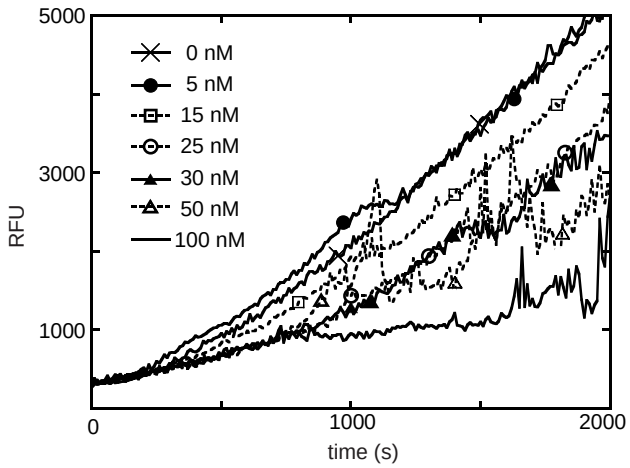
Fig. S4. Modeling an actin filament with both Arp2/3 complex and Crn1. *A.* Model of Coronin1-A (purple ribbons) bound to an actin filament (grey ribbons) based on three dimensional averaging of negative stained filaments saturated with coronin (14). The WD repeat domain of Coronin1-A binds in the seam between two protofilaments, contacting three adjacent actin subunits. *B.* Model of Arp2/3 complex bound to a mother filament of actin based on a three dimensional reconstruction of the electron microscopy images of branch junctions (15). The Arp2/3 complex wraps around the seam between the protofilaments and packs closely against two subunits from one protofilament and loosely against three additional subunits in the second protofilament . Arp3, orange; Arp2, red; ARPC1, green; ARPC2, cyan; ARPC3, magenta; ARPC4, blue; ARPC5, yellow. *C.* Model of Arp2/3 complex and coronin simultaneously bound to an actin filament. Actin subunits from the models in (A) and (B) were overlaid to make this model. The complex clashes with two individual coronin molecules. Arp3 (subdomains 3,4) and ARPC3 clash with one coronin and Arp3 (subdomains 1,2) and ARPC2 clash with another.

Fig. S5. Crn1 activates *Bos taurus* (Bt) Arp2/3 complex. Time course of 2 μM 15% pyrene actin polymerization showing the effect of 0 – 3.0 μM full-length Crn1 (full-length) on nucleation by 10 nM BtArp2/3 complex and 25 nM GST-N-WASp-VCA.

Table S1: Strains used in this study.

Strain	***Alias***	***Genotype***	***Source***
	KEBY88	*MATα ura3-52 leu2-3, 112 his3-Δ200 trp1-901 lys2-801 suc2-Δ9 pep4–3*	17
wt	ScBN056	*MATα ura3-52 leu2-3, 112 his3-Δ200 trp1-901 lys2-801 suc2-Δ9 pep4–3 ΔCrn1::KanMX6 ScCrn1_wt::LEU2 ABP1:: GFP(S65T)-HIS3MX6*	This study
crn1Δ	ScBN059	*MATα ura3-52 leu2-3, 112 his3-Δ200 trp1-901 lys2-801 suc2-Δ9 pep4-3 ΔCrn1::KanMX6 ScCrn1_WD+CC::LEU2 ABP1:: GFP(S65T)-HIS3MX6*	This study
crn1-wd-cc	ScBN063	*MATα ura3-52 leu2-3, 112 his3-Δ200 trp1-901 lys2-801 suc2-Δ9 pep4-3 ΔCrn1::KanMX6 ABP1:: GFP(S65T)-HIS3MX6*	This study
crn1-akk	ScBN065	*MATα ura3-52 leu2-3 ,112 his3-Δ200 trp1-901 lys2-801 suc2-Δ9 pep4-3 ΔCrn1::KanMX6 ABP1::GFP(S65T)-HIS3MX6 ScCrn1_503WEE_AKK::LEU2*	This study
crn1-alee	ScBN066	*MATα ura3-52 leu2-3, 112 his3-Δ200 trp1-901 lys2-801 suc2-Δ9 pep4-3 ΔCrn1::KanMX6 ABP1::GFP(S65T)-HIS3MX6 ScCrn1_480LLKK_ALEE::LEU2*	This study

