Inventory of Supplemental Information:

Supplemental Data: Figure S1, related to Figure 1; Figure S2, related to Figure 2; Figure S3, related to Table 1; Table S1, related to Figure 2; Table S2, related to Figure 3; Movie S1, related to Figure 4. Supplemental Experimental Procedures. Supplemental References.

Supplemental Data

Supplemental Figures

Figure S1. Native WAVE2 and WAVE1 complexes are basally inactive and are not activated by Rac or Nck

(A-B) and (D-G) Actin polymerization assays containing 1 μM actin/pyrene-actin and 30 nM Arp2/3 complex.

(A) The activity of 0.25 nM recombinant WAVE2 protein can be detected in actin polymerization assays. The indicated concentrations of isolated recombinant WAVE2 protein ("rWAVE2") were tested.

(B) Recombinant WAVE2 protein loses activity when heated to 60°C. Isolated recombinant WAVE2 protein was heated for 10 min at the indicated temperatures, cooled down to room temperature and 16 nM was added to the assay.

(C) PAK pull-down assays testing effector binding for GDP-bound wild type ("WT") and GTP-bound constitutively active ("CA"), unprenylated ("u") and prenylated ("p") Rac1 and Rac2. Pull-down assays with the p21-binding domain of PAK fused to GST ("GST-PBD") were done as described in Supplemental Experimental Procedures, and the input ("I"), beads ("B") and supernatant ("S") were analyzed by Western blot for Rac. Assays excluding GST-PBD demonstrate that binding of constitutively active Rac is specific.

(D) Nck protein used in this study is functional, as it can activate recombinant N-WASP synergistically with PIP_2 liposomes. Actin polymerization induced by 100 nM recombinant N-WASP was tested in the absence of activators or upon addition of either 10 μM (total lipid) liposomes composed of 45% (molar fraction) PC, 45% PI and 10% $PI(4,5)P_2$ ("PIP_2"), 200 nM Nck, or both.

(E) Native WAVE1 complex from bovine brain is not activated by Rac alone, even at 7.5 μM. Where indicated, 50 nM native WAVE1 complex ("WAVE1c") was tested in the absence of agonists or with 7.5 μM unprenylated Rac1-GMPPNP ("uRac1-GMPPNP"). A reaction where 10 nM native WAVE1 complex was heated to 57 °C prior to being assayed demonstrates that the complex contains WAVE1 protein capable of activity.

(F) Native WAVE2 complex from EGF stimulated A-431 cells becomes activated during elution with 0.5 M KCl. Immuno-purified WAVE2 complexes were eluted in control elution buffer ("control") or in elution buffer containing 0.5 M KCl as described in Supplemental Experimental Procedures, and 20 nM was tested.

(G) Native WAVE2 complex from EGF stimulated A-431 cells becomes activated during prolonged storage on ice. Immuno-purified WAVE2 complex was eluted as described in Supplemental Experimental Procedures and kept on ice. 5 nM WAVE2 complex was tested in pyrene-actin assays 7 or 10 days after elution.

Figure S2. Prenylated Rac-GTP and acidic phospholipids activate the WAVE2 complex cooperatively

(A-B, E-F) Actin polymerization assays containing 30 nM Arp2/3 complex and either 1 μM actin/pyrene-actin (A, E-F) or 2 μM actin/pyrene-actin (B).

(A) Rac1 and PIP_3 liposomes do not affect the activity of uncomplexed WAVE2 protein. 1.25 nM isolated recombinant WAVE2 protein ("rWAVE2") was tested without further additions or with 50 nM prenylated Rac1-GTP ("pRac1-GTP") and 10 μM PIP_3 liposomes ("PIP_3").
(B) α-FL-WAVE2 polyclonal antibody inhibits the activity of recombinant WAVE2 protein, but not of N-WASP. The activity of either 10 nM recombinant WAVE2 protein ("rWAVE2") or 10 nM recombinant N-WASP activated by 100 nM prenylated Cdc42-GTPγS and 10 μM liposomes composed of 48% PC, 48% PI and 4% PIP_3 ("N-WASP act.") was measured without further additions or in the presence of 0.1 mg/ml of either pre-immune IgG or α-FL-WAVE2 polyclonal IgG.
(C) Activation of WAVE2 complex by prenylated Rac1-GTP and PIP_3 liposomes is inhibited by α-FL-WAVE2 and depends on the Arp2/3 complex. All assays contain 1 μM actin/pyrene-actin, and where indicated, 30 nM Arp2/3 complex, 5 nM WAVE2 complex ("WAVE2c"), 50 nM prenylated Rac1-GTP ("pRac1-GTP"), 10 μM PIP_3 liposomes ("PIP_3") and 0.1 mg/ml α-FL-WAVE2 polyclonal IgG.
(D) Native WAVE2 complex immuno-purified from the *membranes* of EGF stimulated A-431 cells, resolved on a 4-12% Bis-Tris polyacrylamide gel and stained with Coomassie. The main constituent subunits, identified by mass spectrometry, are indicated.
(E) WAVE2 complex purified from *membranes* of EGF stimulated A-431 cells is basally inactive and can be activated by prenylated Rac1-GTP and PIP_3 liposomes. Assays were performed exactly as for WAVE2 complex purified from the cytosol, shown in Figure 2B.
(F) Native WAVE1 complex purified by conventional chromatography from bovine brain is not activated by prenylated Rac1-GTP and acidic phospholipids. Where indicated, 10 nM WAVE1 complex was tested in the absence of agonists or with 12.5 nM prenylated Rac1-GTP and 10 μM PIP_3 liposomes composed of 30% PIP_3, 45% PC and 25% PI. The activity of 10 nM complex heated to 57°C is shown for reference.

Figure S3. Prenylated Rac is efficiently incorporated into liposomes regardless of nucleotide state or lipid composition
(A) Liposome co-sedimentation assays like the ones described in Table 1 were analyzed by Western blot for Rac. Sequential two-fold dilutions of the input, supernatant and pellet were loaded (the sequential dilutions of the pellet are 2x concentrated relative to the input and supernatant).

Supplemental Tables

Table S1. Composition of native WAVE2 complex immuno-purified from the cytosol of EGF stimulated A-431 human epidermoid carcinoma cells

Gel band Figure 2A	UniProtKB / Swiss-Prot	Protein name	Alternative names	# of peptides	Predicted MW (Da)
Pir121 / Sra-1	Q96F07 and Q7L576 †	p53-inducible protein 121 (Pir121) and Specifically Rac1-associated protein 1 (Sra-1)	Cytoplasmic FMR1-interacting protein 2 (CYFIP2) and Cytoplasmic FMR1-interacting protein 1 (CYFIP1)	123	148,399 and 145,182
Nap 1	Q9Y2A7	Nck-associated protein 1 (Nap 1)	p125Nap1; Membrane-associated protein HEM-2	93	128,790
Sra-1*	Q7L576-2	Specifically Rac1-associated protein 1 (Sra-1) (Isoform 2)	Cytoplasmic FMR1-interacting protein 1 (CYFIP1) (Isoform 2)	8	94,467
WAVE2	Q9Y6W5	Protein WAVE-2	Wiskott-Aldrich syndrome protein family member 2	29	54,284
Abi-1 / Abi-2 + IRSp53	Q8IZP0 and Q9NYB9 †	Abl interactor 1 (Abi-1) and Abl interactor 2 (Abi-2)	Abelson interactor 1; e3B1 and Abelson interactor 2	37	55,081 and 55,663
	Q9UQB8	Insulin receptor substrate p53 (IRSp53)	Brain-specific angiogenesis inhibitor 1-associated protein 2	33	60,868
Actin	P60709	Beta-actin	Actin, cytoplasmic 1	9	41,737
WP1x3 (peptide) + HSPC300	Q8WUW1	HSPC300	Probable protein BRICK1	7	8,745

The table lists the most abundant proteins identified by mass spectrometry in each gel band of the WAVE2 complex shown in Figure 2A. The list is not comprehensive.

†In the gel band labeled Pir121/Sra-1, peptides matching both proteins were identified in the sample, and although some peptides matched amino acid sequences specific to only one of these two related proteins, others matched sequences present in both. We therefore list both proteins together followed by the total number of peptides that match either one or both sequences. The same is true for Abi-1 and Abi-2 in the gel band labeled Abi-1/Abi-2 + IRSp53.

