The oncogenic transcription factor c-Jun regulates glutaminase expression and sensitizes cells to glutaminase-targeted therapy

Michael J. Lukey¹, Kai Su Greene¹, Jon W. Erickson², Kristin F. Wilson¹ & Richard A. Cerione¹,²

Many transformed cells exhibit altered glucose metabolism and increased utilization of glutamine for anabolic and bioenergetic processes. These metabolic adaptations, which accompany tumorigenesis, are driven by oncogenic signals. Here we report that the transcription factor c-Jun, product of the proto-oncogene JUN, is a key regulator of mitochondrial glutaminase (GLS) levels. Activation of c-Jun downstream of oncogenic Rho GTPase signalling leads to elevated GLS gene expression and glutaminase activity. In human breast cancer cells, GLS protein levels and sensitivity to GLS inhibition correlate strongly with c-Jun levels. We show that c-Jun directly binds to the GLS promoter region, and is sufficient to increase gene expression. Furthermore, ectopic overexpression of c-Jun renders breast cancer cells dependent on GLS activity. These findings reveal a role for c-Jun as a driver of cancer cell metabolic reprogramming, and suggest that cancers overexpressing JUN may be especially sensitive to GLS-targeted therapies.
The onset of proliferation imposes a range of biosynthetic and bioenergetic demands on mammalian cells, which are met by a fundamental reprogramming of cellular metabolism. The metabolic phenotype of proliferating cells, including cancer cells, typically includes high rates of glucose uptake and glycolysis coupled to lactate secretion (the Warburg effect), elevated de novo nucleotide biosynthesis, and a high flux of mitochondrial glutamine oxidation. Increased nutrient uptake and re-routing of metabolites into anaerobic processes are not passive adaptations to the proliferative state, but instead are tightly regulated by the signal transduction pathways and transcriptional networks that promote cell growth and cell cycle progression. Thus, many of the oncogenic signals that lead to cellular transformation directly impact cancer cell metabolism.

Metabolic reprogramming supports the proliferative state but can render cancer cells ‘addicted’ to certain nutrients, and therefore provides opportunities for novel therapeutic interventions. Some cancer cells show an absolute requirement for an exogenous supply of glutamine, the most abundant amino acid in plasma. Glutamine has many metabolic fates inside the cell, acting as a carbon and nitrogen source for biosynthetic reactions and also contributing to redox homeostasis. However, it is the role of glutamine as an anaplerotic substrate for the tricarboxylic acid (TCA) cycle that underlies the ‘glutamine addiction’ of many rapidly proliferating cells. The sequential conversion of glutamine to glutamate, and then to the TCA cycle intermediate α-ketoglutarate (α-KG), provides a mechanism for replenishing carbon that is lost from the cycle to anabolic pathways. The first reaction is catalysed by the mitochondrial enzyme glutaminase (GLS), and the second reaction by glutamate dehydrogenase or by one of several transaminase enzymes.

Two genes, GLS and GLS2, encode glutaminase enzymes in mammals. The GLS gene encodes two splice variants, referred to as kidney-type glutaminase and glutaminase C (GAC), while the GLS2 gene encodes two proteins through a surrogate promoter mechanism, liver-type glutaminase and GAB. Whereas the GLS2 isoforms are downregulated in several cancers, the GLS isoforms, in particular the GAC splice variant, are frequently upregulated in cancers of the breast, lung, colon, prostate and brain. Recently, two classes of small-molecule inhibitors of GLS have been identified, based on the lead compounds bis-2-((5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES) and 968. Inhibition of GLS by these molecules, or siRNA-mediated knockdown of GLS, severely impacts the proliferation and/or survival of several cancer cell lines, but does not appear to have detrimental effects on non-tumorigenic cells. Thus, there is considerable interest in targeting GLS as a therapeutic strategy against cancer, and the BPTES derivative CB-839 is currently undergoing clinical trials.

