Supplemental Experimental Procedures

Drugs

We used BKM120 (Buparlisib) (Maira et al., 2012); GDC0941 (Folkes et al., 2008); BYL719 (Alpelisib)(Fritsch et al., 2014; Furet et al., 2013); TGX221(Sturgeon et al., 2008); MK2206 (Hirai et al., 2010); GSK690693 (Rhodes et al., 2008)

GSK650394 (Sherk et al., 2008); Rapamycin (Kunz et al., 1993); NSC23766 (Gao et al., 2004); Cytochalasin D (Jijakli et al., 2002); Cytochalasin E (Jijakli et al., 2002); Jasplakinolide(Bubb et al., 1994); CK-666 (Nolen et al., 2009). Details of the drugs including their targets and concentrations used here, unless noted otherwise in the figure legends, are provided in table S1.

Cells and cell culture

Immortalized human mammary epithelial cells (HMEChtert, in short HMEC) were provided by Dr. Robert Weinberg, MIT. HMEC (Hahn et al., 1999), had been generated from primary mammary epithelial cells (MECs) that were immortalized with human telomerase (hTERT) and SV40 Large T antigen (Zhao et al., 2003). MCF10A cells were from the ATCC. MCF-10A and HMEC cells were cultured in DMEM/F-12 with 5% horse serum, 20 ng/ml epidermal growth factor (EGF), 0.5 mg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 μg/ml insulin, as previously described (Debnath et al., 2003). HCC1937 cells, from ATCC, were maintained in DMEM with 10% FBS. For stable expression of Peredox-NLS, MCF-10A cells were transduced using the retroviral pMSCV vector, followed by puromycin selection. MCF-10A cells expressing inducible myristoylated WT HA-AKT1 or myristoylated HA-AKT1 (gift from Dr. Alex Toker) were cultured in MCF-10A medium and treated with doxycycline at 0.5 μg/ml 16 hours prior to lysis to induce AKT1 expression. MCF10-A cells with knock-in of PIK3CA or AKT mutations were generated as described (Gustin et al., 2009; Lauring et al., 2010) and cultured in MCF10A media. HA-Aldolase A WT and HA-Aldolase A R42A in pcDNA3 were used for transient transfection into MCF10A cells. 2 μg of each plasmid or empty vector were transfected using lipofectamine 2000. 48 hours after transfection, cells were lysed for immunoblotting. MCF-10A or MCF7 cells were transiently transfected with pcDNA3.0 Rac1 (WT, T17N, Q61L from Addgene (Subauste et al., 2000)). 2 μg of each plasmid or empty vector were transfected with lipofectamine 2000. 24 hours after transfection, cells were serum starved overnight and then stimulated with insulin with or without 2.5 μM BKM120 for three hours before preparing cell lysates. See table S4 for DNA constructs used.

Cell permeabilization, fractionation and determination of aldolase A levels. 2 x 10^5 MCF10A cells/well were seeded into 6-well plates and treated with as indicated. For permeabilization, cells were washed with 3× PBS and then incubated in 30 μg/ml digitonin/PBS for 5 min at 4^{o}C. After incubation, the supernatant was collected and cells lysed with 200 μl of RIPA buffer for each well. The supernatant was centrifuged at 2000 rpm to remove cellular components. 40 μl of supernatant (8% of total supernatant) or 20 μl (10% of total lysate) of cell lysate were run on the same SDS-page, transferred to PVDF membrane for immunoblotting and scanned using a LI-COR system. Another 40 μl of supernatant was used for an enzymatic aldolase assay. The abundance of aldolase protein was calculated based on the intensity of the aldolase A signal determined with Image J and corrected for the dilution.