*This band appears to be Sra-1 isoform 2 because its apparent molecular weight matches the predicted molecular weight of isoform 2, and all 8 peptides identified match the sequence of isoform 2.

Table S2. WAVE2, Abi-1 and Abi-2 contain multiple phosphorylated sites in both serum starved and EGF stimulated conditions

Sample (gel band in Figure 3B)	Phosphorylated peptide	Phosphorylated residue
WAVE2, serum starved A-431	R**SST**IQDQKLF	Serine 102 or Serine 103 or Threonine 104
	DSAS**S**PSPSFSE	Serine 257
	DSASSP**S**PSFSE	Serine 259
	SSVV**S**PSHPPPAPPLG**S**PPGPK	Serine 296 and Serine 308
	SSVVSP**S**HPPPAPPLGSPPGPK	Serine 298
	SSVVSPSHPPPAPPLG**S**PPGPK	Serine 308
	DEDDW**S**D	Serine 497
WAVE2, EGF stimulated A-431	R**SST**IQDQKLF	Serine 102 or Serine 103 or Threonine 104
	D**T**PPPLNNLTPYR	Threonine 129
	DSAS**S**PSPSFSE	Serine 257
	DSASSP**S**PSFSE	Serine 259
	SSVVSP**S**HPPPAPPLGSPPGPK	Serine 298
	SSVVSPSHPPPAPPLG**S**PPGPK	Serine 308
Abi-1, serum starved A-431	TNPPTQKPP**S**PPMSGR	Serine 183
	TLEPVKPPTVPNDYMT**S**PAR	Serine 216
	LGSQH**S**PGR	Serine 225
	D**S**P**T**PPPPPPP	Serine 392 or Threonine 394
	DD**S**PPPPPPPPVDYE	Serine 410
Abi-1, EGF stimulated A-431	TNPPTQKPP**S**PPMSGR	Serine 183
	TLEPVKPPTVPNDYMT**S**PAR	Serine 216
	LGSQH**S**PGR	Serine 225
	D**S**P**T**PPPPPPP	Serine 392 or Threonine 394
	DD**S**PPPPPPPPVDYE	Serine 410
Abi-2, serum starved A-431	TTPPTQKPP**S**PPMSGK	Serine 183
	NMAPSQQ**S**PVR	Serine 227
	HTPPTIGG**S**LPYR	Serine 368
Abi-2, EGF stimulated A-431	TTPPTQKPP**S**PPMSGK	Serine 183
	GTLGRH**S**PYR	Serine 196
	NMAPSQQ**S**PVR	Serine 227

WAVE2 complexes immuno-purified from serum starved or EGF stimulated A-431 cells were analyzed by mass spectrometry as described in Supplemental Experimental Procedures. The table lists phosphorylated peptides identified for each protein under each condition. Residues in red were confidently assigned as the phosphorylated sites based on fragmentation data, and the residue number for the human protein (UniProtKB/Swiss-Prot Q9Y6W5 for WAVE2, Q8IZP0 isoform 1 for Abi-1 and Q9NYB9 isoform 1 for Abi-2) is indicated in the right column. Residues in blue could not be assigned unambiguously based on fragmentation data, and the residue number for possible phosphorylated sites is indicated in the right column (note: only one of the residues indicated in blue is phosphorylated in each peptide).

Supplemental Experimental Procedures

Commercial and previously published antibodies

The following commercial antibodies were used for Western blot analysis during conventional purification of WAVE2 and WAVE1 complexes: WAVE2 (C-14) goat polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, California; sc-10394); WAVE1 (L-19) goat polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, California; sc-10388). The following antibodies were used for Western blot analysis during PAK pull-down assays (Figure S1C) and liposome sedimentation assays (Figures 4B, 4C, S3A and Table 1): Anti-Rac1 purified mouse monoclonal antibody (BD Transduction Laboratories, BD Biosciences, San Jose, California; Cat. # 610650) recognizes both Rac1 and Rac2; Anti-Abi-1 (clone 1B9) mouse monoclonal antibody, purified IgG (MBL international Corporation, Woburn, Massachusetts; Code D147-3); Anti-p125Nap1 rabbit polyclonal IgG (Upstate, Lake Placid, New York; Cat. # 07-515); Anti-PIR121-1/Sra-1 rabbit polyclonal IgG (Upstate, Lake Placid, New York, Cat. # 07-531); Arp2 (H-84) rabbit polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, California; sc-15389); affinity purified anti-HSPC300 polyclonal antibody, described previously (Gautreau et al., 2004).

Preparation of new antibodies

For Western blot analysis of WAVE2 during liposome sedimentation assays (Figures 4B, 4C and Table 1) and for inhibition of WAVE2 activity (Figures S2B and S2C) we used polyclonal antibodies raised against human recombinant full length WAVE2 protein (α-FL-WAVE2). Recombinant WAVE2 protein produced in 293F cells (described below) was used to immunize rabbits (Cocalico Biologicals, Reamstown, Pennsylvania) and total IgG was purified from the antiserum on Affiprep Protein A (BioRad) using established protocols (Harlow and Lane, 1999). Total IgG purified from pre-immune serum did not inhibit WAVE2 activity (Figure S2B).

For immuno-purification of native WAVE2 complex, polyclonal antibodies were raised against previously described (Gautreau et al., 2004) WAVE2 peptide 1 (WP1) with amino acid sequence (C)NQRGSGLAGPKRSS (synthesized at the Tufts University Core Facility, Boston, Massachusetts). This sequence, excluding the N-terminal cysteine that was added for conjugation, is present in WAVE2 but absent from WAVE1, WAVE3, WASP and N-WASP, making the resulting antibodies specific for WAVE2 (data not shown). Rabbits were immunized with keyhole limpet hemocyanin (KLH) conjugated WP1 (Cocalico Biologicals, Reamstown, Pennsylvania) and specific α-WP1 polyclonal antibodies were affinity purified from the antiserum on WP1 coupled SulfoLink gel (Pierce Biotechnology) according to established protocols (Harlow and Lane, 1999). α-WP1 IgG was eluted with 100 mM glycine (pH 2.5 at 24°C) / 150 mM NaCl, neutralized with 0.05 volumes 1.5 M Tris (pH 8.8 at 24°C), concentrated in Centricon Plus-80 centrifugal filter devices (10,000 MWCO; Millipore), dialyzed through 10,000 MWCO membrane against phosphate buffered saline containing 50% glycerol, and stored at -20°C. A peptide consisting of three tandem repeats of the WP1 sequence, WP1x3, was synthesized (Sigma-Genosys) for elution of bound WAVE2 complex during immuno-purification.

Conventional purification of native WAVE2 complex from pig leukocytes

Pig leukocyte cytoplasmic extract was prepared as previously described (Weiner et al., 2006), with some modifications. Containers were either sterile or washed without detergents and autoclaved to prevent activation of neutrophils. Pig blood was obtained from Blood Farm (Groton, Massachusetts).

Approximately 20 l of fresh blood were collected into polypropylene jugs containing 157.5 ml of sterile ACD anticoagulant (80 mM sodium citrate, 15 mM sodium dihydrogen phosphate [NaH_2PO_4*$2H_2O$], 160 mM glucose, 17 mM citric acid, and 2 mM adenine) per liter of blood. Blood was transported to the laboratory at room temperature. At the laboratory all steps of the leukocyte isolation procedure were carried out at room temperature except where noted. Blood was poured into polypropylene containers and 190 ml of 154 mM NaCl / 3% polyvinylpyrrolidone (MW 360,000) were added per liter of blood plus anticoagulant. After mixing thoroughly, blood was left undisturbed for 30 to 60 min to allow cells to separate into two phases. The pale upper phase, enriched in leukocytes, was carefully decanted avoiding the deep red lower phase containing erythrocytes. Cells were centrifuged at 1,500xg (max) for 8 min at 15°C in a swinging bucket rotor, resuspended in a small volume of calcium-free mHBSS (20 mM Hepes [pH 7.2 at 24°C], 150 mM NaCl, 4mM KCl, 1 mM $MgCl_2$, 10 mM glucose) and pooled. Cells were centrifuged and resuspended in one cell volume of calcium-free mHBSS. Contaminating erythrocytes were lysed by osmotic shock: 20 cell volumes of ddH_2O were added and mixed gently for 20 s, followed immediately by addition of 2.2 cell volumes of 10x calcium-free mHBSS to regain an isotonic solution. Cells were centrifuged and washed twice by resuspension in calcium-free mHBSS and centrifugation. The osmotic shock and washes were repeated, and any large aggregates of cell were discarded during the last wash by allowing them to settle and transferring the remaining cells to another container before centrifugation. Typically 30-50 ml of packed leukocytes were obtained from 20 l of blood.

