SI APPENDIX

SUPPLEMENTAL MATERIAL AND METHODS

Cell Culture

MDA-MB-231 and MDA-MB-468 human breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in Leibovitz's L15 medium (Thermofisher, Carlsbad, CA) supplemented with 10% fetal bovine serum (Cellmax, Lanzhou, China), penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37 ºC in humidified incubators with 5% CO_2.

FSIP1 silencing

MDA-MB-231 and MDA-MB-468 cells ($5x10^5$ cells/well) were cultured in 6-well plates and were transfected at 70% confluency with FSIP1 (5'-CAG UGU UUC AUA CUC AAA TT-3'), AMPK (5'-GAG GAG AGC UAU UUG AUU ATT-3') or control siRNA (5'-UUC UCC GAA CGU GUC ACG UTT-3') using Mission siRNA transfection reagents (Sigma, St. Louis, MO) for 48 h. Efficacy of gene silencing was determined by Western blot. Stable MDA-MB-231 cells expressing control or FSIP1-silencing shRNA were generated following transduction with lentivirus (OriGene, Rockville, MD) at a multiplicity of infection of 10 using polybrene (4 µg/ml, Sigma) and selection with puromycin (10 µg/ml, Sigma).

Generation of FSIP1 knockout MDA-MB-231 cells

Oligonucleotide encoding FSIP1-targeted gRNA (5'-GCT CAG GGG TAA AC ACA ACC-3') was synthesized (Umangen, Shanghai, China) and cloned into the pX330 vector (Addgene, Cambridge, MA) to generate pX330-FSIP1-SgRNA5. MDA-MB-231 cells (2×10^5 cells) were transfected with pX330-FSIP1-SgRNA5 and pPGKpuro (Addgene) using lipofectamine 2000 (Invitrogen). Cells were selected using puromycin (1.0 µg/mL, Sigma) and were cloned by limiting dilution. Colonies were expanded for subsequent characterizations. Their genomic DNA containing the CRISPR/Cas9 target sequence was PCR-amplified to generate FSIP1 knockout (KO) cells. The efficacy of FSIP1 knockout was confirmed by Western blotting.

Cell proliferation assay

Cell proliferation and drug cytotoxicity were determined using CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Cat# G3582, Promega, Madison, WI) according to the manufacturer's instruction. Briefly, MDA-MB-231 and MDA-MB-468 cells expressing FSIP1-silencing or control shRNA ($1x10^3$ cells/well) as well as FSIP1 knockout MDA-MB-231 cells were cultured in 96-well plates for 24 or 48 h. Cells were exposed to 20 µl of freshly prepared 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazine ethosulfate (MTS/PES) solution for 4 hours. Absorbance at 490 nm was measured using a microplate reader (Bio-Rad, Hercules, CA).

Transwell migration assay

The impact of FSIP1 silencing on the migration of breast cancer cells was determined by transwell migration assay. Briefly, control or FSIP1-silenced MDA-MB-231 or MDA-MB-468 cells ($2 x10^4$ cells/well) were loaded on the upper chamber of 24-well transwell plates (8-µm pore size; Corning) in FBS-free medium. The bottom chambers were filled with complete medium. After 24 or 48 h, cells on the upper surface of the membrane were removed using cotton swabs and migrated cells on the bottom surface were stained with 0.1% crystal violet.

The numbers of migrated cells in five randomly selected fields were counted under a phase contrast microscope in a blinded manner.

Protein-protein Docking

The global rigid-body docking for FSIP1 F2 domain (amino acid residues 191-581) complexed with ULK1 was performed by ZDOCK software (1). The FSIP1 F2 domain was modeled with 25,000 decoys using the *ab initio* modeling protocol implemented in the ROSETTA software. The ULK1 crystallographic structure was obtained from the PDB database (PDB ID: 4WNO) (2). All possible FSIP1 F2 domain and ULK1 binding modes with shape complementarity, desolvation energy, and electrostatics were searched using a fast Fourier transform (FFT) algorithm. The quality of modeling results was evaluated by the Rosetta cluster application and DFIRE2 program. The best structure from the cluster was selected for further optimization and structure remodeling. Molecular dynamics was conducted using Gromacs 4.6.7 for the optimization of FSIP1 model over 100 ns. The FSIP1 structure was selected by g_cluster and total energy against the trajectory. The complex structure of FSIP1-ULK1 with the largest interaction area was selected.

