SUPPORTING INFORMATION
Supplementary Figures

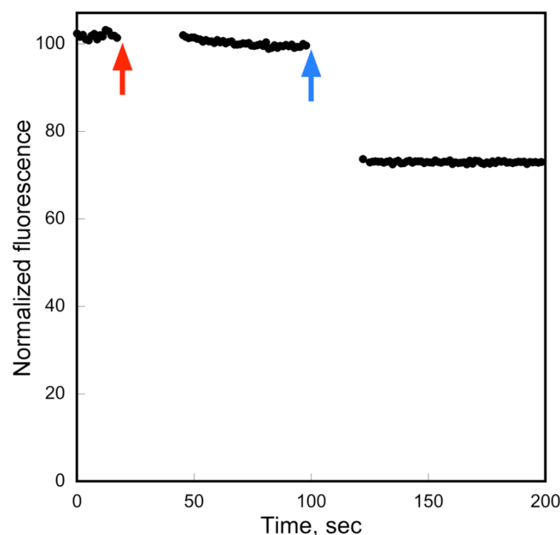

Figure S1. Effect of **2** on fluorescence intensity of polymerized actin. Actin (4 μM, 20% pyrene-labeled) was polymerized overnight. The fluorescence signal intensity was established for 15 sec, then a 1/100th volume of DMSO was added (red arrow) and fluorescence intensity was recorded. At 100 sec, a 1/100th volume of 5 mM compound **2** in DMSO was added (blue arrow) and fluorescence intensity was recorded. Gaps in the plot represent the time required for DMSO addition and mixing (35 sec for DMSO, 26 sec for compound **2**).

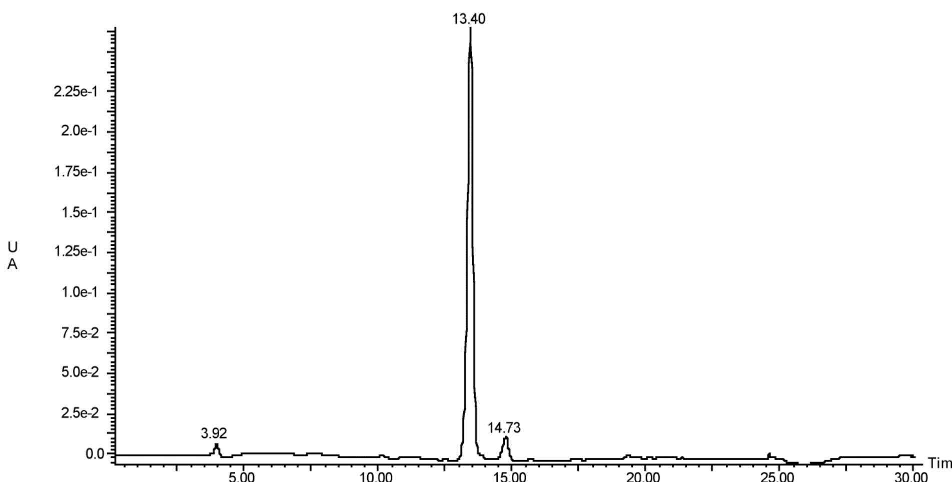

Figure S2. HPLC and ^{1}H NMR analysis of compound **2** (3-(2-amino-5-bromophenyl)-1H-quinoxalin-2-one) obtained from ChemDiv, Inc. (San Diego). LC-MS was performed using a Waters 2545 binary gradient module and a 2487 dual wavelength detector set to 254 and 265, a 2424 ELS detector and a 3100 MS detector. The gradient was linear 5% MeOH (0.05% TFA) 95% H2O (0.05 % TFA) to 95% MeOH (0.05% TFA) 5% H2O (0.05 % TFA) over 20 minutes. The column was a Waters Delta Pak C-18 15u 100A 3.9 x 300mm (catalog number 11797) run at a flow rate of 0.8 ml per minute. ^{1}H NMR was performed on a Bruker WB Advance 300 Mhz instrument using deuterated methanol. ^{1}H NMR (CD_3OD): d = 6.87 (d, 1H,

J=9.3 Hz); 7.22-7.31 (m, 1H); 7.34 (d, 1H, J=8.1 Hz); 7.46 (m, 1H); 7.79 (d, 1H, J=8.4 Hz); 7.94 (dd, 1H, J=2.7 and 9.3 Hz); 9.70 (s, 1H).

