Glutathione biosynthesis is a metabolic vulnerability in PI(3)K/Akt-driven breast cancer

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Cancer cells often select for mutations that enhance signalling through pathways that promote anabolic metabolism. Although the PI(3)K/Akt signalling pathway, which is frequently dysregulated in breast cancer, is a well-established regulator of central glucose metabolism and aerobic glycolysis, its regulation of other metabolic processes required for tumour growth is not well defined. Here we report that in mammary epithelial cells, oncogenic PI(3)K/Akt stimulates glutathione (GSH) biosynthesis by stabilizing and activating NRF2 to upregulate the GSH biosynthetic genes. Increased NRF2 stability is dependent on the Akt-mediated accumulation of p21Cip1/WAF1 and GSK-3β inhibition. Consistently, in human breast tumours, upregulation of NRF2 targets is associated with PI(3)K pathway mutation status and oncogenic Akt activation. Elevated GSH biosynthesis is required for PI(3)K/Akt-driven resistance to oxidative stress, initiation of tumour spheroids, and anchorage-independent growth. Furthermore, inhibition of GSH biosynthesis with buthionine sulfoximine synergizes with cisplatin to selectively induce tumour regression in PI(3)K pathway mutant breast cancer cells, both in vitro and in vivo. Our findings provide insight into GSH biosynthesis as a metabolic vulnerability associated with PI(3)K pathway mutant breast cancers.

To investigate how Akt regulates metabolism in breast cancer, we evaluated metabolic reprogramming induced by an oncogenic Akt mutation identified in breast cancer patients: the E17K mutation, found in both AKT1 and AKT2. We stably expressed wild-type AKT1/2 or AKT1/2(E17K) in non-tumorigenic mammary epithelial MCF10A cells in a doxycycline-inducible manner to assess acute metabolic changes that occur on oncogenic Akt activation. As previously reported, phosphorylation of AKT1(E17K) on Ser473 is only slightly elevated under serum-starved conditions and does not lead to increased phosphorylation of Akt substrates such as PRAS40 and GSK-3β (Fig. 1a). In contrast, AKT2(E17K) is robustly constitutively active, as evidenced by high basal Ser473 phosphorylation, enhanced phosphorylation of downstream substrates, and increased in vitro kinase activity (Fig. 1a and Supplementary Fig. 1a). Although AKT1/2(E17K) does not enhance proliferation in the presence of growth factors (Supplementary Fig. 1b), as reported, only MCF10A AKT2(E17K) cells proliferate in a growth-factor-independent manner (Fig. 1b), demonstrating a functional consequence of constitutive AKT2(E17K) activity. Together, these results indicate that AKT2(E17K) is a more potent oncogenic mutation than AKT1(E17K).

Oncogenic signalling through AKT2(E17K) may support enhanced growth-factor-independent proliferation by reprogramming cellular metabolism. To test this idea, we used targeted liquid chromatography-based tandem mass spectrometry (LC–MS/MS) with selected reaction monitoring to profile steady-state metabolite changes between serum-starved MCF10A AKT2 versus AKT2(E17K) cells and found significant differences between the two cell lines (Supplementary Fig. 2a). Consistent with Akt-mediated regulation of glycolysis, AKT2(E17K) strongly upregulates glycolytic intermediates, including the most upregulated metabolite NADH (Supplementary Fig. 2b,c). Interestingly, the second highest metabolite upregulated by AKT2(E17K) is reduced glutathione (GSH), whereas levels of oxidized glutathione (GSSG) are unchanged, resulting in an increased GSH/GSSG ratio (Fig. 1c).

Elevated GSH levels suggest that AKT2(E17K) controls cellular redox status by increasing the reducing potential of MCF10A cells, a conclusion supported by changes in levels of other metabolites.

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Received 23 September 2015; accepted 11 March 2016; published online 18 April 2016; DOI: 10.1038/ncb3341
First, AKT2(E17K) selectively upregulates metabolites in the oxidative pentose phosphate pathway (PPP), whereas metabolites exclusively in the non-oxidative PPP are unchanged or decreased (Supplementary Fig. 2d). This is consistent with mTORC1 activation downstream of Akt that selectively upregulates the oxidative PPP. Importantly, the oxidative PPP generates reducing equivalents in the form of NADPH, which can be used to reduce GSSG to GSH (Fig. 1d). To further support a change in redox status, the most downregulated metabolite in MCF10A AKT2(E17K) cells is cysteine, the oxidized form of cysteine taken up from growth media (Fig. 1c). This is consistent with a more reducing intracellular environment wherein any cystine taken up is rapidly reduced to cysteine. Interestingly, steady-state cysteine levels do not change (Fig. 1c), suggesting that flux through metabolic processes that utilize cysteine, such as GSH biosynthesis (Fig. 1d), may be increased by AKT2(E17K) as well.

We next asked whether AKT2(E17K) increases flux through the GSH biosynthetic pathway. In this pathway (Fig. 1d), glutamate-cysteine ligase (GCL), which consists of two subunits GCLC and GCLM, first ligates glutamate and cysteine to form γ-glutamylcysteine (γ-GluCys). Glutathione synthetase (GSS) then catalyzes the condensation of γ-GluCys with glycine to form GSH. GSH is also formed from the reduction of GSSG by glutathione reductase (GSR). First, AKT2(E17K) selectively upregulates metabolites in the oxidative pentose phosphate pathway (PPP), whereas metabolites exclusively in the non-oxidative PPP are unchanged or decreased (Supplementary Fig. 2d). This is consistent with mTORC1 activation downstream of Akt that selectively upregulates the oxidative PPP. Importantly, the oxidative PPP generates reducing equivalents in the form of NADPH, which can be used to reduce GSSG to GSH (Fig. 1d). To further support a change in redox status, the most downregulated metabolite in MCF10A AKT2(E17K) cells is cysteine, the oxidized form of cysteine taken up from growth media (Fig. 1c). This is consistent with a more reducing intracellular environment wherein any cystine taken up is rapidly reduced to cysteine. Interestingly, steady-state cysteine levels do not change (Fig. 1c), suggesting that flux through metabolic processes that utilize cysteine, such as GSH biosynthesis (Fig. 1d), may be increased by AKT2(E17K) as well.