After this point, all procedures were carried out at 4°C or on ice. To inactivate serine proteases before lysis, cells were resuspended in 5 cell volumes cold calcium-free mHBSS, and the membrane permeable protease inhibitor diisopropylfluorophosphate was added to a final concentration of 3 mM. Cells were incubated on ice for 30 min, mixing occasionally. Cells were centrifuged and resuspended in 4 cell volumes of cold lysis buffer (20 mM HEPES [pH 7.2 at 4°C], 100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 2 mM PMSF, 10 μg/ml each leupeptin, pepstatin A and chymostatin, Complete EDTA-free protease inhibitor cocktail tablets [Roche Applied Science, Indianapolis, Indiana; 1 tablet per 25 ml of buffer], 1 mM DTT). Cells were lysed by cavitation in a Parr cell disruption bomb (Parr Instrument Company, Moline, Illinois) as follows: cells were pressurized with nitrogen to 350 psi for 25 min while stirring, and released slowly (drop-wise to a small stream) into a sterile container. After successful disruption most cells had lysed but most nuclei remained intact. Immediately after disruption, EGTA was added to a final concentration of 2mM. Disrupted cells were centrifuged at 900xg (max) for 10 min in a swinging bucket rotor, and the crude extract was removed avoiding contamination from the foam layer floating on top or from the nuclear pellet. The extract was clarified by centrifugation at 205,000xg (max) for 1hr in a fixed angle rotor (Beckman Type 45 Ti). The supernatant was removed avoiding contamination from the membrane pellet to obtain cytoplasmic extract (approx. 8mg total protein/ml of extract).

Throughout the purification, WAVE2 immunoreactivity was followed by Western blot using WAVE2 (C14) goat polyclonal antibody. All chromatography media was from GE Healthcare (Piscataway, New Jersey). WAVE2 complex was enriched in a 20-30% ammonium sulfate cut (during each ammonium sulfate step the extract was incubated for at least 1 hr). The 30% ammonium sulfate pellet was resuspended overnight in buffer S (20 mM Hepes [pH 7.0 at 4°C], 90 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT), dialyzed through 10,000 MWCO membrane against the same buffer, and clarified by centrifugation at 33,600xg (max) in a fixed angle rotor. The sample was applied to a 5 ml HiTrap SP Sepharose HP column, and bound proteins were eluted over 10 column volumes with a linear

NaCl gradient from 90 mM to 500 mM. Peak fractions were diluted with 2 volumes of buffer Q (20 mM Hepes [pH 7.5 at 4°C], 90mM NaCl, 1mM $MgCl_2$, 1mM EGTA, 1mM DTT) supplemented with 10% glycerol, and fractionated on a Mono Q HR 5/5 column developed over 15 column volumes with a linear NaCl gradient from 90 mM to 1 M. Fractions were snap frozen in liquid nitrogen and stored at -80°C until further purification. Peak fractions were dialyzed against buffer Q and concentrated on a 1ml HiTrap Q Sepharose HP column by eluting with a step gradient of 500 mM NaCl. The eluate was fractionated by size exclusion chromatography on a Superose 6 HR 10/30 column equilibrated with buffer Q. Peak fractions were concentrated and fractionated on a Mono Q PC 1.6/5 column (operated in the SMART system) in buffer Q, developed over 20 column volumes with a linear NaCl gradient from 90 mM to 400 mM. Peak fractions (approx. 1 mg/ml total protein) were supplemented with 10% glycerol, aliquoted, snap frozen and stored at -80°C. Aliquots were thawed quickly and kept on ice until used in assays.

Conventional purification of native WAVE1 complex from bovine brain

Bovine brain extract was prepared as previously described (Ho et al., 2004), with some modifications. All procedures were carried out at 4°C or on ice. Nine freshly harvested calf brains were stripped of meninges, adherent clots and fibrous white matter, chopped coarsely and washed in ice-cold water. Brain pieces were weighed and mixed with 1.5 volumes (w/v) cold lysis buffer (20 mM Hepes [pH 7.2 at 4°C], 100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 2 mM EGTA, 2 mM PMSF, 10 μg/ml each leupeptin, pepstatin A and chymostatin, 1 mM DTT), blended for 1 min in setting 3 of a Waring commercial blender and homogenized at 400 RPM in a motorized continuous flow overhead teflon homogenizer (LSC homogenizer LH-21, Yamato, Tokyo, Japan). The homogenate was centrifuged at 17,700xg (max) for 30 min in a fixed angle rotor (Beckman JA-10) to remove particulates. The supernatant was centrifuged at 54,000xg (max) for 2.5 hrs in a fixed angle rotor (Beckman Type 19) to generate a clarified extract (approx. 8mg total protein/ml of extract), which was supplemented with 0.1 mM EDTA.

Throughout the purification, WAVE1 immunoreactivity was followed by Western blot using WAVE1 (L-19) goat polyclonal antibody. WAVE1 complex was enriched in a 20-30% ammonium sulfate cut. The 30% ammonium sulfate pellet was resuspended in buffer S/EDTA (20 mM Hepes [pH 7.0 at 4°C], 90 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.1mM EDTA) supplemented with 0.5 mM PMSF and 5 μg/ml each leupeptin, pepstatin A and chymostatin, homogenized in an all-glass Dounce tissue grinder (Kontes), solubilized for 4 hrs, dialyzed through 10,000 MWCO membrane against the same buffer. The sample was diluted to facilitate sedimentation, clarified by centrifugation at 476,000xg (max) for 1 hr in a fixed angle rotor (Beckman Type 70 Ti) and filtered (0.22 μm PVDF). The sample was applied to a 150 ml SP Sepharose HP column, and bound proteins were eluted over 10 column volumes with a linear NaCl gradient from 90 mM to 500 mM. Peak fractions were dialyzed against buffer Q/EDTA (20 mM Hepes [pH 7.5 at 4°C], 90mM NaCl, 1mM $MgCl_2$, 1mM EGTA, 1mM DTT, 0.1 mM EDTA), filtered and fractionated on a HiTrap Q Sepharose HP column (2 columns x 5 ml) developed over 15 column volumes with a linear NaCl gradient from 90 mM to 500 mM. Fractions were supplemented with 10% glycerol, snap frozen and stored at -80°C until further purified. Peak fractions were pooled, dialyzed against buffer Q/EDTA, filtered and concentrated on a 1ml HiTrap Q Sepharose HP column by eluting with a step gradient of 500 mM NaCl. The eluate was fractionated by size exclusion chromatography on a Superose 6 10/300 GL column equilibrated with buffer Q/EDTA supplemented with 10% glycerol. Peak fractions were concentrated and fractionated on a Mono Q PC

1.6/5 column (operated in the SMART system) in buffer Q/EDTA supplemented with 10% glycerol, developed over 20 column volumes with a linear NaCl gradient from 90 mM to 400 mM. Aliquots of the peak fractions (approx. 0.9 mg/ml total protein) were snap frozen and stored at -80°C. For most actin polymerization experiments, aliquots of the Mono Q fractions were thawed quickly and kept on ice until use. The remainder of the Mono Q peak fractions was pooled and purified further in 5-20% sucrose gradients prepared in modified buffer S/D_2O (20 mM Hepes [pH 7.0 at 4°C], 90 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 5 mM β-octylglucoside, made up with deuterium oxide to attain higher density than the Mono Q fractions containing 10% glycerol). Gradients were centrifuged at 284,500xg (max) for 17 hrs in a swinging bucket rotor (Beckman SW 40 Ti) and fractions were collected with an Auto Densi-Flow density gradient fractionator (Labconco). Aliquots of the peak fractions were snap frozen and stored at -80°C.