For isolation of cytoskeletal and soluble cell compartments (Fig. 3A) the ProteoExtract® Cytoskeleton Enrichment and Isolation Kit (Millipore) was used. For immunobotting, GAPDH is used as a cytoplasmic and nuclear compartment control (Tristan et al., 2011), while vimentin is a cytoskeletal compartment control.

siRNA experimentation

Two siRNA duplexes that target P110 α were used. 5,-GCTTAGAGTTGGAGTTTGA-3', and 5,-GCGAAATTCTCACACTATT-3' . The control SiRNA sequence: 5'-UAAGGCUAUGAAGAGAUAC-3'. Two siRNA duplexes that target P110ß were used. The SiRNA1 target sequence is: 5,-CCATAGAGGCTGCCATAAA-3'. The SiRNA2 target sequence is: 5,-GGATTCAGTTGGAGTGATT-3'. Pooled Wave2 SiRNA (M-012141-00-0005) or P34 (M-012081-00-0005) SiRNA from Dharmacon were used for targeting Wave 2 or P34. HiPerFect (QIAGEN) was used for the SiRNA transfection.

Two siRNA duplexes that target Rac1 were used. siRNA1 (5'-AGACGGAGCTGTAGGTAAA-3') targets the coding region of Rac1, and was rescued by the mutant GFP-Rac1 by changing the targeted sequence to 5'-AGATGGTGCAGTTGGAAAG-3'. siRNA2 (5'-CCTTTGTACGCTTTGCTCA-3') targets the 3' UTR of Rac1 and was rescued by the WT GFP-Rac1. The control siRNA sequence is as follows: 5'-UAAGGCUAUGAAGAGAUAC-3'.

HiPerFect (QIAGEN) was used for the siRNA transfection. Eight hours after siRNA transfection, the rescue GFP-Rac1 or control vector were transfected using Lipofectamine 2000 (Invitrogen).

Immunoblotting. Primary monoclonal antibodies against β-actin (sigma, 1:10,000), γ-tubulin (sigma 1:10,000), polyclonal antibody against total Akt (Santa Cruz 1:2,000), monoclonal antibody against GFP (Santa Cruz, 1:2,000), polyclonal antibodies targeting phospho-Akt (S473) (Cell Signaling, 1:2,000), phospho-PRAS40 (T246) (Cell Signaling, 1:2,000), phospho-NDRG1 (T346) (Cell Signaling, 1:2,000), phospho-PAK1 (Ser199/204)/PAK2 (Ser192/197) (Cell Signaling, 1:2,000), Aldolase A (Cell Signaling, 1:2,000 for cell lysate; 1:500 for supernatant), GAPDH (Cell Signaling, 1:2,000 for cell lysate; 1:500 for supernatant), HA tag (1:2000), and monoclonal Rac1 antibody (Millipore, 1:2,000) were used. Standard procedures were used for immunoblotting.

Rac1 GST-PBD-PAK pull down assay. (Benard and Bokoch, 2002) MCF-10A cells or GFP-Rac1 transfected MCF7 cells were starved overnight, indicated inhibitors or vehicle control were added and 15 min later cells were stimulated with insulin. After 3 hours of insulin stimulation, cells were harvested in Mg2+ lysis buffer containing 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, and 10 mM MgCl2, supplemented with 1 mM DTT, 1 mM Na_3VO_4, 10 mM NaF, 10 mM β-glycerol phosphate, and protease inhibitors (Roche). The lysates were precleared with Gluthathione-beads, and then incubated with a GST-PAK1-PBD (p21 binding domain) fusion protein immobilized on glutathione agarose beads for 20 min at 4 °C. Following precipitation, the pellets were washed 3 times within Mg2+ lysis buffer and then analyzed by Western blotting with anti Rac1/2 antibodies (Cell Signaling) to determine levels of active Rac1. GDP was added into the negative control lysate to a final concentration of 1mM immediately before precipitation.