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The three amino acids that constitute GSH are primarily derived from metabolites in growth media: glycine and cysteine are taken up exogenously or synthesized from extracellular glucose and methionine, respectively, and glutamate is derived from extracellular glutamine. Therefore, to compare pathway flux, we used LC–MS/MS to demonstrate that the rate of incorporation of exogenous U-13C5-glutamine into γ-GluCys and GSH is increased in MCF10A AKT2(E17K) cells (Fig. 1e). In contrast, the uptake of U-13C5-glutamine and its incorporation into GSSG is comparable between the two cell lines (Supplementary Fig. 2e,f). Therefore, oncosgenic signalling through AKT2(E17K) stimulates flux through the GSH biosynthetic pathway. Finally, using the NCI-60 metabolomics data set (https://wiki.nci.nih.gov/display/NCIDTPdata/Molecular+Target+Data), which profiles five breast cancer cell lines, we find that relative to two RAS mutant cell lines, three cell lines that harbour PI(3)K pathway mutations have higher levels of GSH (Fig. 1f), suggesting a correlation between elevated GSH levels and PI(3)K pathway mutation status in breast cancer.

We next asked whether enhanced GSH biosynthesis mediated by oncogenic Akt signalling alters the intracellular redox status to increase resistance to oxidative stress. Compared with MCF10A AKT2 cells, apoptosis is suppressed in cells expressing AKT2(E17K) in response to H2O2, as demonstrated by reduced PARP cleavage...
Figure 2 Enhanced GSH biosynthesis confers resistance to oxidative stress. (a–d) Cells were serum-starved for 20–24 h in the presence or absence of 1 μM GSK690693 (a, b) or 50 μM BSO (c, d), followed by treatment with 500 μM H2O2 for 4 h. Cells were immunoblotted for the indicated proteins (data are representative of three independent experiments). (e) Cells were serum-starved for 20–24 h in the presence or absence of 1 μM GSK690693 or 50 μM BSO, followed by treatment with 500 μM tert-butyl hydroperoxide (tBH) or 10 μM LCS-1 for 24 h. Cell viability was measured using a propidium iodide-based plate reader assay (EV, AKT2(E17K)): n = 4; AKT2, PIK3CA: n = 3; PIK3CA(E545K), PIK3CA(H1047R): n = 5; n represents number of biologically independent replicates; see Supplementary Table 1). All error bars represent s.e.m. ***P < 0.001 by a two-sided Student’s t-test. Unprocessed original scans of blots are shown in Supplementary Fig. 6.

(Fig. 2a) and increased cell survival as measured by fluorescence-activated cell sorting (Supplementary Fig. 3a). Resistance to oxidative stress is conferred by oncogenic Akt activity, because the Akt inhibitor GSK690693, which does not induce apoptosis on its own, sensitizes MCF10A AKT2(E17K) cells to H2O2 (Fig. 2a and Supplementary Fig. 3a,d). Similar results are observed in cells expressing oncogenic PIK3CA mutations (Fig. 2b and Supplementary Fig. 3b,c,f). Importantly, this oncogenic PI(3)K/Akt-driven resistance is dependent on enhanced GSH biosynthesis, because buthionine sulfoximine (BSO), a GCL inhibitor, reduces glutathione levels (Supplementary Fig. 3c), selectively sensitizes the mutant cells to H2O2 (Fig. 2c,d and Supplementary Fig. 3d–f). Finally, the PI(3)K pathway mutant cells are also more resistant to two additional inducers of oxidative stress: tert-butyl hydroperoxide and LCS-1, a superoxide dismutase 1 (SOD1) inhibitor that has been shown to inhibit the growth of breast cancer and lung adenocarcinoma cells by generating endogenous ROS13,14 (Fig. 2e). Again, inhibition of either Akt or GSH biosynthesis sensitizes the mutant cells to these agents. Together, these data demonstrate that MCF10A cells harbouring oncogenic PI(3)K pathway mutations are more resistant to oxidative stress, in part owing to the stimulation of GSH biosynthesis by the PI(3)K/Akt pathway.

We determined that one mechanism by which AKT2(E17K) stimulates GSH biosynthesis is through the transcriptional upregulation of the GSH biosynthetic genes GCLC, GCLM, and in particular GSS and GSR (Fig. 3a). These genes are targets of the transcription factor NRF2 (gene symbol NFE2L2), which is the master regulator of the cellular antioxidant response15. Accordingly, NQO1, a canonical NRF2 target, is also upregulated by AKT2(E17K) (Fig. 3a). In support of NRF2 functioning downstream of Akt to stimulate GSH biosynthesis, knockdown of NRF2 reduces total cellular glutathione levels and sensitizes AKT2(E17K) cells to oxidative stress induced by H2O2 (Supplementary Fig. 4a–c). Using a luciferase reporter under the control of the antioxidant response element (ARE), which is recognized by NRF2, we show that AKT2(E17K) strongly activates NRF2-mediated transcription, in a manner dependent on both NRF2 and oncogenic Akt activity (Fig. 3b,f).

