Supplementary Materials and Methods

Western Blot (WB) Antibodies

The following antibodies purchased from Cell Signaling Technologies: pSrc (Y416) (6943), Src (2110), CPT1 (12252), pAMPK (T172) (2535), AMPK (2793), pACC (S79) (3661), ACC (3676), Ki67 (12202), vinculin (4650) and α-tubulin (2125). CPT2 (sc-377294) and β-actin (sc-47778) were purchased from Santa Cruz Biotechnology. ACAA2 (ab128911) was purchased from Abcam.

Western Blot (WB) analysis

Cell lysates in RIPA buffer with protease and phosphatase inhibitor cocktail were separated by SDS-PAGE, transferred to nitrocellulose membranes and stained with 0.1% ponceau in 5% acetic acid in water. After imaging, the membrane was washed with TBST (Tris-buffered saline with 0.1% Tween-20 detergent) and blocked with 5% milk in TBST. After overnight incubation with the primary antibody, the membrane was washed three times with TBST, followed by incubation with the secondary antibody with HRP (Horseradish Peroxidase) for two hours at room temperature. Membranes were visualized using a chemiluminescent detection kit.

Generation of Tamoxifen (Tam) and Fulvestrant (Ful) resistant cell lines

We have previously published the details of the generation of Tam-resistant (TR) MCF7 cell lines TR1 and TR2 (1). To generate MCF7 TRsa cells, MCF7 cells were seeded in low density in a 6-well plate. After overnight culturing, cells were treated with 1μM 4-hydroxy Tam every three days for 30 days. Cells were then reseeded at low density at a 6-well plate and continued to be treated with 1μM 4-hydroxy Tam every three days for another 30 days, until cell growth was no longer

inhibited by 1μM 4-hydroxy Tam. The resistance was confirmed by SRB proliferation assay and clonogenic assay. For T47D Ful resistant (FulR) cells, a similar culturing approach was used with T47D cells in the presence of 1 μM Ful instead of 1μM 4-hydroxy Tam. Except during specific comparative experiments, TR cells (TR1, TR2, and TRsa) were maintained in the complete medium with 4-hydroxy Tam at 1μM, 100nM, and 1μM respectively. Similarly, T47D FulR cells were maintained in the complete medium with 1μM Ful.

AMPK signature analysis

The activity of AMPK in MCF7, TR1, and TR2 cells was quantified using microarray data (deposited to GSE241654). AMPK signature analysis was performed as we previously published (3). Briefly, after robust multichip average (RMA) normalization (4), principal component analysis (PCA) was performed using R 4.2.2 software. The normalized data for each gene underwent log2 transformation, followed by z-score normalization across the cells. PCA for AMPK signature genes was performed with respect to genes and further improved the axes by picking up the most relevant genes. For this, the top 45% of the genes were selected according to the absolute values of the coefficient in the first principal component (PC1). Then, with the redefined AMPK gene set, we redid PCA for the microarray data with respect to cells and plotted the percentage of coefficient in PC1 as the activity.

MTT cell growth assay

Cell viability or proliferation was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) colorimetric assay. Cells were seeded in a 96-well plate and treated with drugs. After incubation, the cells were incubated with MTT solution for 4 hrs. The cells were then

dissolved in DMSO, and the absorbance was determined at 565 nm in an Infinite M200 plate reader (Tecan).

Transwell migration assay

Migration assays were performed using 24-well transwell inserts (Corning, 3464) according to the manufacturer's instructions. Briefly, cells in serum-free media were seeded in the top chamber, and 10% FBS in DMEM was used as a chemoattractant in the bottom chamber. Cells that migrated through the membrane were fixed with 100% methanol and stained with 0.5% crystal violet. The cells were imaged at 4X magnification and analyzed using a Cytation™ 5 (Agilent).

qRT-PCR

RNA was extracted from cells using the RNeasy Plus mini kit (Qiagen, 74134) according to the manufacturer's protocol. RNA (0.5 ~ 1μg) was then converted to cDNA using amfiRivert cDNA Synthesis Platinum Master Mix (Gene Depot, R5600). qRT-PCR was performed using amfiSure Green Q-PCR master mix (Genedepot, Q5603) and 2μL of 10X diluted cDNA. Primers used were ACAA2: F. GGCACTGAAGAAAGCAGGACTG, R. GTGACCCAAAGCAATGGCTCCT and β-actin: F. AGAGCTACGAGCTGCCTGAC, R. AGCACTGTGTTGGCGTACAG.

Clonogenic assay

A clonogenic assay was performed with a slightly modified protocol from a previous publication (5). Briefly, cells were cultured at a low density in a six-well plate for 10-14 days. Cells were treated with drugs every three days starting the day after the cells were seeded. After methanol fixation, cells were stained with crystal violet and imaged using GelCount™ (Oxford Optronix).

The stained crystal violet dye was dissolved in 10% acetic acid, and the absorbance was measured at 590 nm.

Soft agar assay

Soft-agar colony formation assay was performed in triplicate using 6-well cell culture plates as previously described (6). Cells were seeded in 0.35% agarose (top layer) on top of a layer of 0.5% agarose (bottom layer). Colonies were counted at different time points using a GelCount™(Oxford Optronix) colony counter.

Mitochondrial enzymatic activity assay

Mitochondrial electron transport chain (ETC) complex I and Krebs (TCA) cycle citrate synthase (CS) activities were measured using a spectrophotometer (7,8). Briefly, cell pellets were made from MCF7 cells in complete medium and TR cells in complete medium with 1uM Tam. After sonicating the cell pellets, protein concentration was determined using the Bradford assay. The enzymatic activities of complex I and CS were measured using an Infinite M200 plate reader (Tecan). NADH (complex1) and acetyl-coA and oxaloacetate (CS) were used as specific substrates. The enzymatic activities were normalized to protein concentration.

CPT1 activity assay

CPT1 activity assay was conducted by kinetics assay using the spectrophotometric method described previously (9). Briefly, cell pellets were made from cells in the complete medium or medium with 5% lipid depleted FBS (Biowest, S148L). For TR cells, 1uM Tam was added to the medium. After measuring the protein concentration of the cell lysate using the Bradford assay, 100

μg of each sample was pre-incubated with reaction buffer, and activity was measured in the presence of substrates (250 μM Palmitoyl-CoA and 1 mM L-carnitine) at 412 nm. CPT1 activity was calculated based on the change in the slope of the absorption.

Seahorse analysis

Metabolic flux was measured by Cell Mito Stress kit (Agilent, 103010-100) using a Seahorse XFp analyzer (Agilent) as previously described (6). Basal oxygen conception rate (OCR), maximal OCR, and ATP production were calculated using Seahorse wave software (Agilent). Cell number was used for the normalization.

Ki67 Immunohistochemistry

Ki67 immunohistochemistry (IHC) staining of the tumor tissues was performed at the Pathology Core lab facility of the Breast Center at Baylor College of Medicine. After IHC staining, three random areas from each slide were photographed using an OMAX compound microscope and manually counted the Ki67 positive cells.

References

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