Immuno-purification of native WAVE2 complex from A-431 cells

Cytosolic and membrane extracts were prepared from serum starved or epidermal growth factor (EGF) stimulated A-431 human epidermoid carcinoma cells (ATCC cell line designation: A-431, Cat. # CRL-1555). 30 to 90 15 cm round tissue culture dishes of cells were used for each condition. Cells were seeded in 30 ml of growth medium (DMEM, 10% FBS, Pyruvate, Penicilin/streptomycin) at an appropriate density to obtain approx. 70% confluency on the day before harvesting (i.e. cells seeded at 1.8-2 x 10^6 or 3.5-4 x 10^6 cells per ml reached 70% confluency in 4 or 3 days respectively). Cells were grown at 37°C and 10% CO_2, replacing the medium once every 2 or 3 days, and on the day before harvesting they were serum starved overnight in the same medium without FBS. For EGF stimulation, 100 ng/ml human recombinant EGF (Upstate; Cat. # 01-407) was added directly to the medium after serum starvation, and cells were incubated for an additional 15-30 min before harvesting. All procedures after this point were carried out at 4°C or on ice, except where noted. Cells were harvested in batches of 30 dishes, working quickly in the cold with 5 dishes at a time and collecting the cells from each dish sequentially as follows. The medium from each dish was poured off and cells were rinsed once with 50 ml cold PBS. After pouring off the bulk of the liquid, the dish was kept oblique in order to collect and discard as much PBS as possible. For each 30 dish batch, 5 ml of cold lysis buffer (20 mM Tris [pH 7.5 at 4°C], 150 mM NaCl, 10 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 20 µg/ml each leupeptin, pepstatin A and chymostatin, 2 mM PMSF, 2 mM DTT) were added to the first dish. Cells were scraped off (A-431 cells are strongly adherent, so scraping is sufficient to lyse most cells), dispersed by pipetting gently, and transferred sequentially to the following dish of cells.

After harvesting 30 dishes, cells were homogenized 20 strokes (up + down = 2 strokes) in an all-glass Dounce tissue grinder with the small clearance pestle; this procedure disrupted clumps of cells but left most nuclei intact. For preparations larger than 30 dishes, the homogenates were kept on ice until all batches were finished. Nuclei and unbroken cells were removed by centrifugation at 1,000xg (max) for 3 min in a swinging bucket rotor. The supernatant was removed avoiding contamination from the loose pellet. To separate membranes and cytosol, the crude supernatant was carefully layered on discontinuous sucrose gradients prepared in 14x95 mm ultra clear tubes (Beckman) and composed of 2 ml of 5% (w/v) sucrose on top of 1 ml of 70% sucrose in extract buffer (20 mM Tris/HCl [pH 7.5 at 4°C], 150 mM NaCl, 5 mM EDTA, 5 mM NaF, 1 mM Na_3VO_4, 10 µg/ml each leupeptin, pepstatin A and chymostatin, 1mM DTT) (it was helpful to mark the tube at the interface between the crude extract and the 5% sucrose layer because the interface becomes invisible after centrifugation). The gradients were centrifuged at 71,000xg

(max) for 30 min in a swinging bucket rotor (Beckman SW 40 Ti). A thin layer of light membranes floating on the top was carefully aspirated and discarded, and the cytosolic extract above the interface with the 5% sucrose layer was collected. The 5% sucrose layer was aspirated and discarded to avoid contaminating the membranes with any remaining cytosolic extract. The membranes formed a disk that was easily detached from the sides of the tube using the tip of a clean scalpel, and was decanted into a clean tube along with the 70% sucrose, leaving behind any pellet. The loosely packed membranes were easily resuspended by pipetting, and were brought to the same volume as the cytoplasmic extract in extract buffer. To solubilize membrane-associated proteins, 1% Triton-X100 was added to the resuspended membranes from a 20% stock, followed by incubation for 10-20 min with gently rotation. The cytosolic extract was supplemented with 0.2% Triton X-100 to prevent aggregation of the immunoaffinity beads in the subsequent purification. Both cytosolic and membrane extracts were clarified by centrifugation at 39,500xg (max) for 30 min in a fixed angle rotor (Beckman Type 70 Ti) and the clear supernatants were supplemented with 10% glycerol (added from an 80% stock to facilitate mixing).

Dynabeads Protein A (Invitrogen/Dynal Cat. # 100.02D) were crosslinked to saturating α-WP1 polyclonal IgG with dimethyl pimelimidate by standard protocols (Harlow and Lane, 1999) and washed with extract buffer. In order to saturate binding, 40 µl of α-WP1 Dynabeads (bead volumes refer to the original volume of slurry provided by the manufacturer) were added per mg of total protein in the cytosolic extract (approx. 5 mg total protein/ml of extract), and 60 µl of α-WP1 Dynabeads were added per mg of total protein in the membrane extract (approx. 1.5 mg total protein/ml of extract). After approx. 4 hrs of incubation with gentle rotation, the beads were collected on a magnetic particle concentrator. All subsequent washes were done at room temperature by resuspending the beads in buffer and mixing by pipetting, as we found that vortexing led to precipitation of the complex on the beads. Beads were washed three times with two bead volumes of buffer A (20 mM Tris/HCl [pH 7.5 at 24°C], 150 mM NaCl, 5 mM EDTA, 5 mM NaF, 1 mM Na_3VO_4, 10 µg/ml each leupeptin, pepstatin A and chymostatin, 0.5% Triton X-100, 10% glycerol, 1mM DTT), twice with two bead volumes of buffer B (10 mM Hepes [pH 7.9 at 24°C], 100 mM KCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 5 mM EGTA, 0.01% Tween 20, 1 mM DTT), once with two volumes of buffer C (10 mM Pipes [pH 6.5 at 24°C], 100 mM KCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 5 mM EGTA, 0.01% Tween 20, 1 mM DTT), and were resuspended in 0.25 bead volumes of buffer C for elution. 1.7 mg/ml of the competing WP1x3 peptide were added from a 10 mg/ml stock in buffer XB (20 mM Hepes [pH 7.7 at 24°C], 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA), yielding a final pH of 6.8 (the slightly acidic pH helps promote elution). Elution was allowed to proceed for 5 hrs at room temperature with gentle rotation. The eluate was brought to pH 7.7 with 15 mM TAPS [pH 8.5 at 24°C], it was supplemented with 5% glycerol, and small aliquots were snap frozen and stored at -80°C. The final concentration of WAVE2 complex was typically 30-40 µg/ml, but the total protein concentration was approx. 1.6 mg/ml due to the presence of WP1x3 peptide.

In our initial purifications of WAVE2 complex from membrane extracts we noticed an increase in the electrophoretic mobility of WAVE2 in the eluate compared to that on beads before elution, suggesting it was being dephosphorylated by an undefined phosphatase activity; we did not observe the same for complexes purified from cytosolic extracts. Since we later found that phosphorylation was crucial for activation of WAVE2 complex, we adjusted the composition of buffers A, B and C used during washes and elution of the membrane complex to include the following final concentrations of phosphatase inhibitors: 10 mM NaF, 20 mM paranitrophenylphosphate (PNPP), 40 mM β-glycerophosphate and 1 mM

Na_3VO_4. During elution of membrane complex we also added 5 µM okadaic acid and 5 µM Microcystin-LR.

Phosphatase treatment of WAVE2 complex

After incubation with cytosolic extract from serum starved or EGF stimulated A-431 cells, α-WP1 Dynabeads were washed twice with two bead volumes of cold binding buffer (extract buffer supplemented with 0.2% Triton X-100 and 10% glycerol), and three times with two bead volumes of cold phosphatase buffer (20 mM Hepes [pH 7.7 at 4°C], 100 mM KCl, 1 mM $MgCl_2$, 2mM $MnCl_2$, 0.1 mM EDTA, 1mM DTT) supplemented with 0.05% Triton X-100. Before use, lamda protein phosphatase (Upstate, full length, recombinant; Cat. # 14-405) or PP2A (Upstate, human purified enzyme; Cat. # 14-111) was diluted 10-fold with phosphatase buffer (detergent was omitted from the phosphatase buffer to avoid interfering with the phosphatase reaction) and dialyzed through 10,000 MWCO membrane against phosphatase buffer. Beads were resuspended in 0.5 bead volumes of either phosphatase buffer alone or containing 12.5U/µl λPPase, 2.5 mU/µl PP2A, 5 mU/ µl PP2A or 5 mU/µl PP2A pre-incubated for 1 hr with 2.6 µM okadaic acid (sodium salt, Calbiochem 459620, reconstituted in ddH_2O). Beads were incubated for 3 hrs at 30°C followed by 30 min at 4°C with gentle rotation. Washes with buffers A, B and C and elution with WP1x3 peptide followed as described above.