Several mechanisms of NRF2 activation in cancer have been reported16. In the context of oncogene-dependent signalling, mutations in oncogenes such as KRAS and BRAF activate NRF2 by transcriptional upregulation17. In MCF10A AKT2(E17K) cells, however, NRF2 transcript levels do not change relative to wild-type cells (Fig. 3a), suggesting NRF2 activation through a post-transcriptional mechanism. Under basal conditions, the KEAP1–CUL3 E3 ubiquitin ligase complex suppresses NRF2 by ubiquitin-directed proteasomal degradation. Various oxidative cellular stresses disrupt NRF2 ubiquitylation by KEAP1–CUL3, resulting in NRF2 stabilization and activation15. Accordingly, by using cycloheximide, we find that NRF2 is stabilized in MCF10A AKT2(E17K) cells (Fig. 3c), indicating that oncogenic Akt signalling activates NRF2 through stabilization of the NRF2 protein.
Figure 3 AKT2(E17K) activates NRF2 to upregulate the GSH biosynthetic genes. (a) mRNA levels were measured by qRT–PCR and are expressed as fold changes relative to MCF10A AKT2 cells (n=3 biologically independent replicates; see Supplementary Table 1). (b) NRF2 was knocked down over 72 h, and ARE–luciferase activity was assayed in cells serum-starved for 20–24 h (data are from one experiment that was independently repeated two times with similar results; see Supplementary Table 1). (c) Serum-starved cells treated with 20 μg ml⁻¹ cycloheximide (CHX) were immunoblotted for the indicated proteins (data are representative of three independent experiments). (d) Cells serum-starved in the presence or absence of 1 μM GSK690693 for 20–24 h were immunoblotted for the indicated proteins (data are representative of three independent experiments). (e) p21 was knocked down in MCF10A AKT2(E17K) cells over 48 h, with or without the expression of shRNA-resistant HA–FLAG–p21 (indicated by #). ARE–luciferase activity was assayed in cells serum-starved for 20–24 h (data are from one experiment that was independently repeated five times with similar results; see Supplementary Table 1). (f) ARE–luciferase activity was assayed in cells serum-starved for 20–24 h in the presence or absence of 1 μM GSK690693 and 25 μM SB415286 (data are from one experiment that was independently repeated two times with similar results; see Supplementary Table 1). (g, h) Patient tumours from the TCGA BRCA data set were stratified by PI(3)K pathway mutation status (g) and Akt pSer473 levels (h) (see Supplementary Fig. 4d). PIK3CA: PIK3CA mutation; PTEN: PTEN mutation or copy number loss; AKT1: AKT1 mutation; Combined: alterations in PIK3CA, PTEN or AKT1; WT: wild-type PIK3CA, PTEN and AKT1. The heat map represents microarray/RPPA Z-scores for the indicated genes/proteins, which are expressed relative to values in the WT or Akt pSer473 low group. All error bars represent s.e.m. ***P < 0.001, ****P < 0.0001 by a two-sided Student’s t-test. NS, not significant. Unprocessed original scans of blots are shown in Supplementary Fig. 6.

We noted that two Akt substrates are known to regulate NRF2 protein stability. First, p21[Chk1/WAP1] has been reported to directly bind NRF2 and disrupt the KEAP1–NRF2 interaction to induce NRF2 activation19. Consistent with Akt-mediated enhancement of p21 stability20, we find that MCF10A AKT2(E17K) cells express higher levels of p21, in a manner dependent on Akt activity, whereas levels of p27[kip1], another cyclin-dependent kinase inhibitor, are unaffected (Fig. 3d). Importantly, p21 is required for the induction of NRF2 activity by AKT2(E17K) (Fig. 3e). Another Akt substrate, GSK-3β, has been shown to phosphorylate and direct NRF2 towards ubiquitylation and proteasomal degradation after it is released from inhibition by KEAP120. We therefore reasoned that Akt may activate NRF2 through inhibition of GSK-3β. Indeed, GSK690693-mediated inhibition of AKT2(E17K)-induced NRF2 activity is dependent on GSK-3β, because concomitant inhibition of Akt and GSK-3β with GSK690693 and SB415286, respectively, restores NRF2 activity (Fig. 3f). Therefore, GSK-3β is epistatic to and acts downstream of Akt with respect to NRF2 activation. On the basis of these results, we propose that oncogenic PI(3)K/Akt signalling results in p21 accumulation, which disrupts the KEAP1–NRF2 interaction. NRF2 stability is then further enhanced by Akt-mediated inhibition of GSK-3β, leading to NRF2 activation and upregulation of the GSH biosynthetic genes.

Finally, because these results largely rely on the MCF10A system, we asked whether NRF2 activation by oncogenic PI(3)K/Akt signalling functions in human breast tumours. We analysed TCGA Invasive Breast Cancer (BRCA) microarray data21 by stratifying patient tumours according to mutation status: tumours harbouring PIK3CA, PTEN or AKT1 mutations were compared with ‘wild-type’ tumours without these mutations. Analysis of several NRF2 target genes indicates that many of these, and especially GSS and GSR, are...
Next, we explored whether GSH biosynthesis is a metabolic vulnerability associated with PI(3)K/Akt-driven breast cancer progression. Recent studies demonstrated that in RAS mutant cancer cell lines, glutathione depletion by BSO or by inhibitors of the cystine–glutamate antiporter SLC7A11 leads to the inhibition of the glutathione peroxidase GPX4, subsequently inducing a form of cell death termed ferroptosis. However, in MCF10A cells expressing AKT2(E17K), we do not observe an increase in SLC7A11 or GPX4 expression (Supplementary Fig. 5a). Consistently, in two-dimensional (2D) culture conditions, rather than causing a loss in viability, BSO enhances the growth-factor-independent proliferation of MCF10A AKT2(E17K) cells (Supplementary Fig. 5b). Similarly, PIK3CA mutant cells are unaffected by BSO (Supplementary Fig. 5c). In full growth medium, low BSO concentrations stimulate proliferation, and growth inhibition is not observed until \( \sim 1\) mM BSO, at which off-target effects are expected (Supplementary Fig. 5d,e). This is consistent with observations that low BSO concentrations may stimulate proliferation in certain contexts, perhaps owing to the induction of milder levels of oxidative stress that stimulate proliferative signalling. 

