Online Methods

Cell Culture

MCF-10A cells were cultured as described previously[30] and on http://brugge.med.harvard.edu/. MCF-10A cells expressing Bcl-2, ErbB2, E7/Bcl-2, PIK3CA (E545K), Myr-Akt1, IGF-1R or GFP-LC3 were generated as described previously[3]. The pMSCV-Neo–based retroviral vectors encoding wild-type human IGF-IR complementary DNA was a gift from R. Baserga (Thomas Jefferson University, Philadelphia, PA). HMEC cells were immortalised with hTERT and cultured as described previously[31]. BT-474 cells (ATCC) were cultured in RPMI 1640 media plus 10% FBS and pen/strep.

ATP Assays

For the comparison of ATP levels in detached vs. attached cells, the ATPlite assay (PerkinElmer, Waltham, MA) was used. Cells were plated in 96 well poly-HEMA coated (or uncoated) plates at a density of 13,333 cells per well. After 24 hours, ATP assay was conducted according to manufacturer's protocol. For the measure of ATP in detached cells (normalised by protein content), the ATP determination kit (Invitrogen, Carlsbad, CA) was used. Cells were plated in 6-well poly-HEMA coated plates at a density of 400,000 cells per well. After 24 hours, cells were lysed in 1% NP40 (plus protease inhibitors) and lysates were normalised by protein content using BCA Protein Assay (Pierce Biotechnology, Rockford, IL). Lysates were then tested for ATP levels according to manufacturer's protocol. The data from these assays show representative

experiments from more than three independent replicates. For determination of the ATP/ADP ratio, the ADP/ATP Ratio Assay Kit (BioAssay Systems, Hayward, CA) was used according to manufacturer's instructions. The data from the ATP/ADP assay is presented as an average of the ATP/ADP ratio from multiple experiments.

3D Cell Culture

To generate acini, cells were grown in reconstituted basement membrane (Matrigel) as described previously[30] and according the protocol at http://brugge.med.harvard.edu/. For antioxidant treatment, either NAC or Trolox were added to the Matrigel *and* to the overlay media. Immunofluorescence of acini was performed as described previously[30]. The following primary antibodies were used for immunofluorescence: cleaved caspase-3 (#9661, Cell Signaling Technology) and laminin-5 (mab19562, Millipore, Billerica, MA). DAPI (Sigma-Aldrich) was used to counterstain nuclei. For examination of luminal filling, acini were imaged using confocal microscopy to visualise the center of each structure, and then were scored as either clear (~90-100% clear), mostly clear (~50-90% clear), mostly filled (~10-50% clear), or clear (~0-10% clear). The figures including data from these assays show representative experiments from more than three independent replicates.

Reagents

The following reagents were used at the doses indicated and as described in the text/figure legends: methyl pyruvate (Sigma-Aldrich, St. Louis, MO), 2-deoxy-D-glucose (Sigma-Aldrich), DHEA (EMD Biosciences, San Diego, CA), 6-AN (Sigma-Aldrich), N-acetyl-L-cysteine (Sigma-Aldrich), Trolox (EMD Biosciences), etomoxir (Sigma-Aldrich), D-methyl malate (Sigma-Aldrich), Matrigel (BD Biosciences, San Jose, CA), poly(2-

hydroxyethyl methacrylate) (poly-HEMA, Sigma-Aldrich), LY294002 (EMD Biosciences), U0126 (EMD Biosciences), staurosporine (Sigma-Aldrich), and DL-Buthionine-(S,R)-sulfoximine (BSO, Sigma-Aldrich).

siRNA

The following siRNA SMARTpools (Dharmacon, Lafayette, CO) were used: G6PDH (M-008181-01), Beclin-1 (M-010552-00), ATG5 (M-004374-03), and EGFR (M-003114-01). The luciferase GL2 duplex (D-00110-01-20) was used as an siRNA control. Sequence information for each siRNA are as follows:

G6PDH SMARTpool

Duplex 1

Sense: GAGAGUGGGUUUCCAGUAUUU

Antisense: 5'-PAUACUGGAAACCCACUCUCUU

Duplex 2

Sense: CAACAUCGCCUGCGUUAUCUU

Antisense: 5'-PGAUAACGCAGGCGAUGUUGUU

Duplex 3

Sense: CGUGAGGCCUGGCGUAUUUUU

Antisense: 5'-PAAAUACGCCAGGCCUCACGUU

Duplex 4

Sense: GACCUACGGCAACAGAUAUU

Antisense: 5'-PUAUCUGUUGCCGUAGGUCAUU

Beclin-1 SMARTpool

Duplex 1

Sense: CUAAGGAGCUGCCGUUAUAUU

Antisense: 5’-PUAUAACGGCAGCUCCUUAGUU

Duplex 2

Sense: GGAUGACAGUGAACAGUUAUU

Antisense: 5’-PUAACUGUUCACUGUCAUCCUU

Duplex 3

Sense: UAAGAUGGGUCUGAAAUUUUU

Antisense: 5’-PAAAUUUCAGACCCAUCUUAUU

Duplex 4

Sense: GCCAACAGCUUCACUCUGAUU

Antisense: 5’-PUCAGAGUGAAGCUGUUGGCUU

ATG5 SMARTpool

Duplex 1

Sense: GGAAUAUCCUGCAGAAGAAUU

Antisense: 5’-PUUCUUCUGCAGGAUAUUCCUU

Duplex 2

Sense: CAUCUGAGCUACCCGGAUAUU

Antisense: 5’-PUAUCCGGGUAGCUCAGAUGUU

Duplex 3

Sense: GACAAGAAGACAUUAGUGAUU

Antisense: 5’-PUCACUAAUGUCUUCUUGUCUU

Duplex 4

Sense: CAAUUGGUUUGCUAUUUGAUU

Antisense: 5’-P UCAAAUAGCAAACCAAUUGUU

EGFR SMARTpool

Duplex 1

Sense: GAAGGAAACUGAAUUCAAAUU

Antisense: 5'-PUUUGAAUUCAGUUUCCUUCUU

Duplex 2

Sense: GGAAAUAUGUACUACGAAAUU

Antisense: 5'-PUUUCGUAGUACAUAUUUCCUU

Duplex 3

Sense: CCACAAAGCAGUGAAUUUAUU

Antisense: 5'-PUAAAUUCACUGCUUUGUGGUU

Duplex 4

Sense: GUAACAAGCUCACGCAGUUUU

Antisense: 5'-PAACUGCGUGAGCUUGUUACUU

Luciferase GL2 Duplex

5'-CGUACGCGGAAUACUUCGA dTdTdTdT GCAUGCGCCUUAUGAAGCU-5'

For each transfection, 200 nM of siRNA was transfected into cells using oligofectamine (Invitrogen) according to manufacturer's protocol. Knockdown efficiency was examined after 48 hours by western blotting as described below. For experiments involving siRNA in detached cells, cells were plated on poly-HEMA coated plates 24 hours after siRNA transfection and then assays were conducted 48 hours after siRNA transfection. The figures including data employing siRNAs show representative experiments from more than three independent replicates.

Western Blotting

Cells were lysed in 1% NP40 on ice for 20 minutes. Lysates were spun at 14,000 rpm at 4ºC for 30 min and normalised using BCA Assay (Pierce Biotechnology). Lysates were

then subjected to SDS-PAGE on polyacrylamide gels and transfer/blotting were performed as previously described[4]. The following antibodies were used for western blotting: G6PDH (ab993, Abcam, Cambridge, MA), Beclin-1 (#3738, Cell Signaling Technology, Danvers, MA), ATG5 (#2630, Cell Signaling Technology), cytochrome *c* (556433, BD Biosciences), EGFR (#2232, Cell Signaling Technology), P-Akt (#9271, Cell Signaling Technology), P-ERK (44-680G, Invitrogen) and β-actin (A1978, Sigma-Aldrich). The figures including Western blots show representative blots from more than three independent experiments.

Cell Death Assays

Lysates preparation and the measurement of cytochrome *c* release was conducted as described previously[32]. For measuring viability by dye exclusion, detached or attached cells were stained with trypan blue and placed in a hemacytometer. 200 cells were then counted and assessed as either trypan blue positive or negative.

Glucose Uptake Assay

For the analysis of glucose uptake, the Amplex Red Glucose Assay Kit (Invitrogen) was used. Cells were plated at a density of 13,333 cells per well in 96 well poly-HEMA coated (or normal) plates. After 24 hours, media was collected and diluted 1:4000 in water. The amount of glucose in the media was then determined using the Amplex Red Assay according to the manufacturer's instructions. Glucose uptake was determined by subtracting the amount of glucose in each sample from the total amount of glucose in the media (without cells). The data from these assays show representative experiments from more than three independent replicates.

