SUPPLEMENTAL METHODS;

MASS SPECTROSCOPY:

PC3 cells expressing either HA-WASF3 and empty vector were harvested in lysis buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM MgCl2, 10% (v/v) glycerol, 1% (v/v)Triton X-100), proteinase inhibitors (at 1:100 dilution, Fisher, PA), phosphatase inhibitor mixture (P2 and P3, at 1:100 dilution, Sigma, MO), and 1 mM sodium orthovanadate. The cell lysates were centrifuged at 12,000 x g for 15 min, and the cleared lysates were incubated with anti-HA antibody or control rabbit IgG (Santa Cruz, CA) overnight at 4 ºC. The immune complexes were immobilized on the protein A/G ultralink resin (Thermo Scientific Pierce, IL). The beads were washed three times in lysis buffer and recovered by centrifugation, dried in a Genevac EZ-2^{plus} vacuum evaporator (Genevac, Inc., Gardiner, NY) and re-suspended in 20 μL 8M urea/100 mM ammonium bicarbonate (ABC), with 0.6 μL of 100 mM Dithiothreitol (DTT) in 100 mM ABC (i.e. 3 mM DTT). After adjusting to room temperature, 1.5 μL of 200 mM iodoacetamide (IAA) in 100 mM ABC (final concentration of 15 mM IAA) was added. Alkylation was then carried out by incubating the mixture for 1 hour in the dark. Then, 1.5 μL of 200 mM DTT/100 mM ABC was added to remove un-reacted IAA. The urea concentration was reduced to ~1 M by diluting the mixture with 140 μL of 50 mM ABC/2 mM $CaCl_2$. Digestion was carried out by adding 6 μL of 0.40 μg/μL (2.4 μg) of Promega Sequencing Grade trypsin and incubating for 24 h at 37°C. The reaction was stopped by adjusting to pH = 5 with 10% TFA. Samples were then desalted using a manual, Micro Trap desalting cartridge (Michrom BioResources, Auburn, CA). First, the micro Trap was washed with 80 μL of LCMS Solvent B (90/10/0.05% : Acetonitrile/water/heptafluorobutyric acid

(HFBA), followed by equilibration with 80 μL of LCMS Solvent A (2/98/0.05% : Acetonitrile/ water/ heptafluorobutyric acid (HFBA)). Then, 20 μL of the peptide digest was loaded onto the micro Trap and salts were removed by washing with 50 μL aliquots of LCMS solvent A. Tryptic peptides were then eluted from the micro Trap with 16 μL of buffer B. Desalted peptides were evaporated to dryness on Genevac EZ-2^{plus} vacuum evaporator. Nano-HPLC, using Nano Trap Column (Michrom CL5/61241/00); 5μm 200Å Magic C_{18} AQ 75 μm x 150 mm, was carried out using an Agilent 1200 Series Nano Pump (Agilent Technologies, Santa Clara, CA), equipped with a refrigerated autosampler. An Agilent 1200 Series Capillary LC loading pump was used to introduce the sample onto a Captrap cartridge (sample concentration and de-salting) at 5 μL/minute for 5 minutes. An optimized 200-minute water/acetonitrile gradient consisted of the following settings: *Time (min), Flow rate (µl/min), Solvent B (%)* :: (0.00, 0.30, 5.00 :: 5.00, 0.30, 5.00 :: 30.00, 0.30, 15.00 :: 90, 0.30, 40.00 :: 120.00, 0.30, 60.00 :: 180.00, 0.300,90.00 :: 190.00, 0.30, 90.00 :: 195.00, 0.30, 5.00 :: 200.00, 0.30, 5.00. Solvent B was HPLC Grade Acetonitrile (Honeywell Burdick & Jackson, Morristown, NJ) with 0.05% HFBA (Pierce Biotechnologies, Rockford, IL) to achieve the required concentration. Data-dependent MS and MS/MS spectra were acquired on an LTQ Orbitrap Discovery™ (Thermo Fisher Scientific, San Jose, CA). MS/MS spectra were acquired using 2 microscans, a maximum injection time of 200 ms and a precursor isolation width of 2 Da. The normalized collision energy was set at 35%. Six scan events were recorded for each data acquisition cycle. The first scan event, acquired by the FTMS, was used for full scan MS acquisition from 300-2000 *m/z*. Data were recorded in the Centriod mode only (scan event #1 does not permit Profile mode of data acquisition).

The remaining five scan events were used for collisionally activated dissociation (CAD): the five most abundant ions in each MS were selected and fragmented to produce product-ion mass spectra.

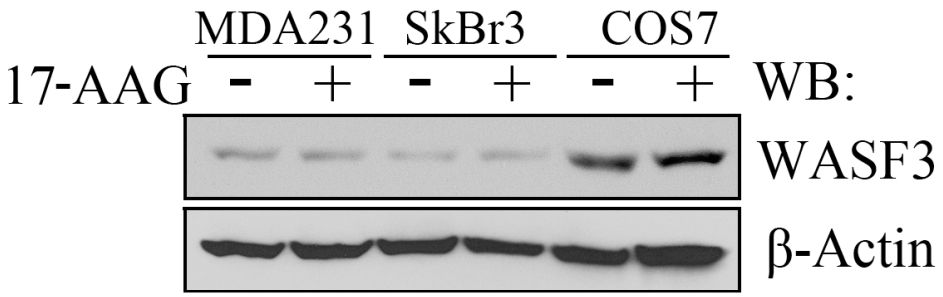

Figure S1. Treatment of breast cancer MDA-MB-231 and SkBr3 cells, as well as COS7 cells, with 1 μM 17-AAG does not affect WASF3 protein levels. β-Actin levels were used as a loading control.