NADH/NAD+ determination with Wide-Field Time-Lapse Microscopy. MCF10A cells stably expressing nuclear-targeted NADH biosensor Peredox-mCitrine were used as described previously (Hung et al., 2011). 1000-4000 cells were plated 1-3 days prior in 96-well plates (Mat-Tek, Matrical), pre-treated with type I collagen (BD Biosciences). For microscopy, cells were placed in DMEM (Life Technologies 31053), supplemented with 0.3% BSA, EGF, hydrocortisone, cholera toxin, and insulin as described (Debnath et al., 2003), and maintained at ~37^{0}C and ~5% CO2. T-Sapphire and YFP images were acquired on a Nikon Eclipse Ti microscope with a 20x 0.75NA objective, 2x2 binning with a Hammamatsu Orca-ER digital camera, at intervals of 5-6 minutes. After about 2 hours of

baseline imaging, cells were treated with vehicle control or inhibitors and imaged for additional ~20 hours. Biosensor signal was calibrated with lactate or with pyruvate and iodoacetate as described (Hung et al. 2011). Analysis was performed using a custom MATLAB (MathWorks) algorithm, which subtracted background, set threshold and cell segmentation. We generated T-Sapphire-to-YFP ratios for a population of ~300-700 cells over time in two independent assays for each condition.

Immunofluorescence

MCF10A cells were serum-starved overnight and then stimulated with or without insulin (5 μ g/ml) for 3 hours. The cells were fixed as indicated below for confocal microscopy, stained against Wave2 (1:50, Cell signaling) or P34 (1:100, Millipore) and examined with a Zeiss Axiovert 200M fluorescence microscope.

Confocal fluorescence microscopy. HMEC cells transfected with HA-Aldolase A, were fixed with a glutaraldehyde fixation protocol (Blanc et al., 2005). Briefly, cells were rinsed with cytoskeleton buffer, fixed in fixation buffer (0.5% Triton/0.25% Glutaraldehyde in cytoskeleton buffer) for 2 minutes and then rinsed with cytoskeleton buffer. After fixation, cells were treated with post -fixation buffer (2% Glutaraldehyde in cytoskeleton buffer,) for 10 minutes at room temperature followed by 5 minutes of NaBH4 (10 mgs in 10 mL of cytoskeleton buffer) 3 times. Then the cells were washed 3 times in cytoskeleton buffer. After fixation, cells were stained with anti-HA antibodies (Cell signaling,1:50) and counterstained with phalloidin for immunofluorescence. Slides were then covered with DAPI containing fluormount and subjected to confocal fluorescence microscopy (Zeiss LSM 510 Inverted Live-Cell Confocal System).

Fluorescence Recovery After Photobleaching. FRAP was performed on a Zeiss LSM 510 confocal microscope at 37°C, using a 63× objective. Bleaching was done using the 488 nm argon ion laser, set to 100% output. Using the manufacturer's software, a laser bleach pulse of 2-4 μm width was aimed on an area within a cell (argon ion laser bleach when recording green fluorescence) excluding the nucleus. Images were taken with at 488 nm emission set to 5-15% attenuation. In a typical experiment, five pre-bleach images were collected, followed by 50 bleach pulses. After bleaching, images were collected every 4 s for 150 s. Each data set consisted of at least 14 HMEC cells from at least two independent experiments. Recovery curves were generated from background-subtracted images, normalized to pre-bleach images and corrected for fluorescence loss (Phair et al., 2004).

The fluorescence intensities of the bleached area I(t) and of the entire cell T(t) were recorded five times before a laser pulse (Iprebleach and Tprebleach) and then every 3 s after the pulse. The intensity of an area without cells was recorded as a measure of background intensity. Calculations were done according to:

$$\frac{(\mathrm{I}\,(\mathrm{t}) - \mathrm{Ibackground}\,(\mathrm{t})/(\mathrm{Iprebleach} - \mathrm{Ibackground})}{(\mathrm{T}(\mathrm{t}) - \mathrm{Ibackground}\,(\mathrm{t}))/(\mathrm{Tprebleach} - \mathrm{Ibackground})} = \mathrm{I\ norm}\,(\mathrm{t})$$

where I (t) is fluorescence intensity in bleached area at time point t; I prebleach is fluorescence intensity in area to be bleached pre-bleach; I background is fluorescence intensity in an area outside the cell either prebleach or for a given timepoint (t); T (t) is fluorescence intensity of the entire cell at time point t; and T prebleach is fluorescence intensity of the entire cell prebleach.