Non-physiological activation of native WAVE complexes

For thermal denaturation experiments, WAVE complex was heated for 10 min at 57°C and cooled to 24°C in a thermocycler before addition to the pyrene-actin assay.

To test if freezing and thawing of native WAVE1 complex without cryoprotectants could generate activity, the complex was exchanged into buffer XB (20 mM Hepes [pH 7.7 at 24°C], 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA) using a Micro Bio-Spin P-6 column (Biorad) and either frozen straight or after addition of 10% glycerol. Samples were thawed and tested in the pyrene-actin assay.

While testing elution conditions for the immuno-purified WAVE2 complex we found that 0.5 KCl activated the native complex. Complexes were eluted in either control elution buffer (10 mM Pipes [pH 6.8 at 24°C], 100 mM KCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 5 mM EGTA, 0.2 % Triton X-100, 1 mM DTT) or in elution buffer containing 0.5 M KCl (10 mM Hepes [pH 7.8 at 24°C], 500 mM KCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 5 mM EGTA, 0.2 % Triton X-100, 1 mM DTT) and tested in pyrene-actin assays.

Preparation of wild type and constitutively active prenylated Rac1 and Rac2

The wild type and constitutively active Q61L forms of both human Rac1 and Rac2 were cloned into a pCS2+ multi-purpose expression vector modified to encode an N-terminal polyhistidine tag (MARGSHHHHHHARGSQHHHHHAGRPT), and each of these constructs was used to transfect 750 ml of mammalian FreeStyle 293-F cells (Invitrogen) following the manufacturer's protocol. Cells were harvested by centrifugation 42-48 hrs after transfection, washed with phosphate buffered saline (PBS), and pellets were snap frozen.

Prenylated Rac was purified as described previously for Cdc42 (Lebensohn et al., 2006), with some modifications. Cell pellets were quickly thawed and resuspended with 4 volumes of cold lysis buffer (20 mM Hepes [pH 7.4 at 4°C], 1 mM DTT, 5 mM $MgCl_2$, 100 mM NaCl, 1 mM PMSF, 10 µg/ml each leupeptin, pepstatin A and chymostatin, Complete EDTA-free protease inhibitor cocktail tablets [Roche; 1 tablet per 50 ml of buffer], 1 mM EDTA) supplemented with 0.1 mM GDP or GTP for wild

type or Q61L Rac, respectively. All subsequent steps were carried out at 4°C or on ice, unless indicated otherwise. Cells were lysed by sonication and an additional 1 mM PMSF was added to the lysate. The lysate was centrifuged at 1,000g for 10 min in a swinging bucket rotor to separate nuclei and unbroken cells in the pellet from cytoplasm and membranes in the supernatant. The supernatant was recovered carefully by pipetting as opposed to decanting in order to avoid contamination from the very loose pellet. The pellet was resuspended in two volumes of lysis buffer, homogenized in an all glass Dounce tissue grinder using the large clearance pestle, centrifuged as before, and the supernatants were pooled. The supernatant was centrifuged at 150,000xg (max) for 30 min in a fixed angle rotor (Beckman Type 45 Ti) to isolate membranes containing prenylated Rac in the pellet. To extract prenylated Rac from the membranes, the pellet was resuspended in extraction buffer (20 mM Hepes [pH 7.8 at 4°C], 1 mM DTT, 5 mM MgCl2, 100 mM NaCl, 1 mM PMSF, 10 µg/ml each leupeptin, pepstatin A and chymostatin, 1% cholate, 1 mM EDTA, 20 mM imidazole), supplemented with 0.1 mM GDP or GTP for wild type or Q61L Rac, respectively, and homogenized in an all glass Dounce tissue grinder using the small clearance pestle. Extraction was allowed to proceed for 60 min with gentle rotation. The homogenate was then centrifuged at 400,000xg (max) for 30 min in a fixed angle rotor (Type 70 Ti) to obtain extracted prenylated Rac in the supernatant.

Prenylated Rac was purified by nickel affinity chromatography. The supernatant was filtered and applied to a 1ml HisTrap HP column (GE Healthcare) equilibrated with HisTrap buffer (20 mM Hepes [pH 7.8 at 4°C], 1 mM DTT, 5 mM MgCl2, 100 mM NaCl, 1% cholate, 0.1 mM EDTA, 20 mM imidazole). After washing with 10 column volumes of HisTrap buffer containing 120 mM imidazole, Rac was eluted over 10 column volumes with a linear imidazole gradient from 120 mM to 400 mM. Rac containing fractions were pooled, concentrated in Amicon Ultra 4 centrifugal filter devices (Ultracel 10K, Millipore), dialyzed through 10,000 MWCO membrane against storage buffer (20 mM Hepes [pH 7.8 at 4°C], 1 mM DTT, 5 mM $MgCl_2$, 100 mM NaCl, 1% cholate, 10% glycerol, 0.1 mM EDTA), aliquoted, snap frozen and stored at -80°C. The final protein concentration was approx. 0.25-0.5 mg/ml. Since the guanine nucleotide bound to wild type Rac is quickly hydrolyzed to GDP, while GTP bound to Rac Q61L cannot be hydrolyzed, there was no need to re-load the purified proteins with nucleotide (see Figure S1C).

Preparation of prenylated Cdc42

Prenylated GST-Cdc42 was purified from baculovirus infected Sf9 cells and loaded with GTPγS while bound to glutathione sepharose beads as described previously (Lebensohn et al., 2006). The beads were washed and resuspended in cleavage buffer (20 mM Hepes [pH 7.8 at 4°C], 1 mM DTT, 5 mM MgCl2, 100 mM NaCl, 1% cholate), and the fusion protein was cleaved with 10 units of thrombin (Amersham) per mg of bound protein, incubating 14 hrs at room temperature. Thrombin was removed from the eluate by incubation with benzamidine sepharose 4 FF (GE Healthcare). Cleaved Cdc42 (approx. 1.2 mg/ml) was supplemented with 10% glycerol, aliquoted, snap frozen and stored at -80°C.

Preparation of wild type and constitutively active unprenylated Rac1 and Rac2

The wild type and constitutively active Q61L forms of both human Rac1 and Rac2 were cloned into a pET28 vector (encoding an N-terminal hexahistidine tag) and each of these constructs was used to transform BL21 Star (DE3) *E.coli*. 1 l cultures were grown in terrific broth until they had reached an OD(600nm) of 1.5-2 absorbance units, at which time they were induced with 0.3 mM IPTG for 3 hr at 37°C. Cells were harvested by centrifugation, washed with PBS, and pellets were snap frozen.

Cell pellets were quickly thawed and resuspended with 4 volumes of cold lysis buffer (20 mM Tris [pH 8.0 at 4°C], 5 mM $MgCl_2$, 150 mM NaCl, 1 mM DTT, 1 mM PMSF, Complete EDTA-free protease inhibitor cocktail tablets [Roche; 1 tablet per 50 ml of buffer], 0.01 mM GDP, 1% Triton X-100, 0.1 mg/ml lysozyme). All subsequent steps were carried out at 4°C or on ice, unless noted otherwise. Cells were lysed by sonication and the lysate was clarified by centrifugation at 186,000xg (max) for 1 hr in a fixed angle rotor (Beckman Type 45 Ti).

Unprenylated Rac was purified by nickel affinity chromatography. The supernatant was filtered and applied to a 1ml HisTrap HP column equilibrated with HisTrap buffer (30 mM Hepes [pH 8.0 at 4°C], 5 mM $MgCl_2$, 500 mM NaCl, 0.01mM GDP, 1 mM DTT, 20 mM imidazole, 10% glycerol). After washing with 20 column volumes of HisTrap buffer, Rac was eluted over 15 column volumes with a linear imidazole gradient from 20 mM to 350 mM. Rac containing fractions (peak fractions approx. 8-11 mg/ml) were snap frozen and stored at -80°C.

To load wild type, unprenylated Rac1 with GMPPNP, fractions in HisTrap buffer were supplemented with an additional 20 mM Hepes and 20 mM EDTA [pH 8.0], and 1.2 μM GMPPNP (Sigma G0635) was added. Loading was allowed to proceed for 15 min at 30°C, followed by addition of 30 mM $MgCl_2$ and further incubation for 15 min on ice. Fractions were dialyzed through 10,000 MWCO membrane against cold assay buffer (20 mM Hepes [pH 7.7 at 4°C], 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT), centrifuged at 20,000xg (max) for 15 min to remove precipitated material and the supernatant was aliquoted, snap frozen and stored at -80°C.