As certain phenotypes differ in cells grown in 3D culture, which more accurately recapitulates the morphology of tumours growing in vivo, we evaluated how BSO affects proliferation in three dimensions (Fig. 4a). The number and morphology of spheroids formed by cells expressing empty vector control, wild-type PIK3CA, or wild-type AKT2 are only slightly affected by BSO. In contrast, the number of spheroids formed by cells expressing oncogenic PIK3CA or AKT2(E17K) is markedly reduced by BSO treatment, and spheroids that do grow are smaller and do not form multi-acinar structures observed in untreated cells. Therefore, in contrast to 2D culture, under 3D culture conditions that model both tumour initiation and maintenance we find that BSO selectively prevents tumour proliferation in certain contexts, perhaps owing to the induction of milder levels of oxidative stress that stimulate proliferative signalling.

We also assessed whether enhanced GSH biosynthesis is required for mutant cells to resist anoikis and undergo anchorage-independent growth in soft agar. Although AKT2(E17K), like AKT1(E17K), does not drive colony formation, PIK3CA mutant cells form colonies in a manner sensitive to BSO treatment (Fig. 4b). Therefore, enhanced GSH biosynthesis is necessary, but not sufficient, for
BSO synergizes with CDDP to selectively induce cell death and tumour regression in PI(3)K pathway mutant breast cancer cells. (a) Cells were treated with vehicle or 50 μM BSO for 48 h before treatment with CDDP for 48 h, in the presence or absence of 50 μM EUK-134 or 1 mM N-acetyl cysteine (NAC). Cell viability was measured using a propidium iodide-based plate reader assay (n = 3 biologically independent replicates; see Supplementary Table 1). (b,c) T47D (b) or MDA-MB-231 (c) xenografts were grown in nude mice treated with vehicle (T47D, n = 5; MDA-MB-231, n = 5), BSO (T47D, n = 4; MDA-MB-231, n = 4), CDDP (T47D, n = 4; MDA-MB-231, n = 4), or a combination of one week of BSO pre-treatment followed by CDDP (T47D, n = 5; MDA-MB-231, n = 7) (n represents number of biologically independent tumours; see Supplementary Table 1). All error bars represent s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001 by a two-sided Student’s t-test.

Figure 5

anchorage-independent growth. As cells experience increased levels of oxidative stress when detached from their matrix, stimulation of GSH production by oncogenic PI(3)K/Akt signalling is necessary for PIK3CA mutant cells to overcome this elevated oxidative stress and undergo anchorage-independent growth.

Finally, because BSO sensitizes PIK3CA and AKT2 mutant cells to oxidative stress (Fig. 2), we reasoned that targeting GSH biosynthesis in combination with agents that induce oxidative stress, such as chemotherapy or radiation, may be a viable therapeutic strategy for treating breast cancer cells harbouring a PI(3)K pathway mutation. In particular, oxidative stress is a major mechanism involved in cisplatin (CDDP) toxicity. Moreover, elevated GSH levels have been associated with CDDP resistance, and GSH has been suggested to form a conjugate with CDDP to facilitate its export and elimination from cells. Therefore, we reasoned that BSO would sensitize PI(3)K pathway mutant breast cancer cells to CDDP. We identified three breast cancer cell lines that are resistant to CDDP as a single agent in vitro (Fig. 5a). BSO potently synergizes with CDDP to induce cell death in T47D and ZR-75-1 cells, which harbour a PIK3CA and PTEN mutation, respectively. Importantly, this cell death is partially rescued by EUK-134, a hydrogen peroxide scavenger, and is fully rescued by the antioxidant N-acetyl cysteine, indicating that the synergistic effect is mediated by GSH depletion. In contrast, MDA-MB-231 cells, which lack a PI(3)K pathway mutation, are insensitive to this combination. These results were corroborated in vivo: whereas BSO and CDDP only inhibit the growth of T47D xenografts in nude mice as single agents, CDDP induces T47D tumour regression in mice that have been pre-treated with BSO for a week (Fig. 5b). In contrast, tumour regression is not induced by the combination in MDA-MB-231 xenografts (Fig. 5c), further suggesting a PI(3)K/Akt-pathway-dependent effect of this drug combination. Given the renewed interest in using platinum-based agents for breast cancer treatment, these results provide insight for inhibiting GSH biosynthesis to maximize the efficacy of platinum-based chemotherapies specifically for the treatment of breast tumours harbouring a PI(3)K pathway mutation.

Taken together, our results show that the stimulation of GSH biosynthesis by NRF2 activation constitutes a part of the metabolic reprogramming mediated by oncogenic PI(3)K/Akt signalling in breast cancer. By integrating analyses from a mammary epithelial cell line model, a panel of breast cancer cell lines, tumour xenografts, and TCGA breast cancer data, we provide pre-clinical evidence that may inform future therapeutic approaches for treating PI(3)K pathway mutant breast cancer cells by exploiting GSH biosynthesis as a metabolic vulnerability.
Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper.

