Supplemental Figure Legends

Supplemental Figure 1. (A) Reference guide for the phospho-protein array. **(B)** Representative fluorescent arrays for whole cell lysates from MDA-MB-231 cells cultured at pH 7.4 or pH 6.7 for 72 hours. **(C-D)** Quantitative analysis of fluorescence intensity for all phosphorylated proteins. Data represent the mean ± S.D. of two replicate arrays for each sample.

Supplemental Figure 2. Histological analysis of LC3 spatial expression patterns in relation to pimonidazole hydrochloride (Hypoxia) and Glut1 expression. MDA-MB-231 MFP tumor was histologically stained for LC3, Glut1, and pimonidazole hydrochloride. Pixel analysis was performed to highlight the areas of highest expression. High spatial concordance was observed for all three histological markers. Pixel analysis was performed using Aperio^{TM} Positive Pixel Count v9 (Red – strong positive).

Supplmental Figure 3. Histological analysis of *in-vivo* LC3 expression in HS766T subcutaneous tumors buffered with sodium bicarbonate. **(A)** Whole cell lysates from HS766T cells cultured at neutral pH 7.4 or pH 6.7 for 48 hours were analyzed for LC3-II expression. **(B)** Positive pixel analysis was completed for LC3 staining on whole tissue cross sections from HS766T tumors treated with tap or $NaHCO_3$. An overall significant decrease in total positive and strong positive LC3 pixels was observed in $NaHCO_3$ treated samples. The data are plotted as the mean ± standard deviation of three whole tumor cross sections from each treatment group. **(C)** A 5x magnification of representative tissue regions from HS766T tumors stained for LC3.

Supplemental Methods

FACS analysis:

MDA-MB-231/7.4 cells were seeded in 100 mm tissue culture dishes 24 hours prior to treatment. Cultures were grown in low pH 6.7 medium for 24 hours. MDA-MB-231/6.7ext cells were seeded directly in pH 6.7 medium. Following treatment, the samples were trypsinized, pelleted by centrifugation, washed once in PBS, re-suspended in PBS, and fixed by an equal volume of ethanol (4°C). On the day of analysis, the fixed samples were pelleted, resuspended, and incubated for two hours in PBS containing 100 U/ml RNAse A and 50 mg/ml propidium iodide. A minimum of 10^4 cells/sample was analyzed to determine the percentage of apoptotic cells and cells in G_1, S, and G_2/M phase (MODFIT; Variety Software). Stained samples were analyzed with a Beckton Dickinson FACScalibur. The data is a representation of three independent experiments.

mRNA preparation and gene expression analysis:

MCF-10A human benign breast epithelial cells were maintained in DMEM/F-12 (Sigma Aldrich) supplemented with 5% horse serum (Life Technologies), 5 ng/ml epidermal growth factor (EGF), and 100 μg/ml insulin (Sigma Aldrich). MCF10.DCIS, SUM102 and SUM225 human mammary DCIS cell lines were maintained in DMEM/F-12 (Sigma Aldrich) supplemented with 5% horse serum and Ham's F-12 (Sigma Aldrich) supplemented with 10% fetal bovine serum, respectively. N1ME normal breast myoepithelial cells (a kind gift of K. Polyak from the Dana Farber Cancer Institute, Boston, MA) were maintained in Medium 171 phenol red-free media supplemented with Mammary Epithelial Growth Supplement (Life Technologies). WS-12Ti human breast

tumor-associated fibroblasts were maintained in DMEM/F-12 (Sigma Aldrich) supplemented with 10% fetal bovine serum (HyClone Laboratories). Three-dimensional cultures of MCF10A, MCF10.DCIS, SUM102, SUM 225 and N1ME cells were generated by seeding 4.5 x 10^5 cells of each line in duplicate on 100 mm plates that had been coated with 600 µl of reconstituted basement membrane (rBM; Cultrex, Trevigen). The cells were then overlaid with 2% rBM diluted in growth media as specified above. WS-12Ti fibroblasts (4.5x10^5 each) were seeded in duplicate on 100 mm plates that had been coated with 4 ml of collagen I and a 2% overlay diluted in their growth media was applied. All media were changed at day 4. At day 8, media were switched to pH buffered DMEM/F12 containing the 2% overlay for an additional incubation of 72 hours. To insure maintenance of the desired pH, the media was buffered with 2 g/l sodium bicarbonate, 25 mM of both PIPES and HEPES, the pH adjusted to either 6.8 or 7.4 and then incubated overnight in 5% CO_2. Before adding to the cultures, the pH was again adjusted to the desired pH. The cells, with the exception of the WS-12Ti fibroblasts, were harvested for RNA isolation in PBS with 5 mM EDTA. WS-12Ti fibroblasts were harvested with 0.1% collagenase. RNA was isolated using an RNeasy Mini kit from QIAGEN (Valencia, CA). Processed RNA was hybridized to U133 Plus 2.0 arrays (Affymetrix Corp.) using established protocols that have been previously published (1).