Preparation of Nck

A glutathione *S*-transferase (GST) fusion of human Nck1 was expressed in *Escherichia coli*, affinity purified on glutathione-Sepharose beads (GE healthcare) and GST was cleaved from Nck using PreScission protease (Amersham Pharmacia Biotech), as described previously (Rohatgi et al., 2001).

Preparation of recombinant WAVE2 protein

Human full length WAVE2 was cloned into a modified pCS2+ vector encoding an N-terminal ZZ-TEV_4 tag (two IgG binding domains of protein A followed by four tobacco etch virus (TEV) protease cleavage sites (Stemmann et al., 2001)). This construct was used to transfect 1.75 l of 293-F cells. Cells were harvested by centrifugation 48 hrs after transfection, washed with PBS and resuspended with 9 volumes of cold lysis buffer (20 mM Hepes [pH 7.7 at 4°C], 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, Complete EDTA-free protease inhibitor cocktail tablets [Roche; 1 tablet per 50 ml of buffer]).

After this point, all procedures were carried out at 4°C or on ice, unless noted otherwise. Cells were lysed by cavitation in a Parr cell disruption bomb (pressurized with nitrogen to 450 psi for 25 min), or alternatively by homogenizing them 10 strokes in an all glass Dounce tissue grinder using the small clearance pestle, followed by brief sonication. Lysates were supplemented with 1mM PMSF, clarified by centrifugation at 186,000xg (max) for 1 hr in a fixed angle rotor (Beckman Type 45 Ti) and filtered.

The ZZ-TEV_4-WAVE2 fusion protein was purified in batch on IgG sepharose 6 fast flow (GE Healthcare) and washed sequentially at room temperature with buffer A (20 mM Hepes [pH 7.7 at 24°C], 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT), buffer B (20 mM Hepes [pH 7.7 at 24°C], 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 400mM NaCl), buffer C (20 mM Hepes [pH 7.7 at 24°C], 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 1% Triton X-100), and cleavage buffer

(30mM Tris [pH 7.5 at 24°C], 500 mM NaCl, 5 mM β-ocytlglucoside, 1 mM DTT, 10% glycerol). WAVE2 was eluted at room temperature by cleaving it from the ZZ tag using TEV protease fused to GST, and GST-TEV was removed by adding glutathione sepharose 4B. The eluate was aliquoted, snap frozen and stored at -80°C.

PAK pull-down assays

Unprenylated and prenylated wild type and constitutively active Rac1 and Rac2 were tested for effector binding in pull-down assays with the p21-binding domain (PBD) of p21-activated kinase 1 (PAK) fused to GST as described previously (Benard et al., 1999), with some modifications. Rac proteins were diluted to 5 μg/ml in binding buffer (25 mM Tris [pH 7.5 at 4°C], 40 mM NaCl, 30 mM $MgCl_2$, 1 mM DTT, 1% cholate, 1% β-octylglucoside, 2 mg/ml bovine serum albumin), samples of the input were removed for analysis and 50 μl (250 ng) were incubated with 10 μl of packed glutathione sepharose 4B beads (GE healthcare) and 10 μg of GST-PBD. For constitutively active Rac, assays excluding GST-PBD were also conducted to control for non-specific binding of Rac to the beads. After incubation for 30 min at 4°C the supernatant was removed, beads were washed four times with 0.5 ml of cold binding buffer and resuspended in the same buffer. The input, supernatant and beads were boiled in sample buffer, resolved on 4-12% Bis-Tris polyacrylamide gels, transferred to nitrocellulose and analyzed by Western blot for Rac.

Preparation of liposomes

Liposomes were prepared as described (Lebensohn et al., 2006) and extruded 21 passes through a 0.1 μm membrane. For fluorescent liposomes used in microscopy experiments and liposome sedimentation assays, dried lipids were resuspended in lipid buffer supplemented with 20% (w/v) sucrose. To assess their reproducibility, liposomes were routinely tested for their ability to induce actin polymerization in Xenopus egg extracts as described previously (Lebensohn et al., 2006). All lipids were from Avanti Polar Lipids, Inc.: 1-Stearoyl-2-Arachidonoyl-*sn*-Glycero-3-Phosphoinositol-3,4,5-trisphosphate (Tetra-ammonium Salt) (Cat. # 850166P, MW 1,195.20); 1-Stearoyl-2-Arachidonoyl-*sn*-Glycero-3-[Phosphoinositol-4,5-Bisphosphate] (Tri-ammonium Salt) (Cat. # 850165P, MW 1,098.19); 1-Stearoyl-2-Arachidonoyl-sn-Glycero-3-[Phosphoinositol-3,5-Bisphosphate] (Tri-ammonium Salt) (Cat. # 850164P, MW 1,098.19); L-α-Phosphatidylinositol (Liver, Bovine-Sodium Salt) (Cat. # 840042C, MW 909.12); L-α-Phosphatidylserine (Brain, Porcine-Sodium Salt) (Cat. # 840032C, MW 812.05); L-α-Phosphatidylethanolamine (Brain, Porcine) (Cat. # 840022C, MW 746.06); L-α-Phosphatidylcholine (Egg, Chicken) (Cat. # 840051C, MW 760.09); 1,2-Dioleoyl-*sn*-Glycero-3-Phosphoethanolamine-N-(Lissamine Rhodamine B Sulfonyl) (Ammonium Salt) (Cat. # 810150C, MW 1,301.73). $PI(3,4,5)P_3$, $PI(4,5)P_2$ and $PI(3,5)P_2$ were obtained in powder form, resuspended in a solution of chloroform : methanol : water (65:35:8 v/v) (Avanti Cat. # 690013X) to make a stock solution of 0.2 mg/ml, and used within 3 months (Avanti determined that phosphoinositolphosphate solutions are unstable). The rest of the lipids were obtained as chloroform stock solutions, as these are unaffected by long term storage. All lipid stock solutions were stored at -20°C.

Preparation of proteins used in actin polymerization assays

Actin was purified from rabbit skeletal muscle as described (Zigmond, 2000). Pyrene actin was labeled as described (Zigmond, 2000) or purchased from Cytoskeleton (Cat. # AP05), reconstituted as instructed,

snap frozen in small aliquots and stored at -80°C. Arp2/3 complex was purified from bovine brain as described (Ho et al., 2006). Peak fractions eluting from the final Superdex 200 column were supplemented with 10% (w/v) sucrose, snap frozen and stored at -80°C; side fractions were pooled, loaded onto a 1ml hydroxylapatite column equilibrated with hydroxylapatite buffer (20 mM phosphate [pH 7.2 at 4°C], 0.2 mM $MgCl_2$, 0.2 mM ATP, 1 mM DTT), eluted with a linear phosphate gradient from 20 to 400 mM, dialyzed into buffer XB (20 mM Hepes [pH 7.7 at 24°C], 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA) supplemented with 0.5 mM ATP, 1 mM DTT and 10% glycerol, snap frozen and stored at -80°C. Both the Superdex 200 and hydroxylapatite fractions were used in pyrene actin assays throughout the course of this study. Recombinant N-WASP was prepared as described (Ho et al., 2006).

Data analysis of actin polymerization assays

For kinetic analysis of actin polymerization assays the baseline fluorescence was offset by subtracting the fluoresce of the earliest acquired time point from the fluorescence of all other points. Because direct comparisons were only made between reactions conducted in the course of a few days using the same stock of actin/pyrene-actin, the steady-state plateau did not vary significantly and we generally deemed it unnecessary to normalize the data in any way. Hence, both fluorescence intensity and maximum actin polymerization rates are expressed in arbitrary units. The maximum actin polymerization rate was determined from the slopes of lines fitted (using the least squares method LINEST function in Microsoft Excel) to groups of ten consecutive measurements (encompassing 105 sec intervals) throughout the entire pyrene actin fluorescence trace. The data in Figure 2H, depicting maximum actin polymerization rate as a function of the concentration of WAVE2 complex, was fitted by least squares to the hill equation, $V=Vmax\ E^n/(E^n+K^n)$, using the MATLAB statistical toolbox. V is the maximum actin polymerization rate (determined as described above), E is the concentration of WAVE2 complex, Vmax is the maximum actin polymerization rate at saturation (10 nM WAVE2 complex), and the values for the equilibrium constant K and Hill coefficient n were obtained from the best fit.