Acknowledgements
We thank J. Brugge, B. Manning, J. Blenis, A. Beck, I. Harris and members of the Toker and Cantley laboratories for suggestions; A. Baldwin (Lineberger Comprehensive Cancer Center, University of North Carolina School of Medicine, USA), Y. R. Chin (Department of Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School, USA) and G. DeNicola (Department of Medicine, Weill Cornell Medical College, USA) for critical reagents; and M. Yuan and S. Breitkopf for technical assistance with mass spectrometry. Research support was derived in part from the National Institutes of Health (R01CA177910 (A.T.), P01CA120964 (J.M.A.), P30CA06516 (J.M.A.), R01GM041890 (L.C.C.)). E.C.L. is a pre-doctoral fellow of the NSF graduate research fellowship programme (NSF DGE1144152). C.A.L. is financially supported in part by the Pancreatic Cancer Action Network as a Pathway to Leadership Fellow and through a Dale F. Frey Breakthrough award from the Damon Runyon Cancer Research Foundation.

Author Contributions
E.C.L., C.A.L., L.C.C. and A.T. designed the study and interpreted the results. E.C.L. and H.H. assisted with the LC-MS/MS metabolomic studies and data interpretation. A.J. and A.T. wrote the manuscript. E.C.L. performed the experiments. J.M.A. and C.A.L. designed the study and interpreted the results. E.C.L.

Competing Financial Interests
L.C.C. owns equity in, receives compensation from, and serves on the Board of Directors and Scientific Advisory Board of Agios Pharmaceuticals. Agios Pharmaceuticals is identifying metabolic pathways of cancer cells and developing drugs to inhibit such enzymes to disrupt tumour cell growth and survival.

Published online at http://dx.doi.org/10.1038/ncb3341
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METHODS

Cell lines. MCF10A, MDA-MB-231, T47D and ZR-75-1 cells were obtained from the American Type Culture Collection (ATCC) and authenticated using short tandem repeat (STR) profiling. No cell lines used in this study were found in the database of commonly misidentified cell lines that is maintained by ICLAC and NCRI Biosample. MCF10A cells were maintained in DMEM/Ham's F12 (CellGro) supplemented with 5% (w/v) nonfat dry milk for 1 h, and then incubated with the TBST buer (10 mmol l⁻¹ Bio-Rad) at 100 V for 90 min. The blots were blocked in Tris-buerc saline by SDSPAGE and transferred electrophoretically to nitrocellulose membrane.

In vitro cocktail, 50 nmol l⁻¹ methionol (Sigma-Aldrich), 50 nmol l⁻¹ glutamine, 50 nmol l⁻¹ pyruvate, and 50 nmol l⁻¹ sodium pyrophosphate. Cells were maintained in the indicated growth medium or serum-free medium every two days.

Inhibitors. All antibodies except the anti-HA and anti-NRF2 antibodies were purchased from Cell Signaling Technology. Anti-HA (E545K) and pJP1520HAGFP, pJP1520HA AKT2/2 (HAGFP) were obtained from the pTRIPZ lentiviral vector (Thermo Scientific). Expression of each Akt variant was induced by treatment of cells with 1 μM doxycycline for 48 h. pJP1520HAGFP, pJP1520HA AKT1/2 lentiviral expression vector (W. Harper, Harvard Medical School). The shRNA-resistant pHAGECHAFLAGp21 was generated into the pHAGECHAFLAG lentiviral expression vector (W. Harper, Harvard Medical School). The shRNA-resistant pHAGECHAFLAGp21 was generated using site-directed mutagenesis (Qiagen).

METASTATIC POTENTIAL. MCF10A cells expressing empty vector (EV), HA AKT1/2, or HA-AKT1/2(E17K) were serum-starved for 16 h and lysed as described above in EBC buer (0.5% NP-40, 120 mmol l⁻¹ NaCl, 2 mmol l⁻¹ EDTA, 2 mmol l⁻¹ EGTA, 50 mmol l⁻¹ Tris-HCl (pH 7.4), proteinase inhibitor cocktail, 50 mmol l⁻¹ calcium, 1 mmol l⁻¹ sodium pyrophosphate, and 20 mmol l⁻¹ sodium fluoride). HA-AKT1/2 or HA-AKT1/2(E17K) was immunoprecipitated from cell extracts with an anti-HA antibody and incubated with 300 ng GSK-3β fusion protein peptide (Cell Signaling Technology) in the presence of 150 mmol l⁻¹ cold ATP in a kinase buffer (Cell Signaling Technology) for 40 min at 30 °C. The kinase reaction was terminated by the addition of SDS-PAGE sample buffer.

Proliferation assays. For WST-1 assays, MCF10A cells were seeded into 96-well plates (Corning) at a density of 1,500 cells per well in 100 μl medium. The medium was changed to either regular growth medium or serum-free medium after 16 h. Cell viability was measured at the indicated time points after the medium change using the water-soluble tetrazolium salt WST-1 assay (Clontech) according to the manufacturer's protocol.

For sulphorhodamine B assays, MCF10A cells were seeded into 12-well plates (Corning) at a density of 5 x 10⁴ cells per well in 1 ml medium. The medium was changed to either regular growth medium or serum-free medium every two days. Cell number was measured at the indicated time points using sulphorhodamine B staining, as previously described.