Fluorescence recovery curves represent the median of the fluorescence recovery of at least 14 cells.

Curves were modeled using GraphPad Prism software and the one phase exponential decay algorithm.

Aldolase enzymatic assay. The aldolase enzymatic assay was performed based on Boyer's modification of the hydrazine assay (Jagannathan et al., 1956) in which 3-phosphoglyceraldehyde reacts with hydrazine to form a hydrazone which absorbs at 240 nm. Before performing aldolase enzymatic assay on cells grown in 6-well plates, the cell supernatant or cell lysate was mixed with 3 µl EDTA (0.01M), 6 µl iodoacetate (0.01M), 200 µl hydrazine (0.0035M), and appropriate volume of lysis buffer to make a final volume of 300 µl. A blank read was taken at 240 nm, 10 µl of 0.12 M FBP were added and absorption was detected at 240 nm in 5 min intervals for three readings. Mean enzymatic activity was determined according to:

$$\frac{\{[A(15\ \text{min})-A(10\ \text{min})]+[A(10\ \text{min})-A(5\ \text{min})]+[A(5\text{min})-A(0\ \text{min})]\}}{3}.$$

For the detection of aldolase activity in whole cells, cells were grown in UV-transparent 96-well plates, treated for three hours, and lysed in 30 µl Digitonin solution (100 µg/ml PBS), then 60 µl of the EDTA/Iodoacetate/hydrazine solution were added followed by addition of 2.5 µl FBP and absorption was read in a BioTek plate reader at 240 nm. Enzymatic activity was determined in triplicate, normalized to the control, and means and standard deviations calculated.

FDG-PET Scanning. A total of 0.3 to 0.4 mCi of FDG was injected intravenously through the retro-orbital vein of the anesthetized mouse. After a "washout" period of 1 hour, the mouse was imaged on a NanoPET/CT (Bioscan/Mediso) scanner. The NanoPET/CT is a high-resolution small-animal multimodality scanner consisting of 12 lutetium-yttrium oxyorthosilicate (LYSO) detector blocks. The blocks comprise a total of 39,780 crystals each with a dimension of 1.2 mm × 1.2 mm × 13 mm.

Images were acquired in 3 dimensions. The mice remained supine and maintained their position throughout the procedure. First, a computed tomography (CT) scan was conducted, and second, a whole-body FDG-PET scan was acquired covering the same area as the CT scan. Counts per minute (cpm) were obtained, converted to becquerels (Bq), and values were normalized for region of interest volume and injected dose. To correct for metabolic and injected activity variability between exams and to determine tumor-specific uptake changes, FDG-uptake rates were corrected for calculated according to the following formula: (activity in tumor in Bq) / ((injected activity in Bq)/(mouse weight in cm3)). For studies involving repeat scanning, the change in tumor-specific FDG uptake was determined in percentage [1 − (FDG-uptake post/FDG-uptake pre) × 100]. Animals were housed in the Longwood Small Animal Imaging Facility between scans.

Hyperpolarized NMR studies. NMR data were acquired using a 9.4T horizontal bore MRI scanner (Bruker Biospec 94/20, Bruker, Billerica, MA). After acquisition of proton images and the selection of a slice covering the tumor, the magnetic field homogeneity within the tumor was optimized using a point-resolved spectroscopy (PRESS) sequence to iteratively minimize the proton linewidth. After local shimming, the proton linewidths over the tumors ranged from 33 to 103 Hz (full width, half maximum). A slice-selective 13C spectroscopic scan was prescribed with 7.5-degree tip angle, 2s TR, 6.5kHz spectral width and 8192 spectral points. Immediately prior to injection, a series of 64 scans was initiated, and spectra were acquired from the tumor with 2s temporal resolution before, during, and after injection. By the end of the series of scans, the metabolite signals had decayed away to an unobservable level. Spectral lines from pyruvate and lactate were integrated using a time-domain fitting method (AMARES in jMRUI (Naressi et al., 2001)). The integrated signals from pyruvate and

lactate were then summed over the 64 scans to compute the time integrals of each metabolite signal. The ratio of the lactate integral to the pyruvate integral was then computed. This 'area under the curve' analysis provides a measure of the rate constant for formation of lactate from pyruvate (Hill et al., 2013).