Liposome co-sedimentation assays

Reactions were set up exactly as for actin polymerization assays, but using liposomes labeled with lissamine rhodamine PE and prepared with buffer containing 20% sucrose (w/v) as described above. We omitted actin/pyrene-actin in the liposome co-sedimentation assays. Samples were removed for Western blot analysis of the input and the rest was incubated at room temperature for 25 min and then centrifuged at 109,000xg (max) for 15 min in a fixed angle rotor (Beckman TLA-100) at 4°C. The supernatant was removed immediately without disrupting the liposome pellet, and the pellet was resuspended to half of the input volume. The input, supernatant and pellet, along with sequential 2-fold dilutions, were resolved on 4-12% Bis-Tris polyacrylamide gels, transferred to nitrocellulose, and analyzed by Western blot for Pir121, Nap 1, WAVE2, Abi-1, Arp2 and Rac1 using the Odyssey infrared imaging system (LI-COR). For the immunoprecipitation experiment shown in Figure 4C, the pellet was resuspended to half the input volume with cold assay buffer (20 mM Hepes [pH 7.7 at 24°C], 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT) supplemented with 1% Triton X-100 to solubilize the liposomes, and centrifuged at 109,000xg (max) for 15 min in a fixed angle rotor (Beckman TLA-100) at 4°C to remove insoluble material. The supernatant was carefully recovered, a sample was removed for Western blot analysis of the IP input, and WAVE2 was immunoprecipitated with α-WP1 Dynabeads (described above). Immunoprecipitation was allowed to proceed for 20 min at 4°C, the supernatant was removed

immediately, and the beads were resuspended to the same volume as the input. The IP input, supernatant and beads, along with 2-fold dilutions, were analyzed by Western blot for all the components of the WAVE2 complex and Rac1. The stoichiometry of the different subunits in the liposome pellet (Figure 4B) or in the WAVE2 IP beads (Figure 4C) was calculated from the ratio of the fluorescence in the liposome pellet or IP beads and the respective input. For the co-sedimentation assays reported in Table 1, the percentage of WAVE2 bound was calculated from the ratio of the fluorescence in the supernatant and the respective input (we analyzed the input and supernatant as opposed to the pellet in order to avoid introducing error due to possible losses when handling the pellets of the different samples).

Microscopy

Reactions were set up exactly as described for actin polymerization assays, but using liposomes labeled with lissamine rhodamine PE and prepared in buffer containing 20% (w/v) sucrose. Assays were supplemented with 0.1 μM Alexa Fluor 488 conjugated rabbit muscle actin (Molecular Probes, Invitrogen Cat. # A12373). Actin polymerization was measured by pyrene actin fluorescence, and when the steady state fluorescence level had been reached (typically ~1000 sec), an aliquot was transferred (using a cut pipette tip to avoid damaging structures) to a glass slide and covered with a No. 1.5 coverslip for confocal microscopy, or to a chambered No. 1.0 borosilicate coverglass system (Lab-Tek, Nalge Nunc International Cat. # 155411) for widefield microscopy (this system helped preserve the structures and enabled a more accurate three dimensional view).

Confocal images in Figure 4A were collected in the Nikon Imaging Center at Harvard Medical School with a Yokogawa spinning disk confocal on a Nikon TE2000-U inverted microscope equipped with a 100x Plan Apo NA 1.4 objective lens. Alexa Fluor 488 actin fluorescence was excited with the 488nm line (selected with a 488/10 filter, Chroma # Z488/10x) from a 100mW Melles Griot argon krypton laser and collected with a triple band pass dichroic mirror (Chroma # 53055) and a 525/50 emission filter (Chroma # ET525/50m). Lissamine rhodamine PE fluorescence was excited with the 568 line (selected with a 568/10 filter, Chroma # Z568/10x) from the same laser and collected with the same triple band pass dichroic mirror and a 620/60 emission filter (Chroma # ET620/60m). Images were acquired with a Hamamatsu ORCA ER cooled CCD camera (Model C4742-80-12AG) controlled with MetaMorph software. Z-series optical sections were collected with a step size of 0.3 microns and are displayed as maximum z-projections rendered in MetaMorph.

Widefield images in Movie S1 were collected on a Nikon TE2000-E inverted microscope equipped with a 60x Plan Apo NA 1.4 objective lens. Fluorophores were excited with a mercury lamp (X-Cite 120 Fluorescence Illumination System) and Alexa Fluor 488 actin fluorescence was collected with a FITC filter cube (HQ:F Nikon # 96320) while lissamine rhodamine PE fluorescence was collected with a rhodamine filter cube (G-2E/C Nikon # 96312). Images were acquired with a Hamamatsu ORCA ER cooled CCD camera (Model C4742-95-12ERG) controlled with MetaMorph software. Z-series were collected with a step size of 0.35 microns, and are displayed as a 360 degree 3-D reconstruction rendered in Metamorph.

Mass Spectrometry

Protein sequence and phosphorylation analysis was done by LC-MS/MS at the Taplin Mass Spectrometry Facility at Harvard Medical School. Excised gel bands were cut into approximately 1 mm^3 pieces. The samples were reduced with 1 mM DTT for 30 min at 60°C and alkylated with 5 mM iodoacetamide for 15

min in the dark at room temperature. Gel pieces were subjected to a modified in-gel trypsin digestion procedure (Shevchenko et al., 1996). Gel pieces were washed and dehydrated with acetonitrile for 10 min followed by removal of acetonitrile. Pieces were dried completely in a speed vac. Gel pieces were rehydrated with 50 mM ammonium bicarbonate solution containing 12.5 ng/µl modified sequencing-grade trypsin (Promega, Madison, WI) at 4°C. Samples were then kept overnight at 37°C. Peptides were extracted by removing the ammonium bicarbonate solution, and washed once with a solution of 50% acetonitrile, 5% acetic acid (v/v). The extracts were dried in a speed vac for 1 hr and stored at 4°C.

Samples were reconstituted in 5 µl of HPLC solvent A (2.5% acetonitrile, 0.1% formic acid). A nano-scale reverse-phase HPLC capillary column was created by packing 5 µm C18 spherical silica beads into a fused silica capillary (100 µm inner diameter, 12 cm length) with a flame-drawn tip (Peng and Gygi, 2001). The column was equilibrated, each sample was pressure-loaded off-line and the column was reattached to the HPLC system. Peptides were eluted with a gradient of solvent B (97.5% acetonitrile, 0.1% formic acid).

Eluted peptides were subjected to electrospray ionization and fed into an LTQ-Orbitrap mass spectrometer (ThermoFinnigan, San Jose, CA). Eluting peptides were detected, isolated, and fragmented to produce a tandem mass spectrum of specific fragment ions for each peptide. Peptide sequences, and hence protein identity, were determined by matching protein or translated nucleotide databases with the acquired fragmentation pattern with the software program Sequest (ThermoFinnigan, San Jose, CA). The modification of 79.9663 mass units to serine, threonine, and tyrosine was included in the database searches to determine phosphopeptides. Each phosphopeptide determined by the Sequest program was also inspected manually in ensure confidence assignment.