ROS and Glutathione Assays

Cells were plated at a density of 13,333 cells per well in 96 well poly-HEMA coated (or normal) plates. After 24 hours, carboxy-H_2DCFDA (Invitrogen) was added to each well at a concentration of 10 μM and mixed well. Carboxy-H_2DCFDA is a cell-permeant indicator for reactive oxygen species that is retained in the cell after deacetylation and nonfluorescent until oxidation occurs within the cell. After 2-3 hours, absorbance was monitored on a fluorimeter. The figures including data from these assays show representative experiments from more than three independent replicates. Alternatively, reduced glutathione (GSH) was measured as a proxy for ROS using 7-amino-4-chloromethylcoumarin (CMAC, Invitrogen)[33]. CMAC was added to detached or attached cells at 40 μM and fluorescence was measured at an excitation of 360 nm and an emission of 460 nm. For the measurement of ROS in MCF-10A acini, the acini were stained with 25 μM carboxy-H_2DCFDA and 1 μM Hoescht 33342 for 1 hour at 37ºC. Acini were then immediately imaged using confocal microscopy.

Fatty Acid Oxidation Assay

Cells were plated at a density of 100,000 cells per well in 12 well poly-HEMA coated (or normal) plates. After ~3-4 hours, 100 μM oleic acid (Sigma-Aldrich) was added to each well. After 24 hours, 1 μl of 1 μl/ μCi [1-14C]-Oleic Acid (American Radiolabeled Chemicals, St. Louis, MO) was added to each well and incubated at 37ºC for 1 hour. To release $^{14}CO_2$, we then added 150 μl of 3M perchloric acid (Fisher Scientific, Pittsburgh, PA) to each well and immediately covered each well with phenylethylamine (Sigma-Aldrich) saturated Whatman paper. Plate was then incubated at room temperature overnight and subsequently Whatman paper was removed, placed into Ready-Safe Liquid Scintillation Fluid (Beckman Coulter, Fullerton, CA), and ^{14}C counts were read on

scintillation counter. The figures including data from these assays show representative experiments from more than three independent replicates.

NAD(P)H Fluorescence analyses

A two-photon excited fluorescence microscope was established on the basis of a Leica SP2 confocal microscope. It is equipped with a Ti:Sa laser (Spectral-Physics Broad Band Mai Tai) with a tunable wavelength range of 710 – 990 nm, a pulse frequency of 80 MHz, and a pulse width (FWHM) of 100 femtoseconds. Excitation wavelength was set to 730 nm. In the emission light path a SP700 short pass and BG39 filters were used to block any residual excitation photons. The fluorescence signal was filtered through a 505DCXR dichroic beam splitter (Chroma Technology) and wavelengths shorter than 505 nm were channeled to a Hamamatsu MCP-PMT detector. The effective spectral bandwidth of the detected fluorescence signal is 400 – 500 nm, corresponding to the maximum NAD(P)H emission[34]. A 63X (HCX PL APO 63X NA 1.2 W) water immersion objective lens was used for all the experiments. For each image, the emitted photons were collected for 5 minutes in the single photon counting mode (Becker & Hickl, SPC-830). All instrument settings and imaging parameters were maintained throughout the experiments. The images of photon counts are displayed on the same intensity and spatial scales allowing for direct visual comparison.

MCF-10A cells were seeded on Day 0 at a density of 5000 cells per well in a cover slip bottom 8-well chamber. On Days 4 and 8, NAD(P)H fluorescence in 3D MCF-10A structures was imaged at the equatorial cross sections using two-photon fluorescence microscopy while being maintained on stage in a 37°C chamber with 5% CO2. For the antioxidant experiments (Fig. 4b) the two-photon excited native fluorescence images were acquired through non-descanned detection with a broad band emission filter (400-700 nm) and 730 nm excitation. Images were deconvolved by

Huygens Professional (Scientific Volume Imaging, Hilversum, The Netherlands). The images of more than 90 acinar structures were acquired on Day 8 for either control or Trolox (50 μM) treatment. The randomized, blinded images were evaluated by five independent observers for the presence of NAD(P)H dichotomy (native fluorescence intensity difference between the inner and outer cells based on images from the center of each structure). For acini with filled lumen, an average from all five observers was derived from the positive scoring of a criterion. The standard deviation indicates the variation in a score among the five observers from this experiment.

Soft Agar Assays

Cells (4 X 10^4) were added to 1.5 ml (+/- antioxidants) of growth media plus 0.4% low melt agarose (Sigma-Aldrich) and layered onto a 2 ml bed of growth media plus 0.5% low melt agarose. Cells were fed every 2-3 days with 1 ml of growth media (+/- antioxidants). At the indicated times, growth media was removed and viable colonies were stained with iodonitrotetrazolium chloride (Sigma-Aldrich). Colony number and colony size were determined using ImageJ. The figures including data from these assays show representative experiments from more than three independent replicates.

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