Two-photon fluorescence correlation spectroscopy. MCF10A cells expressing eGFP-labeled aldolase were grown on a coverglass and mounted onto a custom-made heating stage with temperature controlled by thermocouple. During the experiments, samples were kept in FluoroBrite DMEM at 37 °C and placed on an inverted microscope. eGFP fluorophores were excited by two-photon excitation: 3 mW at 850 nm, generated by a mode-locked Ti-sapphire laser. The laser beam was aligned to backfill the 40 × water-immersion objective, which focuses the light into the cytoplasmic region of the sample. Fluorescence emitted from the sample was collected by the same objective and detected on a GaAsP hybrid detector with an emission filter by a non-descanned detection scheme. Every photon detected was tagged with the time relative to the starting time of each FCS measurement by time-correlated single photon counting (TCSPC) module (SPC-152, Becker &Hickl GmbH) and autocorrelated with the others in-situ to generate a FCS curve by B&H software. The FCS measurement time was 20 seconds.

Cells with eGFP-aldolase were scanned by fast galvano mirrors. Cells expressing amounts of eGFP-aldolase enough to provide a good signal-to-noise ratio, without over expression, were selected for FCS measurements. FCS curves were acquired over a 2 hour time period at 15 min intervals. At time zero, cells were not treated with any drug nor stimulated. After the initial measurement at time zero, cells were stimulated with or without drugs. The FCS curves obtained during the experiments were fit by a model function for freely diffusing particles and a 3-dimensional Gaussian confocal volume. The confocal volume in the microscope system was calibrated using Alexa fluor 488 dye. Post-experimental analyses were conducted using custom-written Matlab routines.

Seahorse assay

An XF24 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA, USA) was used to determine the effects of the inhibitors on MCF10A or Myr-MCF10A cells. 20,000 MCF10A cells/well were seeded into the 24-well Seahorse XF24 culture plates within MCF10A medium overnight. Cells were washed with PBS and then incubated for 1 hour at 37°C in Seahorse incubation medium containing 1 μM glucose, 2 mM L-glutamine with or without different inhibitors. Incubations were performed in a CO_2-free incubator to ensure accurate measurements of extracellular pH. Measurements of extracellular acidification rate (ECAR) were performed according to the manufacturer's instructions. After every 3 measurements in 8 min intervals, glucose, oligomycin or 2DG were added into the wells at the indicated time points to a final concentration of 10 mM, 10 μM or 50 mM for each. ECAR is presented as mean ± standard deviation (S.D.) of experimental triplicates.

Actin precipitation assay

MCF10A cells were transfected with WT GFP-*ALDOA* or R42A GFP-*ALDOA* A. 48 hours after transfection, cell lysates were prepared with RIPA buffer (pH7) and adjusted to a protein concentration of 3mg/ml. Then MgCl and KCl were added into the cell lysate to a final concentration of 2 mM and 50 mM for each. The cell lysates were aliquoted for F-actin or G-actin precipitation. Immediately before actin precipitation, ATP was added into cell lysate of actin precipitation to a final concentration of 10mM. 10 μg human platelet F-actin or G-actin (1mg/ml, Cytoskeleton, Inc) were and into the 200

µl cell lysate and incubated at room temperature for 1 hour. The actin containing lysate was then centrifuged at 150, 000 x g for 1.5 h at room temperature. The pellet and supernatant were collected for immunoblotting.

Glucose uptake assays

MCF-10A cells were cultured to ~50% confluence in continuous log phase, in growth media on 6 cm dishes. A complete media change was performed three hours prior to metabolite collection. The cells were treated with 1 mM ^{13}C labeled 2DG for 30 seconds and metabolites collected with 70% methanol extraction for LC-MS/MS. Alternatively, glucose uptake was measured with the a kit from abcam (ab136955).

Supplemental References

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