Supplementary Information

Legends to Supplementary Figures

Supplementary Figure 1. Withaferin A inhibits growth of breast cancer cells. (**A**) Breast cancer cells were treated with various concentration of withaferin A (WFA) as indicated and subjected to anchorage-dependent clonogenicity assay**.** (**B**) Cell viability of MCF7, MDA-MB-231 and MDA-MB-468 cells treated with various concentration of WFA. *, $P<0.001$, compared with vehicle-treated controls (denoted with letter "C").

Supplementary Figure 2. Withaferin A inhibits growth of breast cancer cells. (**A-D**) SUM159, SUM149, T47D and BT474 cells were treated with various concentrations of WFA as indicated followed by MTT assay. * $P< 0.01$, compared with control.

Supplementary Figure 3. Withaferin A inhibits growth of breast cancer cells and reduces ATP levels. **(A-D)** MDA-MB-468, SUM 159, T47D and SKBR3 breast cancer cells were treated with various concentrations of WFA as indicated followed by MTT assay. * $P< 0.01$, compared with control. **(E)** MDA-MB-468, SUM 159, T47D and SKBR3 breast cancer cells were treated with various concentrations of WFA as indicated and intracellular ATP production was measured. Relative ATP levels are expressed with respect to the control. * $P< 0.001$, compared with control.

Supplementary Figure 4. Withaferin A inhibits growth of breast cancer cells. (**A-D**) MCF7, MDA-MB-231, MDA-MB-468 and SKBR3 cells were treated with various concentrations of WFA as indicated followed by trypan blue exclusion assay. * $P< 0.005$, compared with control.

Supplementary Figure 5. Withaferin A increases apoptosis in breast cancer cells. (**A-C**) MCF7, MDA-MB-468 and MDA-MB-231 cells were treated with various concentrations of WFA as indicated followed by Hoechst 33342 staining apoptosis detection. Mean number of apoptotic cells are presented in bar graphs. * $P< 0.01$, compared with control.

Supplementary Figure 6. Inhibition of autophagy does not impact WFA-mediated inhibition of clonogenicity. (**A, B**) MCF7 and MDA-MB-231 cells were treated with 5 μM WFA alone or in combination with 200 nM Baf,

25 μM CQ and 4 mM 3MA as indicated and subjected to clonogenicity assay. Representative duplicate wells are shown for each treatment.

Supplementary Figure 7. Inhibition of AMPK hinders WFA-mediated growth inhibition while AMPK potentiates it. (**A**) Total protein lysates from MDA-MB-231-derived xenograft tumors from vehicle-treated and WFA-treated mice were examined for the expression of phospho-PRKAA1. ACTB was used as a loading control. (**B-E**) MCF7, MDA-MB-231, SUM149 and SUM159 cells were treated with WFA (5 μM), compound C (Com. C) and AICAR alone and in combination as indicated and subjected to XTT assay. Bar graph shows fold change in cell viability. * $P< 0.01$, compared with control; ** $P< 0.01$, compared with WFA alone; # $P< 0.005$, compared with WFA alone.

Methods

Materials and methods are given in main manuscript. Additional details are provided here.

Clonogenicity assay

Anchorage-dependent clonogenicity assay[71] was conducted in the presence of WFA. Single-cell suspension of breast cancer cells were plated in 12-well plates at a density of 1000 cells per well followed by treatment with WFA. The medium was removed after treatment-period and colonies were fixed with formalin and stained with crystal violet (0.1% in 20% methanol) (Sigma-Aldrich, C3886) and colony numbers were assessed visually. Colonies containing >50 normal-appearing cells were counted and pictures were taken using a digital camera.

Cell-viability assay

Viability of breast cancer cells was examined using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay (Thermo Fisher Scientific, M6494) following the manufacturer's instructions. Breast cancer cells were plated in 96-well plates at an initial density of 5×10^3 cells/well for 24 h followed by WFA treatment and were subjected to MTT assay. Cell densities used in experiments performed were within the linear range of the MTT assay. A standard curve was prepared using cell densities from 1×10^3 to 1×10^6, and the results were calculated with respect to the number of cells.

Transmission Electron Microscopy

Breast cancer cells were treated with WFA or vehicle-control for 3 or 6 h and fixed with electron microscopy fixing buffer (2.5% glutaraldehyde, 3 mM $MgCl_2$, in 0.1 M sodium cacodylate buffer, pH 7.2) at 4^oC overnight. Cells were rinsed with 3 mM $MgCl_2$, 3% sucrose (Sigma, S0389), 0.1 M sodium cacodylate, thrice, and postfixed in reduced 1% osmium tetroxide with 0.8% potassium ferrocyanide, 0.1 M sodium cacodylate for 2 h on ice in the dark. Cells were rinsed 3 times with 0.1 M Maleate buffer (pH 6.2) followed by staining with 2% uranyl acetate (0.22 µm filtered, 1 h, dark) in 0.1 M maleate buffer and dehydration through a graded series of ethanol (30 to 100%). Specimens were embedded in EPON, sectioned, stained and examined with an H7600 transmission electron microscope (Hitachi, Tokyo, Japan).