SFig. 1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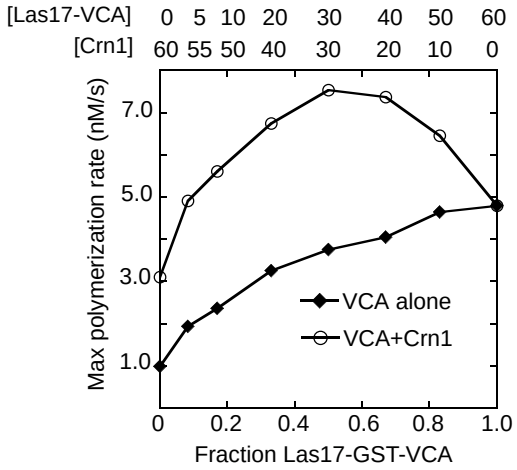
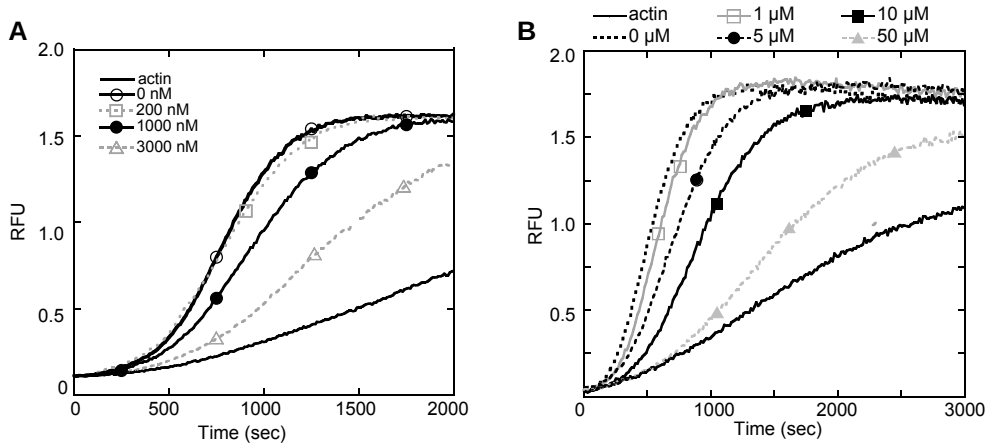
3000
1000
RFU
0
1000
2000
time (s)
0 nM
5 nM
15 nM
25 nM
30 nM
50 nM
100 nM

SFig. 2

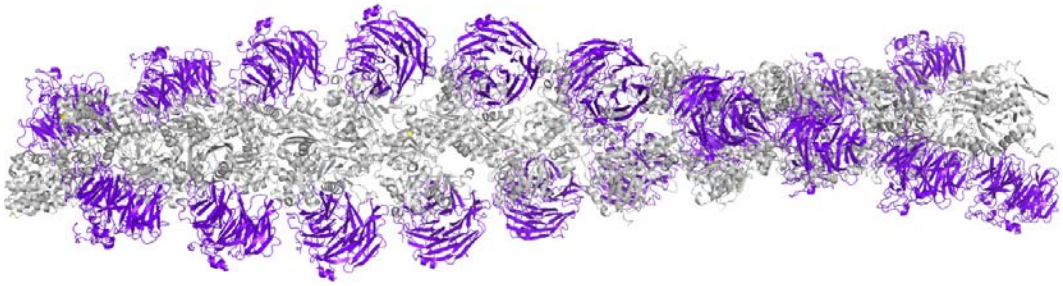

[Las17-VCA] 0 5 10 20 30 40 50 60
[Crn1] 60 55 50 40 30 20 10 0
Max polymerization rate (nM/s)
7.0
5.0
3.0
1.0
VCA alone
VCA+Crn1
0
0.2
0.4
0.6
0.8
1.0
Fraction Las17-GST-VCA

SFig. 3


A
actin
0 nM
200 nM
1000 nM
3000 nM
RFU
Time (sec)
B
actin
0 μM
1 μM
5 μM
10 μM
50 μM
RFU
Time (sec)

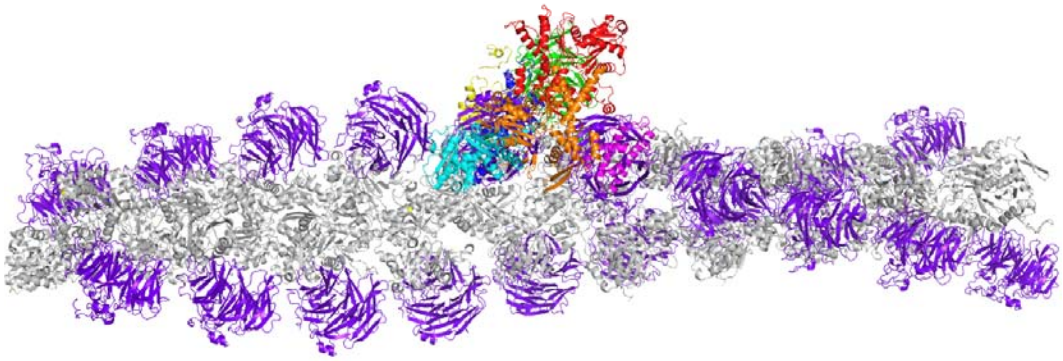
SFig. 4

A

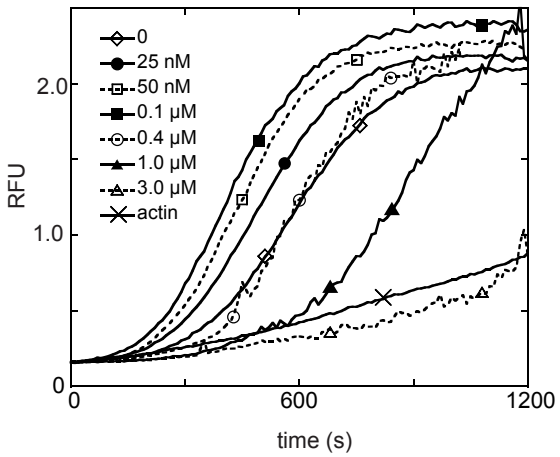


B

C



SFig. 5


0
25 nM
50 nM
0.1 μM
0.4 μM
1.0 μM
3.0 μM
actin
RFU
2.0
1.0
0
0
600
1200
time (s)