Real Time quantitative RT-PCR:

RNA extraction and Real Time PCR were performed as previously described (2). Cultures of MDA-MB-231 cells were maintained in either normal pH (7.4) or low pH (6.7) medium for 72 hours with RNA isolated using an RNeasy Mini kit from QIAGEN.

Primer sets were determined to distinguish *ACTB*, *ATG5*, and *BNIP3* mRNA. Real Time RT-PCR was conducted using a Cephid (Sunnyvale, CA) Smart Cycler and the QuantiTect SYBR Green RT-PCR kit (QIAGEN) format. Raw mRNA expression values were determined as being 2^{-C_T}, where C_T is the number of cycles required for the SYBR green I fluorescence to cross the threshold of 30 arbitrary fluorescence units.

3-Methyladenine Toxicity Assay:

MDA-MB-231/7.4 and MDA-MB-231/6.7ext were seeded at a density of 10,000 cells/well. Cultures were treated with 10 mM 3-methyladenine (Sigma Aldrich) for 48 hours. CyQuant (Life Technologies), a fluorescent DNA probe, was used to determine cell number at T=0, 24, and 48 hours. Fluorescence intensity was determined with a Perkin Elmer (Waltham, MA) 2030 VictorX4 multilabel plate reader with fluorescein filters set at ~485 nM excitation and ~535 nM emission.

Immunocytochemistry Image Analysis:

Approximately ten confocal images of anti-LC3 staining from MDA-MB-231 cells cultured at neutral pH (7.4) and low pH (6.7) for 48 hours were quantified for average number of LC3 puncta per cell. The counting of LC3 puncta and nuclei in the confocal image was performed using fully automated method in Definiens Developer XD (Definiens) based on cognition network technology. A specific rule set was written to automatically detect, segment and count the LC3 puncta and nuclei. The detection and segmentation of objects was performed primarily based on the green and blue layer, mean brightness and the size. The classification of image objects was carried out from pixel

level to the required image objects by merging smaller objects based on their respective properties like layer value, relation to other objects or classes and size. The rule set outputs the numbers of the classified objects for the respective classes.

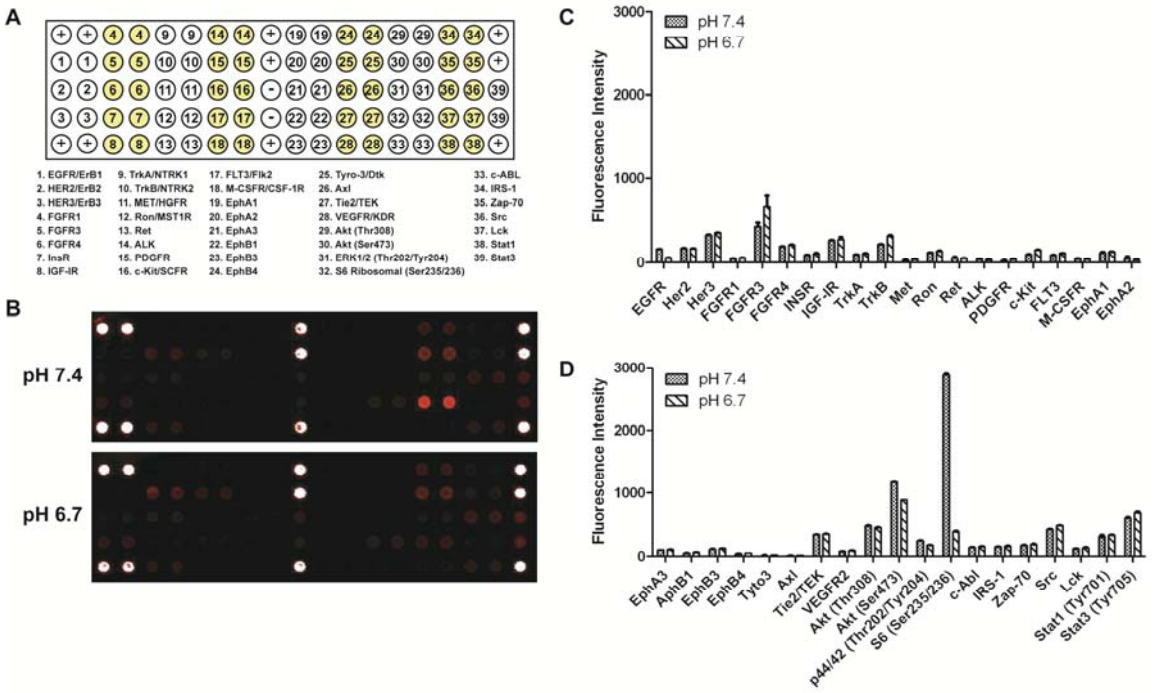
Immunohistochemical image analysis:

Image analysis for the pimonidazole hydrochloride, LC3, and BNIP3 stained slides has been performed using a optimized Aperio PositivePixelCount ® v9.0 algorithm with the following optimized thresholds [HueValue=0.1; HueWidth=0.5; Iwp(High)=220; Iwp(Low)/Ip (High)=175; Ip(Low)/Isp (High)=100; Isp(Low)=0; Inp(High)=-1] to segment positive pixels of various intensities. The percent of positivity has been quantified by the number of cells exhibiting positive stain as a percentage of total tumor cell count. The staining intensity can be thresholded into negative (0), low (1+), moderate (2+) and strong positive (3+) by mean stain density (0-255 dynamic range) for each tumor cross section.