Immunofluorescence and confocal imaging

Breast cancer cells were fixed and immunofluorescently stained[72] followed by imaging using a Zeiss LSM510 Meta (Zeiss, Dublin, California, USA) laser scanning confocal system conFig.d to a Zeiss Axioplan 2 upright microscope (Zeiss, Dublin, California, USA). Intralysosomal function was measured using LysoTracker Red. Breast cancer cells were cultured on coverglass slide chamber and treated with vehicle or WFA followed by incubation with 50 nM LysoTracker Red DND-99, and the fluorescence intensity was observed under the confocal microscope. Multiple view-fields were examined and representative cells from 3 view-fields were photographed. For the *mRFP-EGFP-LC3B assay*, breast cancer cells were seeded in 6-well plates with microscope cover glasses, transfected with mRFP-EGFP-LC3B (Addgene, 21074; deposited by Tamotsu Yoshimori) using Fugene (Promega, E2311) for 24 h followed by treatment with vehicle-control or WFA. At the end of treatment, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS; FisherScientific, 70-013-032) and examined with a confocal microscope. Cells with GFP-$LC3B^+$ puncta (green) or mRFP-$LC3B^+$ (red) or GFP^+ $mRFP^+$ (yellow) puncta were examined, imaged and at least 50 to 100 cells/sample were counted in triplicates.

Immunoblotting

Breast cancer cells were scraped in modified RIPA buffer to prepare whole cell lysates.[73,9] Total protein was quantitated using Bradford Reagent (BioRad, 5000006) and equal amounts of lysate protein were resolved on

sodium-dodecyl sulfate polyacrylamide gels, and transferred to PVDF membrane followed by western blot analysis. Immunodetection was performed using an enhanced chemiluminescence HRP antibody detection system (Denville Scientific, E2500) according to the manufacturer's instructions. The immunoblots are representative of multiple independent experiments and bar diagrams are included showing quantification of western blot signals.

RNA isolation and RT-PCR

For RNA isolation and RT-PCR,[74,75] total cellular RNA was extracted using the TRIzol Reagent (ThermoFisher Scientific, 15596026). RT-PCR was performed using specific sense and antisense PCR primers. *ATG5*: Forward 5' TTG ACG TTG GTA ACT GAC AAA GT-3', Reverse 5'- GCT CTT CCT TGG AAC A-3'; *BECN1*: Forward 5'- ACC TCA GCC GAA GAC TGA AG-3', Reverse 5'- TGT CAG AAC TAC AAA CGC TGT T-3'; *ATG7*: Forward 5'- AAG CAA GAG AAA GCT GGT CAT C-3', Reverse 5'- AGT AGC AGC CAA GCT TGT AAC C-3'; *ACTB*: 5'- ACC ATG GAT GAT GAT ATC GC-3', Reverse 5'- ACA TGG CTG GGG TGT TGA AG-3'.

Breast tumorigenesis assay

MDA-MB-231 cells xenografts from our previously published study[9] were used to determine the effect of withaferin A on autophagy. MDA-MB-231 xenografts were generated, grouped in 2 experimental groups (8 mice/group) and treated with intraperitoneal injections of either vehicle (10% DMSO, 40% cremophor-EL, and 50% PBS) or vehicle containing 4 mg Withaferin A (ChromaDex Inc., Irvine, CA)/kg body weight 5days/week for 5 weeks. The dose and route of WFA administration were selected from previous study documenting *in vivo* efficacy of WFA. Tumors were collected after 4 weeks of treatment. In this study, WFA administration resulted in a statistically significant decrease in tumor growth[9]. Tumors from this study were subjected to analysis by immunohistochemistry and western blotting. At least four random, nonoverlapping representative images from each tumor section from eight tumors of each group were captured using ImagePro software for quantitation of LC3B and SQSTM1 expression. All animal studies were in accordance with the guidelines of Johns Hopkins University IACUC.