LC-MS/MS metabonomic profiling. Cells were maintained in the indicated growth medium, and fresh medium was added 3 h before the experiment. For metabolite extraction, medium from biological triplicates was aspirated, and 80% (v/v) methanol at dry-ice temperatures was added. Cells and the metabolite-containing supernatants were collected, and the insoluble material in lysates was centrifuged at 4,000g for 5 min. The resulting supernatant was evaporated using a refrigerated SpeedVac. Samples were re-suspended with 20 μl HPLC-grade water for mass spectrometry. Ten microfolds was injected and analysed using a 5500 QTRAP hybrid triple quadrupole mass spectrometer (AB/SCIEX) during an Apromine UFLC HPLC system (Shimadzu) with selected reaction monitoring (SRM) of a total of 254 endogenous water-soluble metabolites for steady-state analysis of samples. Some metabolites were targeted in both positive and negative ion mode for a total of 285 SRM transitions using positive/negative switching. ESI voltage was +4,900 V in positive ion mode and −4,500 V in negative ion mode. The dwell time was 4 ms per SRM transition and the total cycle time was 1.89 s. Approximately 9–12 data points were acquired per detected metabolite. Samples were delivered to the MS through normal-phase chromatography using a 4.6 mm i.d. × 10 cm Amide Xbridge HILIC column (Waters Corp.) at 300 μl min⁻¹. Gradients were run starting from 85% buffer B (HPLC grade acetonitrile) to 42% B from 0 to 5 min; 42% B to 0% from 5 to 15 min; 0% B to 16 from 16 to 24 min; 8% B to 85% B from 24 to 25 min; 85% B was held for 7 min to re-equilibrate the column. Buffer A was comprised of 20 mM ammonium hydroxide/20 mM ammonium acetate (pH = 9.0) in water/acetonitrile (95:5). Peak areas from the total ion current for each metabolite SRM transition were integrated using MultiQuant v2.0 software (AB/SCIEX). For 13C-labeled experiments, SRMs were created for expected 13C incorporation in various forms for targeted LC-MS/MS. Data analysis was performed in MatLab. For state- steady profiling, metabolite peak area intensities represent the normalized (by the average of cell number and cellular protein content), integrated total ion current from a single SRM transition and are expressed as relative values across all samples. For flux analyses, metabolite abundances represent the normalized (by cell number), integrated total ion current from a single SRM transition.

Isotope labelling. MDM lacking glucose, glutamine and pyruvate (CellGro) was supplemented with 10 mM glucose and 2 mM U-¹³C-glutamine (Cambridge Isotope Labs). Labelled medium was added to the cells, and cellular metabolites were extracted as described above at 1, 3 and 8 h.

IGF-1, hydrogen peroxide and tert-butyl hydroperoxide treatments. For IGF-1 stimulation, cells were serum-starved for 20–24 h and stimulated with 100 μg ml⁻¹ IGF-1 (R&D Systems) for 10 min. For H₂O₂ and tert-butyl hydroperoxide treatments, cells were serum-starved for 20–24 h in DMEM lacking pyruvate and then stimulated with the indicated concentrations of H₂O₂ (Fisher) or tert-butyl hydroperoxide (Sigma-Aldrich) in Hank’s balanced salt solution (HBSS; Gibco).

Cell death assay. Cells were trypsinized at 37 °C, followed by staining with 7-AAD (BD Pharmingen). Briefly, cells were washed twice with PBS and stained with 5 μl 7-AAD in binding buffer for 15 min. FACS analysis was performed with FACS Calibur (Becton-Dickinson) and FlowJo (TreeStar software).

Propidium iodide viability assay. Cell viability was assayed with a propidium iodide- based plate reader assay, as previously described. Briefly, cells in 96-well plates were treated with a final concentration of 30 μM propidium iodide for 60 min at 37 °C. Cell death was measured by quantifying the fluorescence intensity of the cells using a SpectraMax M5 (Molecular Devices) at 530 nm excitation/620 nm emission. Dicitonin was then added to each
well at a final concentration of 600 μM. After incubating for 30 min at 37°C, the final fluorescence intensity was measured. The fraction of dead cells was calculated by dividing the background-corrected initial fluorescence intensity by the final fluorescence intensity. Viability was calculated by (1 – fraction of dead cells).

Total glutathione measurements. Cellular metabolites were collected by methanol extraction and concentrated by SpeedVac evaporation, as described above. Total glutathione levels within these samples were measured according to the manufacturer’s protocol by using the Glutathione Assay Kit from Cayman Chemical, which is based on the Tietze method.38

Quantitative real-time PCR. Total RNA was isolated with the RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. Reverse transcription was performed using the Quantitect Reverse Transcript Kit (Qiagen). Quantitative real-time PCR was performed using an ABI Prism 7700 sequence detector. GCLC primer: sense, 5'-TTGATCATCTCCTGGCCGACG-3', antisense, 5'-TGTCTGAGTTTGAGCAGGGG-3'; GCLM primer: sense, 5'-CTCCTGCTTGATGACTCA-3', antisense, 5'-CTCCTGCGCTTGAATGTCAG-3'; GSS primer: sense, 5'-ATGCCCAGAAATTCTGCTTG-3', antisense, 5'-CACCTTTCTAGTCCCGAGCCAAA-3'; GSR primer: sense, 5'-CAAGAGCTTTTACCCCGATG-3', antisense, 5'-TCGGTGTCCTCATCTTCACT-3'; NQO1 primer: sense, 5'-GACAGAAGGGAATTTGCTCAGA-3', antisense, 5'-CAAAAGCTGACCAAGAGTGG-3'; GPX4 primer: sense, 5'-GAGCCAGGCCAGGGAAG-3', antisense, 5'-GGTGAAGTCCTACCTTGATGCG-3'. PCR reactions were carried out in triplicate. Quantification of mRNA expression was calculated by the ΔCT method with 18S rRNA as the reference gene.

ARE–luciferase assay. Luciferase assays were conducted using Promega’s luciferase assay system, as previously described.34 Luciferase activity was normalized by cellular protein content. The lentiviral ARE–luciferase reporter plasmid (Qiagen) was a gift from G. M. DeNicola.