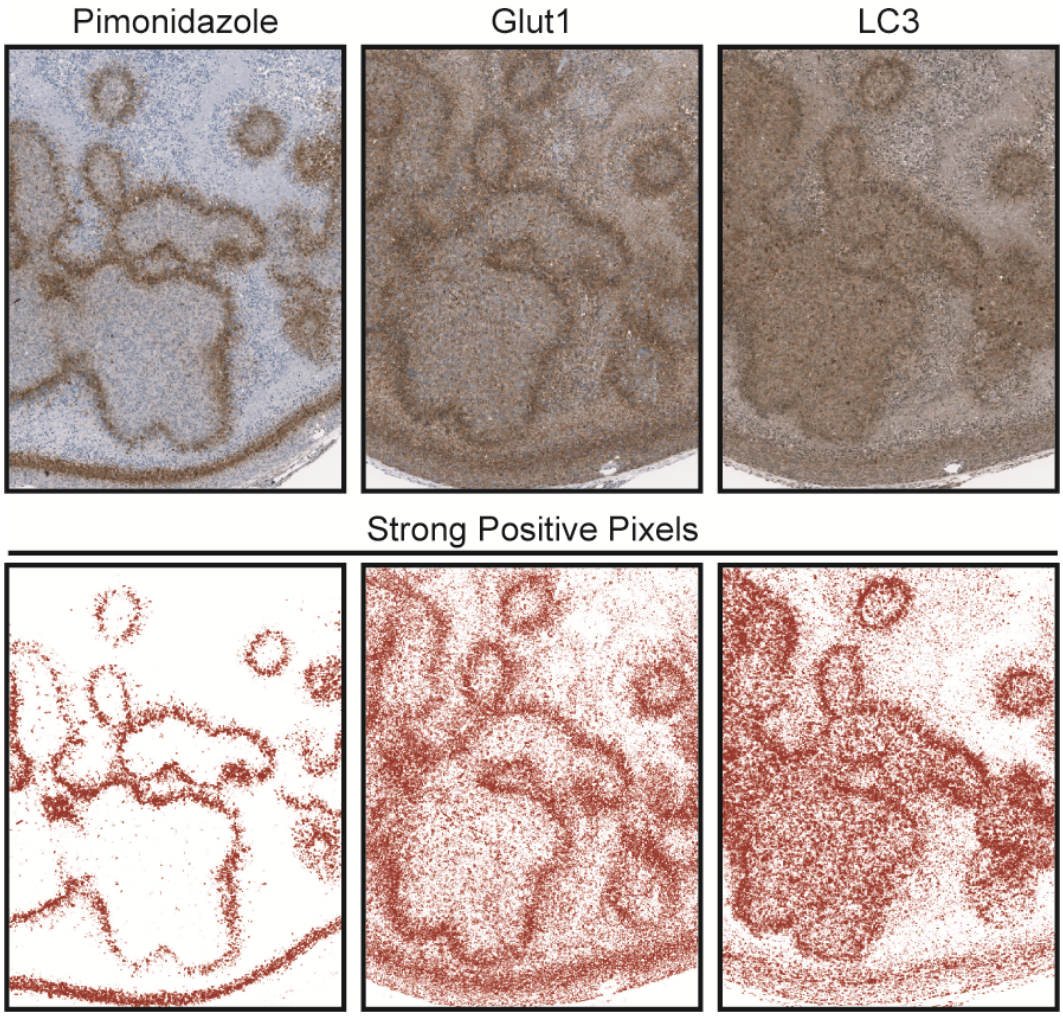
References

1. Dobbin, K.K., Beer, D.G., Meyerson, M., Yeatman, T.J., Gerald, W.L., Jacobson, J.W., et al. Interlaboratory comparability study of cancer gene expression analysis using oligonucleotide microarrays. Clin Cancer Res 2005;11:565-72.
2. Morse, D.L., Carroll, D., Weberg, L., Borgstrom, M.C., Ranger-Moore, J., Gillies, R.J. Determining suitable internal standards for mRNA quantification of increasing cancer progression in human breast cells by real-time reverse transcriptase polymerase chain reaction. Anal Biochem 2005;342:69-77.

Suplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3