STK11/LKB1 stable knockdown using lentiviral short hairpin RNA

We purchased five premade lentiviral *STK11/LKB1* short hairpin RNA (shRNA) constructs and a negative control construct created in the same vector system (pLKO.1) from Open Biosystems (RHS4533). Paired *STK11/LKB1* stable knockdown cells were generated following our previously established protocol.[76] Transient lentivirus stocks were prepared following the manufacturer's protocol. 1.5 x 10^6 293T cells were cotransfected with shRNA constructs (3 µg), 3 µg pCMV-dR8.2 dvpr [pCMV-dR8.2 dvpr was a gift from Bob Weinberg (Addgene, 8455)] and 0.3 µg pCMV-VSV-G [pCMV-VSV-G was a gift from Bob Weinberg (Addgene, 8454)] helper constructs using Lipofectamine 2000 (ThermoFisher Scientific, 11668027) or Fugene (Promega, E2311). Transfected cells were incubated for two days followed by harvesting of viral stocks from the culture medium and filtered to remove nonadherent 293T cells. Selection of MCF7 and MDA-MB-231 cells stably expressing shRNA constructs: MCF7 and MDA-MB-231 cells were plated at subconfluent densities and infected with a cocktail of 1 mL of virus-containing medium, 3 mL of regular medium, and 8 µg/mL polybrene (Sigma-Aldrich, TR-1003). Selection with 0.5 to 2 µg/mL of puromycin (Sigma-Aldrich, P9620) was started 48 h after lentivirus infection. After 4 weeks of selection for MCF7 and MDA-MB-231 cells, monolayers of stably infected pooled clones were harvested for use and cryopreserved.

Apoptosis

Cells were cultured on chamber slides, treated with WFA followed by Hoechst 33342 staining (Thermo Fisher Scientific, H1399) according to the manufacturer's protocol. Apoptotic cells with condensed pycnotic nuclei were counted in 8-10 randomly selected, non-overlapping fields.

Acridine Orange staining

Breast cancer cells were cultured on 6-well plates and treated with vehicle or 2.5 uM or 5 uM WFA for 24 h followed by incubation with acridine orange (Sigma-Aldrich, A9231) for 15-30 min. At the end of incubation, cells were washed twice with PBS, fixed with 4% paraformaldehyde and were examined with confocal microscope.

ATG7 and BECN1 knockout with CRISPR/Cas9

Following oligos were use: *ATG7* (NM_006395.2): set1-Forward 1: CAC CGA ATC AAG TAT GAT GAG AAC A and Reverse 1: AAA CTG TTC TCA TCA TAC TTG ATT C; set2- Forward 2: AAA CTG TTC TCA

TCA TAC TTG ATT C and Reverse 2: AAA CGG ACG ACT CAC AGT GCA CTG C. *BECN1* 1(NM_003766.3): set1-Forward 1: AAA CGG ACG ACT CAC AGT GCA CTG C and Reverse 1: AAA CGC ATG GTG CTG TTG TTG GAC C; set2- Forward 2: CAC CGG CCA ACA GCT TCA CTC TGA T and Reverse 2: AAA CAT CAG AGT GAA GCT GTT GGC C. Digested and purified LentiCRISPRv2 plasmid [lentiCRISPRv2 was a gift from Feng Zhang (Addgene, 52961)] was incubated with phosphorylated, annealed oligos for *ATG7* and *BECN1* in a ligation reaction, transformed into Stbl3 bacteria (ThermoFisher Scientific, C7373-03). Plasmid DNA was purified and sequenced for verification of correct insertion. lentiCRISPR with inserted sequences were cotransfected into HEK293T cells with packinging plasmids pVSVg [pVSVg was a gift from Tannishtha Reya (Addgene, 14888)] and psPAX2 [psPAX2 was a gift from Didier Trono (Addgene, 12260)]. MCF7 cells were transfected twice, and selected for a week. MCF7 cells were examined for the *ATG7* and *BECN1* knockout using immunoblot analyses.

Survival analysis

We assessed the correlation between AMPK and LDHA expression and survival using an independent multicentre gene expression database established as described previously (PMID: 23836010). All samples were measured using Affymetrix HGU133A or HUG133 plus 2 gene chips. For AMPK and LDHA, the probe sets 207709_at and 200650_s_at were used, respectively. Both probe sets have eleven probes and a complete coverage of all known transcript variants. The entire combined dataset contains 4,374 patients, of which 696 were estrogen receptor (ER) negative and HER2 negative (hence on termed as TNBC cohort). The mean follow-up of all patients was 71.5 months. ER and HER2 status were determined using the probe sets 205225_at and 216836_s_at, respectively because receptor status determined by immunohistochemistry was not available for all patients. For estrogen receptor, positivity was set for patients having a normalized expression over 500, the cutoff for HER2 was 4800. We performed Cox proportional hazards regression analysis to correlate expression and relapse-free survival and Kaplan-Meier plots were drawn for visualization.