Supplemental Figure

Supplemental Fig. 1. **NWASP L229P and L232P proteins are constitutively active.** Western blot analysis of Sepharose beads coated with each WASP/NWASP construct after incubation with cell lysates. Blots were probed with antibodies against GST, actin, or Arc34 (component of the Arp2/3 complex). The samples were loaded as follows: Lane 1 (purified GST-WASP and purified actin), Lane 2 (rat NWASP WT), Lane 3 (rat NWASP L229P), Lane 4 (rat NWASP L232P), Lane 5 (mouse WASP WT), Lane 6 (mouse WASP L272P), Lane 7 (human WASP S272P), and Lane 8 (GST-only beads). Note the enhanced levels of actin and Arc34 associated with NWASP L229P and L232 compared to WT NWASP (and mWASP L272P and hWASP S272P compared to WT mWASP), indicating an enhanced level of actin polymerization

Supplemental Fig. 2**. NWASP-deficient fibroblasts expressing NWASP phospho-regulatory or constitutively active constructs result in increased migration.** Stable cell line monolayers were wounded using a pipette tip and images at 19h post injury are depicted. The boundary of the original wound is overlayed onto the image to indicate the amount of wound closure that has occurred. Note the increase in migration of all mutant constructs compared with WT NWASP.

Supplemental Fig. 3. **Modified Plaque Assay.** Stable cell line monolayers were infected with GFP-expressing *Shigella*. After 48h, a 20x22 grid of images was taken with a 10x objective and combined to form a montage image. The central image shows the montage image with the GFP channel (*Shigella* plaques) overlayed on the phase images (MEFs). The two insets depict one image from the montage containing a single *Shigella* plaque. The upper panel is the GFP image overlayed onto the phase image, and the lower image is the GFP only image.

Supplemental materials and methods

Actin polymerization bead assay

Assay was performed as described previously [11]. GST-WASP/NWASP constructs were loaded onto glutathione-coated beads as described above. U937 cells (1×10^7) were lysed in 1 mL polymerization buffer, PB (1% NP40, 130 mM NaCl, 20 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1% aprotinin, 10 µg/mL leupeptin, 1 mM phenylmethanesulfonyl fluoride), and clarified by centrifugation at 20,000*g* for 10 minutes. WASP/NWASP-coated beads (25 µL) were incubated with 250 µL U937-PB supplemented with 5 mM MgCl2 at room temperature for 1 hour with rotation. As a negative control, 2 µM cytochalasin D was added to the lysate to inhibit F-actin polymerization. Beads were washed twice with PB/MgCl2, resuspended in NuPAGE LDS sample buffer (Invitrogen), and heated to 70°C for 10 minutes. Bound proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using 4% to 12% NuPAGE Bis-Tris gels (Invitrogen), blotted, and stained with antibodies against GST (B-14: sc-138 monoclonal mouse anti-GST; Santa Cruz Biotechnology, Santa Cruz, CA) to reflect the WASP/NWASP, or actin (AC-15 monoclonal mouse anti–β-actin; Sigma).

Migration Assay. A confluent monolayer of cells was scratched using a pipette tip. The same region of the scratch was imaged using a 4x objective at 0, 12, and 19 hours after the injury. The area of the scratch devoid of cells was measured using ImageJ software. Three scratches per monolayer were measured for each experiment. The experiment was completed three times.

Modified plaque assay. Exponential phase bacteria expressing GFP were centrifuged at 1000 rpm for 15 min onto a confluent monolayer of cells seeded in a 6-well plate at an MOI of 1. Cells were

incubated for 1 hour at 37ºC with 5% CO_2. Cells were washed three times with PBS, incubated for 48 hours in media containing 50µg/ml gentamicin. Cells were washed 1 time with PBS and covered with DMEM. Plates were image on a microscope with an automated stage (IX-70; Olympus Optical). Using a 10x objective, a 20 image x 22 image grid was captured, in both phase and GFP channels, and stitched together. Using Slidebook software, the GFP image was analyzed and objects greater than 25 voxels in size (about 50µm in diameter) were considered plaques. This parameter was arbitrarily chosen because it sufficiently removed background signals. Analysis with a threshold of 20 or 30 voxels yielded similar results.