TCGA data analysis. Mutation, copy number analysis, microarray, and RPPA data from the published TCGA Invasive Breast Cancer data set46 were downloaded from cBioPortal. Analysis of the data was conducted in MatLab.

3D cultures. 3D cultures were prepared as previously described. Briefly, chamber slides were coated with growth-factor-reduced, phenol-free Matrigel (Corning) and allowed to solidify for 30 min. Cells (2,000–4,000) in assay medium were seeded on coated chamber slides. Assay medium contained DMEM/Ham’s F12 supplemented with 5% equine serum (Gibco), 10 mg mL−1 insulin (Life Technologies), 500 ng mL−1 hydrocortisone (Sigma-Aldrich), 5 mg mL−1 EGF (R&D Systems), and 100 ng mL−1 cholera toxin (Sigma-Aldrich). The assay medium was replaced every four days. Doxycycline (150 ng mL−1) was added every two or three days. The indicated concentrations of BSO (Sigma-Aldrich) were added one day after seeding and re-added every two or three days.

Soft agar assays. Cells (4 × 10^4) were added to 1 mL of growth medium plus 0.4% noble agar (BD Biosciences), with or without BSO (Sigma-Aldrich). The resulting mixture was layered onto a 2 mL bed of growth medium plus 0.8% noble agar in 6-well plates (Corning), with or without BSO. Cells were fed every three to six days with 0.5 mL of growth medium (± BSO). After four weeks, the growth medium was removed, and viable colonies were stained with iodonitrotetrazolium chloride (Sigma-Aldrich). Colony number was determined using MatLab.

In vitro combination assays. MDA-MB-231, T47D and ZR-75-1 cells were seeded at a density of 10,000, 13,000, and 10,000 cells per well, respectively, in 96-well plates (Corning). Cells were treated with 50 μM BSO (Sigma-Aldrich) for 48 h, followed by treatment with cisplatin (Tocris) at a range of concentrations for 48 h, in the presence or absence of 50 μM EUK-134 or 1 mM N-acetyl cysteine. Cell viability was measured using a propidium iodide-based plate reader assay.

Xenograft studies. Female nude mice (5–6 weeks old) were purchased from Taconic and maintained and treated under specific pathogen-free conditions. All procedures were approved by the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center (BIDMC) and conform to the federal guidelines for the care and maintenance of laboratory animals. No statistical method was used to predetermine sample size. These experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment. The mice were injected subcutaneously with 5 × 10^6 T47D cells (n = 20 mice) or 2.5 × 10^6 MDA-MB-231 cells (n = 20 mice) in medium with 50% growth-factor-reduced, phenol red-free Matrigel (Corning). For mice bearing T47D xenografts, 17β-oestradiol was administered daily using a verapamil oral method.39 Tumour formation was examined every two to three days for the duration of the experiment. When tumours reached a size of 5–6 mm in diameter, they were divided into a control group and treatment groups of BSO alone, CDDP alone, and BSO in combination with CDDP (at least n = 4 per group). L-SR-BSO (Fisher) was pre-treated with BSO for seven days before treatment with CDDP, 10 mg per kilogram per week. For the combination treatment group, mice were pre-treated with BSO for seven days before treatment with CDDP. Tumour volume = (π/6)(W^2)L, where W represents width, and L represents length.

Statistics and reproducibility. Sample sizes and reproducibility for each figure are denoted in the figure legends, and raw data from independent replicate experiments can be found in the Statistics Sources Data. Unless otherwise noted, all western blots and microscopy images are representative of at least three biologically independent experiments. Statistical significance between conditions was assessed by two-tailed Student’s t-tests. All error bars represent s.e.m., and significance between conditions is denoted as * P < 0.05; ** P < 0.01; and *** P < 0.001. Statistical data for each quantitative experiment, including mean, s.e.m., and exact P values can be found in the Statistics Source Data.

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Supplementary Figure 1. Characterization of AKT1(E17K) and AKT2(E17K).

a, Anti-HA immunoprecipitates from serum-starved cells were assayed for their ability to phosphorylate a GSK-3β fusion peptide in vitro (data is representative of two independent experiments). b, Proliferation of cells grown in the presence of serum and growth factors was determined using the WST-1 assay (data are from one experiment that was independently repeated two times with similar results (Supplementary Table 1)). Unprocessed original scans of blots are shown in Supplementary Figure 6.
Supplementary Figure 2  Metabolomic profiling of serum-starved MCF10A cells expressing AKT2(E17K) vs. AKT2.  