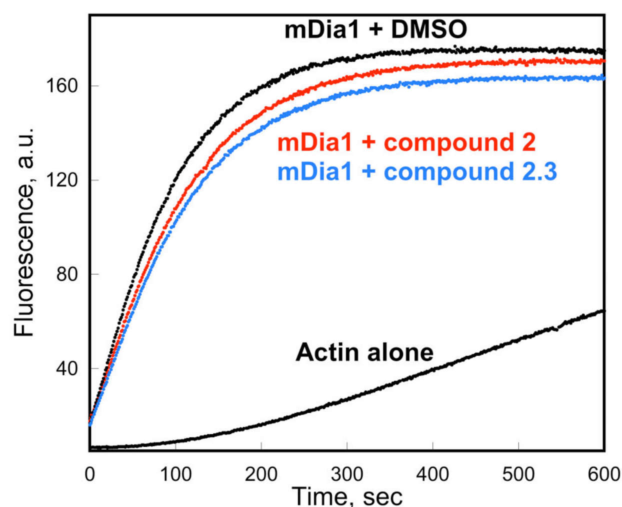

Figure S3. Inhibition by **2** and **2.3** is reversible on compound dilution. mDia1-FH2 (1.25 μM) was mixed with DMSO, **2**, or **2.3** (50 μM of compound) in polymerization buffer for 5 min at 23˚C. This mixture was diluted 1/1000 into a polymerization reaction containing 4 μM actin (5% pyrene label), and actin polymerization kinetics were recorded.

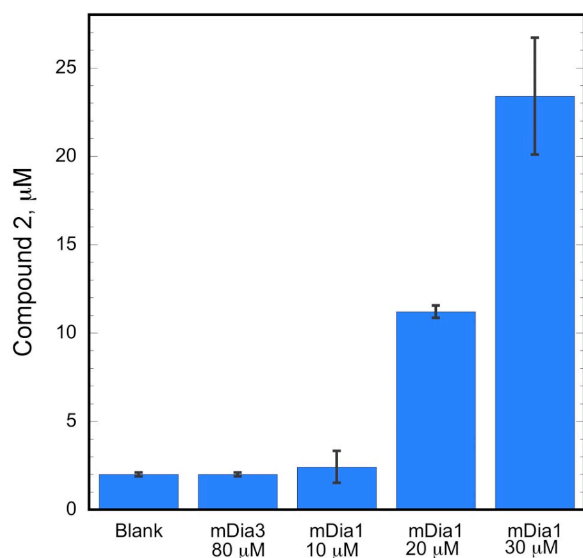

Figure S4. Equilibrium dialysis of compound 2 with mDia1 and mDia3. Proteins at the indicated concentrations were dialyzed (0.5 mL of each sample) against 1 L of dialysis buffer containing 2 μM compound 2 for 72 hrs at 4˚C. The concentration of compound 2 in each dialyzed sample was measured by absorbance at 380 nm.

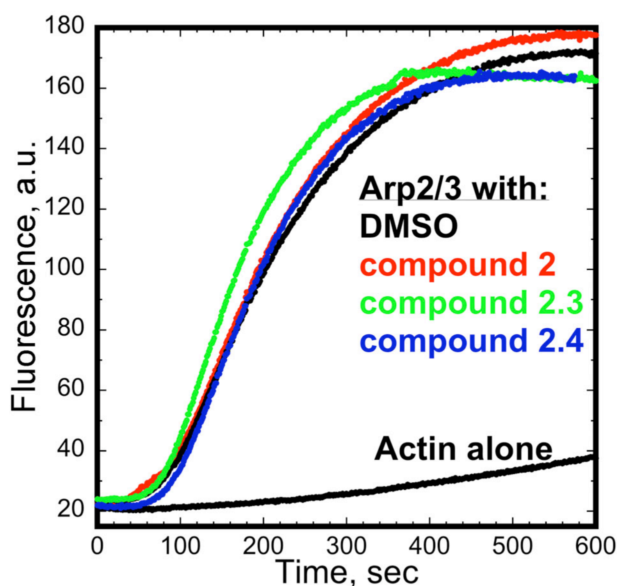

Figure S5. Effect of compounds on Arp2/3 complex activity.
Compounds 2, 2.3, and 2.4 were tested at 50 μM for effects on Arp2/3 complex (2.5 nM) in the presence of 50 nM GST-VCA. Actin concentration 4 μM (5% pyrene label).

Materials and Methods

Recombinant proteins

Murine mDia1-FH2 (amino acids 748-1175) and mDia2-FH2 (amino acids 612-1034) were expressed and purified from *E. Coli* as a GST-fusion protein and the GST was removed by thrombin cleavage as described (*1*). Murine mDia3-FH2 (amino acids 676-1100) was expressed and purified following the same method as for mDia1 and mDia2. Murine INF2-(FH1-FH2) (amino acids 538-993) was expressed and purified from *E. Coli* as a 6xHis-tagged protein, and the 6xHis tag was removed by thrombin cleavage as described (*2*). Murine FRL1-(FH1-FH2) (amino acids 449-1094) was expressed and purified from *E. Coli* as a GST-fusion protein and the GST was removed by thrombin cleavage as described (*3*). Expression and purification of GST-VCA and N-WASP have been described (*4*).

Compound sources

Compounds **1**, **2**, **2.4**, and **2.9** were obtained from ChemDiv, Inc. **2.3** was from Ryan Scientific, Inc. **2.5**, **2.6**, **2.8**, and **2.10** were purchased from ChemBridge, Inc. **2.7** was obtained from InterBioScreen Ltd.