Western blot analysis

Cells were lyzed in RIPA buffer containing PMSF, protease and phosphatase inhibitors (KEYGEN, Nanjing, China). Protein concentrations were measured using a BCA Protein Assay Kit, according to the manufacturer's instructions (Pierce). Total cell lysates (50 μg/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.025% Tween 20 (TBST), and incubated overnight at 4°C with primary antibodies. The antibodies included mouse anti-human FSIP1 (OriGene, TA806085), rabbit anti-human LC3 (Proteintech, 12135-1-AP), anti-ATG5 (Proteintech, 10181-2-AP), anti-ATG7 (Proteintech, 10088-2-AP), anti-Beclin1 (Proteintech, 11306-1-AP), anti-SQSTM1 (Proteintech, 18420-1-AP), anti-AXIN1 (Proteintech, 16541-1-AP), anti-TCF7L2 (Proteintech, 13838-1-AP), anti-GSK3β (Proteintech, 24198-1-AP), anti-UAP56 (Proteintech, 14798-1-AP), anti-GAPDH (Proteintech, 10494-1-AP), anti-β-Tubulin (Proteintech, 10068-1-AP), anti-ULK1 (Signalway, 43772), anti-p-ULK1^{Ser317} (Cell Signaling Technology, 12753s), anti-p-ULK1^{Ser757} (Cell Signaling Technology, 6888), anti-p-GSK3β (Cell Signaling Technology, 9323), anti-β-Catenin (Wanleibio, WL0962a), anti-mTOR (Cell Signaling Technology, 2972), anti-p-mTOR (Cell Signaling Technology, 5536), anti-AMPK (Cell Signaling Technology, 2532), anti-APC (Boster, BA0645), and anti-p-AMPK (Cell Signaling Technology, 8208). The bound antibodies were detected with horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, at 1:10,000 dilution). Protein signals were visualized using enhanced chemiluminescent reagents and quantified by densitometry using ImageJ (NIH).

Immunoprecipitation

MDA-MB-231 cells were harvested and lyzed in cold RIPA lysis buffer containing protease inhibitors, followed by centrifuging. The cell lysates (50 μg/tube) were incubated with anti-FSIP1, anti-ULK1 or control isotype IgG (2 μg) with gentle agitation at 4 ºC overnight. Subsequently, the reactive mixtures in individual tubes were added with 70 μl of protein G microbeads and incubated at 4 ºC for 4 h. After centrifugation, the microbeads were washed with TBST and the bound proteins were eluted with 2x SDS loading buffer. The eluted proteins were subjected to SDS-PAGE and immunoblotting with anti-ULK1 and anti-FSIP1 as well as anti-tubulin, respectively.

Seahorse assay

The impact of FSIP1 silencing on the mitochondrial respiration in MDA-MB231 cells was determined by the Seahorse assay in an XF24 extracellular flux analyzer (Seahorse Bioscience, North Billerica, MA), according to the manufacturer's instruction (3). Briefly, control and FSIP1-silenced MDA-MB-231 cells were cultured overnight in XF24 (V7) microplates ($2x10^4$/well). The cells were cultured in basic medium containing glucose, glutamine and sodium pyruvate at 37 ºC in a non-CO_2 incubator for 1 h and the basal oxygen consumption rates (OCR) of individual wells of cells were measured three times at 5-min intervals. Subsequently, cells were sequentially treated with oligomycin (an ATP synthase inhibitor), FCCP (a stimulator of oxygen consumption), and rotenone/antimycin A (Krebs cycle inhibitors, all from Sigma) to measure the ATP-linked, proton leak, maximal respiration, and spare respiratory capacity, respectively, after each treatment using the software equipped, normalized to protein concentrations measured by BCA assay.

Transmission electron microscopy (TEM)

Autophagosome formation in control and FSIP1-silenced MDA-MB-231 cells was examined by TEM. Briefly, cells were harvested, fixed in 2.5% glutaraldehyde and embedded. Ultra-thin 70-nm sections were prepared using a microtome, mounted on a copper grid, and stained with 4% aqueous uranyl acetate (10 min) and Reynolds lead citrate (2 min). Autophagosomes were imaged under a transmission electron microscope (JEM-2000EX, JEOL, Sagamihara, Japan).

***In vivo* tumor xenograft and treatment**

The experimental protocol was approved by the Animal Research and Care Committee of China Medical University, Shenyang, China. Female BALB/c nude mice at 6 weeks of age (Hunan SilaikeJingda Laboratory Animal, Changsha, China) were housed in a specific pathogen-free facility with free access to autoclaved food and water. To establish xenograft tumors *in vivo*, individual mice were first injected with $1x10^6$ control or FSIP1-silenced MDA-MB-231 cells into their flanks (n=5-8 per group). When subcutaneous tumors grew to 100-200 mm^3, the mice were randomized and treated intraperitoneally with vehicle or 10 mg/kg docetaxel on day 1, 7 and 14. The growth of tumors and the body weights of mice were monitored for 21 days, following which tumors were dissected and weighed. LC3, ATG5 and ATG7 expression levels in the tumor sections were determined by immunohistochemistry.

Statistical analysis

Data are expressed as mean ± SEM. Difference among groups was analyzed by one-way ANOVA followed by post-hoc Newman-Keuls test using SPSS Windows v.17.0. A p-value of <0.05 was considered statistically significant.

References

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3. Zhou Y*, et al.* (2017) Mitochondrial Spare Respiratory Capacity Is Negatively Correlated with Nuclear Reprogramming Efficiency. *Stem Cells Dev* 26(3):166-176.