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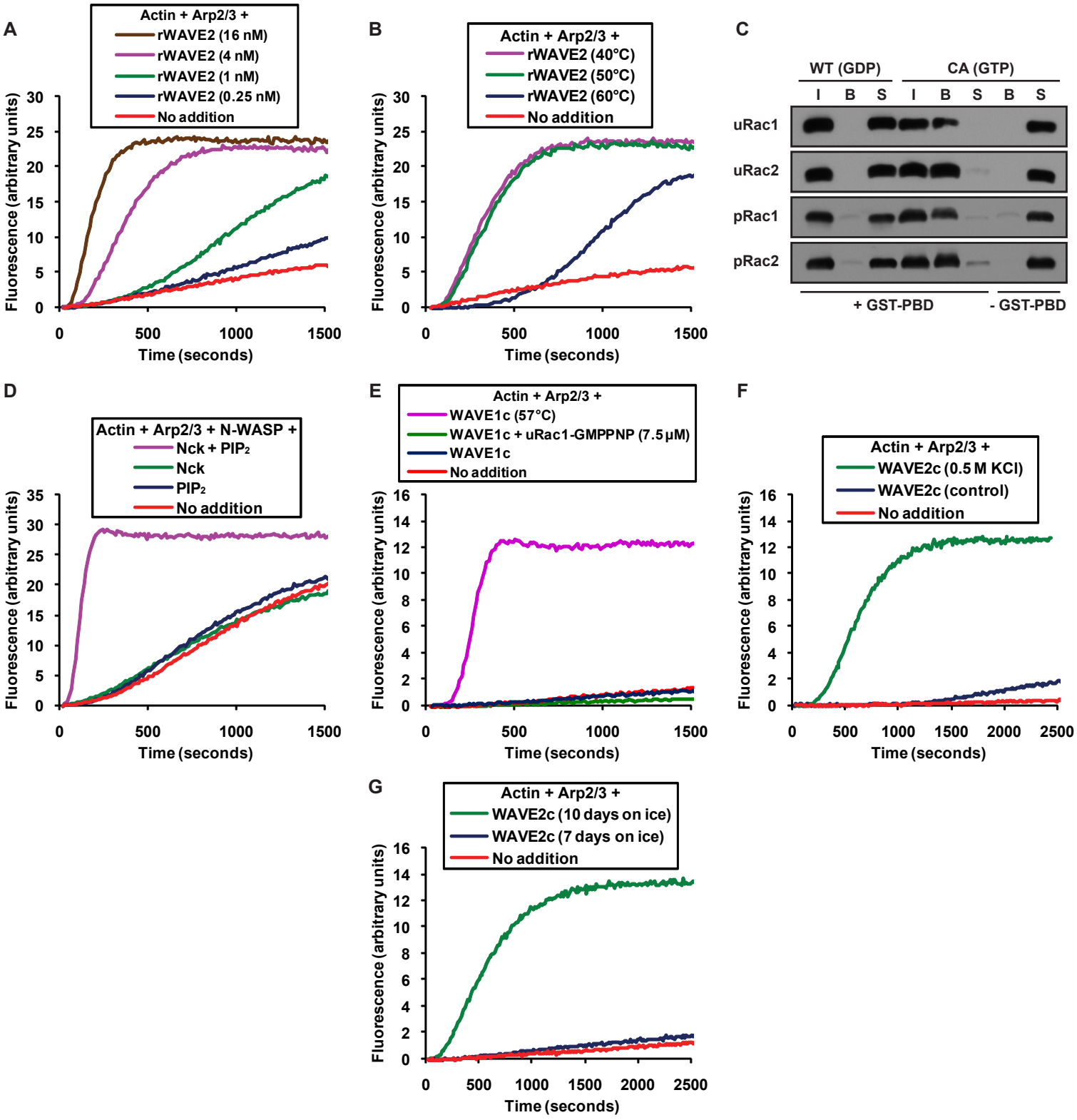
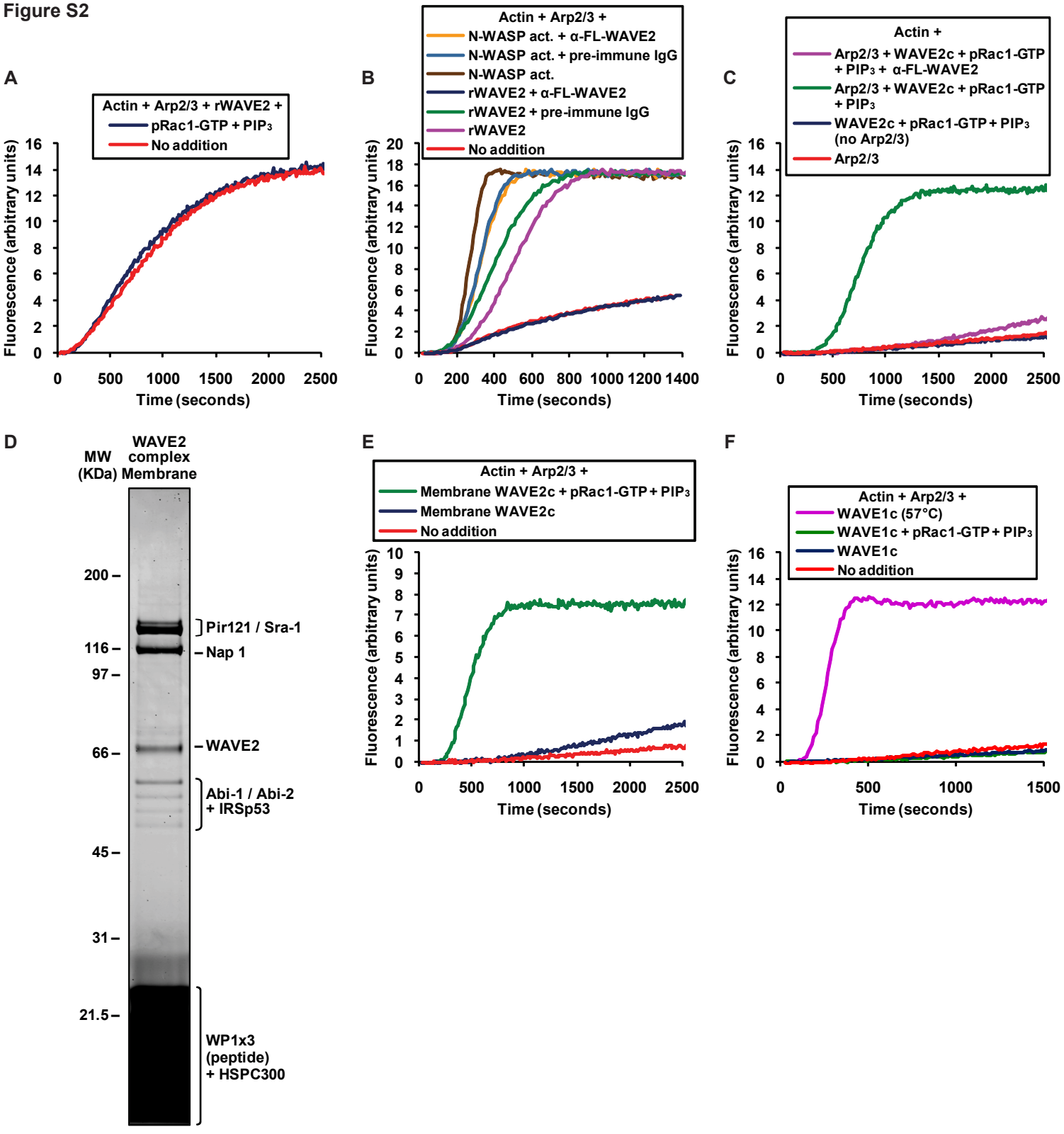
Supplemental Figure S1

Figure S1



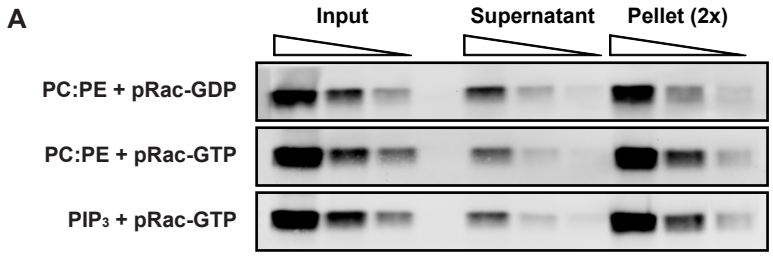
Supplemental Figure S2

Figure S2



Supplemental Figure S3

Figure S3



Supplemental Movie S1. Activation of the WAVE2 complex takes place on the membrane surface

Merged 360 degree 3-D reconstruction of actin structures (pseudocolored green) resulting from activation of the WAVE2 complex on PIP_3 liposomes (pseudocolored red) containing prenylated Rac2-GTP. Fluorescence images of a reaction where 5 nM WAVE2 complex from serum starved A-431 cells was activated by 50 nM prenylated Rac2-GTP and 2.5 μM liposomes composed of PC, PI, PIP_3 and lissamine rhodamine PE (44:45:10:1 molar percentage) in an assay containing 0.7 μM actin, 0.2 μM pyrene-actin, 0.1 μM Alexa 488 actin and 30 nM Arp2/3 complex. Image acquisition and processing are described in Supplemental Experimental Procedures.