Actin and Arp2/3 complex preparation

Rabbit muscle actin was purified from acetone powder (*5*) and labeled with pyrenyliodoacetamide (*6*). For single cuvette assays, both labeled and unlabeled actin was gel filtered on S-200 column (*7*). For high-throughput screen, actin was used without gel filtration. Arp2/3 complex was purified from bovine brain as described (*4*).

High-throughput screen
Recombinant mDia1-FH2 was diluted to 10 nM in Dilution Buffer (64 mM KCl, 1.6 mM $MgCl_2$, 1.5 mM EGTA, 15 mM imidazole pH 7.0, 1.7 mM Tris HCl pH 8.0, 0.425 mM DTT, 0.17 mM ATP, 0.009% sodium azide, 0.02% Thesit) and 20 µl was aliquotted into each well of a 384-well black reaction plate (Corning #3820) using a BioTek microFill. A library of structurally diverse, pre-plated compounds was obtained from ChemDiv, Inc (San Diego). Individual compounds (~20 nl) were then added by pin transfer from stock plates containing 5 mM stocks of each compound solubilized in dimethyl sulfoxide (DMSO) using a CyBi-Well (CyBio). In each plate, 16 wells (column 2) received DMSO alone as a negative control and 16 wells (column 23) received no mDia-FH2 (buffer only, positive control). The reaction was initiated by the addition of 10 µl of 3 µM monomer actin (~30% pyrene-labeled) in G buffer (2mM Tris-HCl pH8, 0.5mM DTT, 0.2mM ATP, 0.1mM $CaCl_2$, 0.01% Sodium Azide) by microFill. Immediately after actin addition, plates were transferred to an Envision plate reader (Perkin Elmer) and fluorescence intensity was measured kinetically over ~10 minutes at room temperature with no delay between reads using excitation filter Umbelliferone (355 nm /40 nm bandwidth), emission filter MOCA (400 nm/25 nm) and dichroic mirror Umbelliferone FP D400. Under these conditions each well was measured 16 times over the 10 minutes. Kinetic data was exported to Microsoft Excel and maximum actin assembly rates during the timecourse were calculated from the slope of the pyrene fluorescence curve by linear interpolation within a 2.5 min sliding window across the 10 min reaction. Compounds that depressed the maximum actin assembly rate by greater than 3 standard deviations below that of the DMSO control reactions in the same plate where considered "hits." All compounds were screened in duplicate and those that were not identified as hits in both replicates were not considered further.

HTS "robustness" is described quantitatively by the Z' factor, which compares variability of an assay relative to its dynamic range (*8*). Low assay variability with respect to dynamic range (a high Z' value) is critical for statistically significant discrimination between "hits" and assay variability. Z' values were calculated for each plate using positive and negative control wells and plates with unacceptably low Z' values (< 0.5) were rejected. The average Z' value for screening plates across the entire HTS campaign was > 0.75, indicative of an excellent assay.

Cuvette-based pyrene-actin assay
This method has been described in detail (*9*). The final polymerization buffer contained 10 mM Imidazole pH 7.0, 50 mM KCl, 2 mM Tris-HCl pH 8.0, 1 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, 0.1 mM ATP, and 0.2 mM thesit. Thesit is the common name for the non-ionic detergent nonaethylene glycol monododecyl ether (Sigma-Aldrich P-9641). A stock of actin monomers, containing 13.33 µM total actin (with 5% of the actin being labeled with pyrene) was made in G-buffer (2 mM Tris pH 8.0, 0.5 mM DTT, 0.2 mM $CaCl_2$, 0.1 mM ATP) and kept on ice. Four minutes before the polymerization assay was started, the compound and the formin (or Arp2/3 complex and GST-VCA) were mixed in 1.5x polymerization buffer at 23˚C. Two minutes before the polymerization assay was started, the actin stock was mixed with a $1/10^{th}$ volume of 10 mM EGTA, 1 mM $MgCl_2$ at 23˚C. Polymerization was started by mixing 2 volumes of formin/compound mix with 1

volume of actin. Pyrene fluorescence (excitation 365 nm, emission 407 nm) was monitored in a PC1 spectrofluorometer (ISS, Champaign, IL) or an LS50B spectrofluorimeter (Perkin Elmer). The time between final mixing and start of data collection ranged between 10 and 15 s for each assay.

Equilibrium dialysis

All proteins were pre-dialyzed in polymerization buffer prior to experiment. For the experiment, samples (0.5 mL) were placed in Slide-A-Lyzer® dialysis cassettes (Thermo Scientific, 0.1-0.5 mL capacity, 10,000 MWCO) and dialyzed against 1 L of polymerization buffer containing 2 μM compound 2 for 72 hrs at 4˚C with rapid stirring of dialysis buffer. The concentration of compound 2 in dialyzed samples was quantified by absorbance at 380 nm, blanking with polymerization buffer (extinction coefficient of compound 2 is 10,000 $M^{-1}cm^{-1}$ at 380 nm).

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