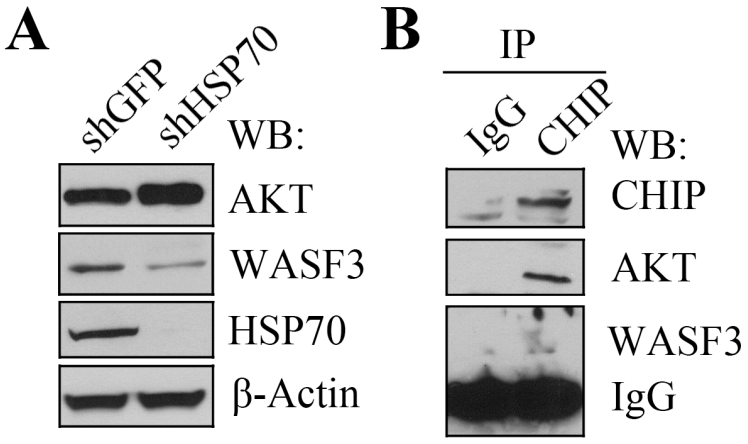

Figure S2. HSP70 regulates WASF3 and AKT proteins by different molecular mechanism. (A) Knockdown of HSP70 in MDA-MB-231 cells leads to a decrease in WASF3, but increases AKT levels. (B) IP from MDA-MB-231 cells shows that AKT, but not WASF3, is present in the CHIP immunocomplex.

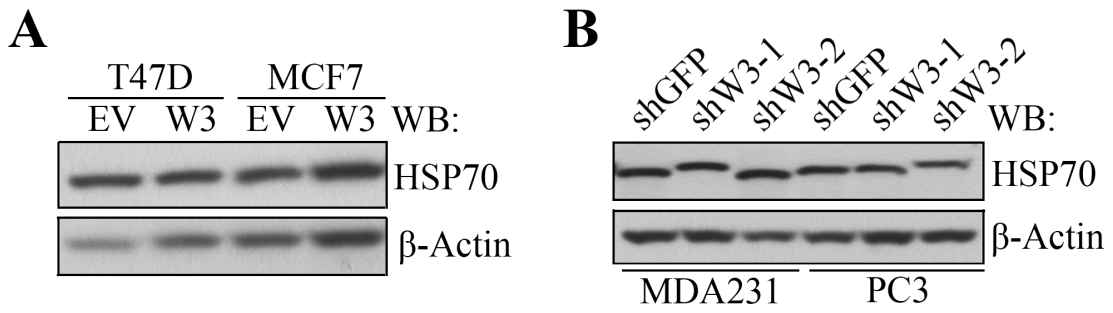

Figure S3. WASF3 does not influence HSP70 levels. Extracts from WASF3 knockdown MDA-MB-231 and SkBr3 cells (A) or WASF3 overexpressing MCF7 and T47D cells (B) were subjected to Western blot analysis using anti-HSP70 antibodies. Neither overexpression nor knockdown of WASF3 affects HSP70 expression levels.

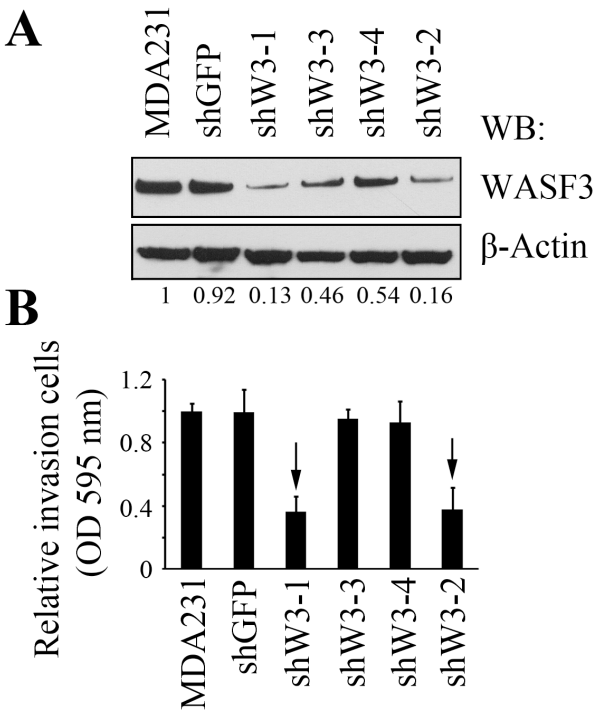

Figure S4. Only when WASF3 is suppressed > 80% is invasion in breast cancer cells reduced. (A) Western blot analysis shows shW3-1 has the greatest WASF3 knockdown effect compared with the other shRNA targeting *WASF3*. Numbers below the lanes indicate the relative (normalized % of MDA231 parental cells) protein levels following ImageJ quantitation. (B) Transwell assays show only those cells stably expressing shW3-1 and shW3-2 lead to reduced invasion potential. In this experiment, shGFP was used as a non-specific control and shW3-1, shW3-2, shW3-3 and shW3-4 represent shRNAs targeting different sequences within the *WASF3* gene.