a, Unbiased hierarchical clustering of relative metabolite abundances in serum-starved MCF10A cells expressing AKT2(E17K) vs. AKT2.  
b, Fold changes of metabolite abundances in serum-starved MCF10A cells expressing AKT2(E17K) vs. AKT2 (data are from one metabolomics experiment that was independently repeated two times with similar results).  
c, Relative abundances of glycolytic intermediates (n = 3 technical replicates from a single independent metabolomics experiment; the experiment was repeated twice with similar results (Supplementary Table 1)).  
d, Relative abundances of metabolites in the pentose phosphate pathway (n = 3 technical replicates from a single independent metabolomics experiment; the experiment was repeated twice with similar results (Supplementary Table 1)).  
e, f, Incorporation of U-13C5-glutamine into glutamine and GSSG over 1, 3, and 8 h in serum-starved cells (n = 3 technical replicates from a single metabolomics experiment (Supplementary Table 1)). All error bars represent s.e.m. *P < 0.05, **P < 0.01 by a two-sided Student’s t-test.
Supplementary Figure 3 Enhanced GSH biosynthesis confers resistance to oxidative stress. a, b, Cells were serum-starved for 20-24 h in the presence or absence of 1 µM GSK690693, followed by treatment with 1 mM H₂O₂ for 6 h. Cell death was assessed by 7-AAD staining followed by FACS (data are from one experiment that was independently repeated two times with similar results (Supplementary Table 1)). c, Total glutathione levels in cells serum-starved for 20-24 h in the presence or absence of 50 µM BSO (n = 3 biologically independent replicates (Supplementary Table 1)). d-f, Cells were serum-starved for 20-24 h in the presence or absence of 1 µM GSK690693 or 50 µM BSO, followed by treatment with 500 µM H₂O₂ for 4 h. Cells were immunoblotted for the indicated proteins (data is representative of three independent experiments). All error bars represent s.e.m. **P < 0.01, ***P < 0.001 by a two-sided Student’s t-test. Unprocessed original scans of blots are shown in Supplementary Figure 6.
Supplementary Figure 4 Nrf2 is necessary for Akt2(E17K)-mediated stimulation of GSH biosynthesis and resistance to oxidative stress. NRF2 was knocked down over 72 h. a, Nrf2 knock-down was confirmed by immunoblot analysis (data is representative of three independent experiments). b, Total glutathione levels in serum-starved cells (data are from one experiment that was independently repeated two times with similar results (Supplementary Table 1)). c, Serum-starved cells were treated with 500 µM H$_2$O$_2$ for 4 h (data is representative of three independent experiments). d, Distribution of RPPA Z-scores for Akt pS473 phosphorylation in patient tumors from TCGA BRCA data set. Breast tumors with Akt pS473 levels greater than 2 standard deviations from the mean were classified as “Akt pS473 high”, while tumors with Akt pS473 levels less than 1 standard deviation from the mean were classified as “Akt pS473 low”. Unprocessed original scans of blots are shown in Supplementary Figure 6.
Supplementary Figure 5 Inhibition of GSH biosynthesis does not robustly inhibit the growth of MCF10A cells expressing AKT2 or PIK3CA variants in 2D culture conditions. 

a, mRNA levels were measured by qRT-PCR and are expressed as fold changes relative to MCF10A AKT2 cells (n = 3 biologically independent replicates (Supplementary Table 1)).

b, c, Proliferation of cells grown in the absence of serum and growth factors, and in the presence or absence of 50 µM BSO, was determined with the SRB assay (data are from one experiment (Supplementary Table 1)).

d, e, Viability of cells grown in full growth media for 48 h in the presence of increasing doses of BSO was measured using the WST-1 assay. Viability is expressed relative to the lowest BSO concentration (data are from one experiment (Supplementary Table 1)). All error bars represent s.e.m. **P < 0.01 by a two-sided Student’s t-test.
Supplementary Figure 6 Unprocessed scans of western blots accompanied by size markers. Images were obtained by enhanced chemiluminescence detection.
Figure 1a

Supplementary Figure 6 continued
**Figure 2a**

Supplementary Figure 6 continued
Figure 2a - Different exposure, same molecular weight and lane markers as Page 3

Supplementary Figure 6 continued
Figure 2a - Different exposure, same molecular weight and lane markers as Page 3
Figure 2a

Supplementary Figure 6 continued
Figure 2a - Different exposure, same molecular weight and lane markers as Page 6

Supplementary Figure 6 continued
Figure 2a

Supplementary Figure 6 continued
Figure 2b

Supplementary Figure 6 continued
Figure 2b - Different exposure, same molecular weight and lane markers as Page 9
Figure 2b

Supplementary Figure 6 continued
Figure 2b

Supplementary Figure 6 continued
Figure 2c

Supplementary Figure 6 continued
Figure 2c - Different exposure, same molecular weight and lane markers as Page 13
Figure 2c

Supplementary Figure 6 continued
Figure 2c

Supplementary Figure 6 continued
Figure 2d

Supplementary Figure 6 continued
Figure 2d - Different exposure, same molecular weight and lane markers as Page 17
Figure 2d

Supplementary Figure 6 continued
Figure 2d

Supplementary Figure 6 continued
Figure 3c

Supplementary Figure 6 continued
Figure 3c - Different exposure, same molecular weight and lane markers as Page 21

Supplementary Figure 6 continued
Figure 3d

Supplementary Figure 6 continued
Figure 3e (left)

Supplementary Figure 6 continued
Figure 3e (right)

Supplementary Figure 6 continued
Supplementary Figure 1a

Supplementary Figure 6 continued
Supplementary Figure 1a - Different exposure, same molecular weight and lane markers as Page 26

Supplementary Figure 6 continued
Supplementary Figure 3d-f

Supplementary Figure 6 continued
Supplementary Figure 3d-f - Different exposure, same molecular weight and lane markers as Page 28
Supplementary Figure 3d-f - Different exposure, same molecular weight and lane markers as Page 28

Supplementary Figure 6 continued
Supplementary Figure 3d-f - Different exposure, same molecular weight and lane markers as Page 28

Supplementary Figure 6 continued
Supplementary Figure 3d-f

Supplementary Figure 6 continued
Supplementary Figure 3d-f

Supplementary Figure 6 continued
Supplementary Figure 4a
Supplementary Figure 4a

Supplementary Figure 6 continued
Supplementary Figure 4c

Supplementary Figure 6 continued
Supplementary Figure 4c - Different exposure, same molecular weight and lane markers as Page 36

Supplementary Figure 6 continued
Supplementary Figure 4c - Different exposure, same molecular weight and lane markers as Page 36

Supplementary Figure 6 continued