One regulator of GLS expression and glutamine catabolism is the transcription factor c-Myc. In P493 Burkitt’s lymphoma and PC3 prostate cancer cell lines, c-Myc upregulates GLS through an indirect mechanism involving transcriptional repression of micro-RNAs miR-23a/b, which target the 3’-UTR of the GLS transcript and suppress its translation. However, the relationship between c-Myc and glutamine metabolism is complex and tissue specific, and tumour-specific alternative polyadenylation of the GLS transcript can cause a switch of the 3’-UTR, allowing it to escape c-Myc/miR-23-mediated regulation. An apparent uncoupling of c-Myc and GLS has therefore provided opportunities for novel therapeutic interventions.

We previously reported that mitochondrial glutaminase activity becomes elevated during Rho GTPase-mediated cellular transformation. Here we show that the oncogenic transcription factor c-Jun is essential for this signalling outcome, and also acts as a primary regulator of GLS expression in human breast cancer cells. Moreover, we demonstrate that overexpression of the JUN proto-oncogene is sufficient to sensitize breast cancer cells to glutaminase-targeted therapy.

Results
Rho GTPases can drive glutamine-dependent transformation. We previously reported that oncogenic-Dbl, a guanine nucleotide exchange factor and potent activator of Rho GTPases, signals to upregulate mitochondrial GLS activity in NIH/3T3 cells. This is an essential event for maintaining Dbl-induced cellular transformation. To explore further the signalling connections that link Rho GTPases with GLS, we utilized an inducible, tetracycline-off, system to control the expression of oncogenic-Dbl in mouse embryonic fibroblasts (MEFs). When doxycycline (0.6 µg ml⁻¹) is present in the culture medium, HA-tagged oncogenic-Dbl is undetectable by western blot analysis of whole-cell lysates. Removal of doxycycline triggers a robust expression of oncogenic-Dbl within 10 h that remains elevated through 72 h (Fig. 1a). This is accompanied by a corresponding increase in GLS protein levels, which peak 24–48 h following induction (Fig. 1a). We isolated mitochondria from uninduced and induced MEFs and assayed the preparations for glutaminase activity as described previously. This confirmed that induction of oncogenic-Dbl results in elevated glutaminase activity (Fig. 1b). We then tested whether GLS was upregulated at the transcriptional level. Cells that were either uninduced or induced (24 h) were analysed by real-time PCR (RT–PCR), which revealed that induction of oncogenic-Dbl expression leads to an ~12-fold increase of the GLS transcript (Fig. 1c).

Induction of oncogenic-Dbl caused MEFs to acquire transformed characteristics, including greatly increased saturation density, and the ability to proliferate in low serum (0.5% fetal bovine serum (FBS)) medium and to undergo anchorage-independent growth. Since these changes were accompanied by an increase in GLS levels, we tested whether cellular transformation was dependent on an exogenous supply of glutamine, and/or on GLS enzymatic activity. After 8 days of culture in medium supplemented with 10% FBS ± 4 mM glutamine, saturation density was assessed by fixing and staining cells with crystal violet (Fig. 1d). Prior to fixation, cells were imaged at × 100 magnification (Supplementary Fig. 1a). The increased saturation density of cells expressing oncogenic-Dbl was completely abolished in glutamine-free medium, but could be partially rescued by supplementation with a cell-permeable analogue of α-ketoglutarate (dimethyl-α-KG), a downstream metabolite of the GLS reaction. We compared cell viability under high-glutamine (4 mM) and low-glutamine (0.2 mM) conditions, and found that uninduced cells remained fully viable following glutamine depletion, whereas induced cells showed an ~5-fold increase in cell death (Fig. 1e). The proliferation rate of both uninduced and induced cells decreased on glutamine deprivation, although the inhibition was ~2-fold greater in induced cells (Fig. 1f).

We then used BPTES, a small-molecule inhibitor of GLS, to test whether MEFs transformed by oncogenic-Dbl were dependent on GLS activity. Saturation density of induced cells was decreased by BPTES treatment in a dose-dependent manner, and partially rescued by supplementation with dimethyl-α-KG (Fig. 1g and Supplementary Fig. 1b). Cell proliferation assays illustrated the remarkable difference in BPTES sensitivity between uninduced and induced MEFs (Fig. 1h). Proliferation of induced cells was potently inhibited by BPTES with an IC₅₀ of 8 µM, whereas uninduced cells were unaffected even by 20 µM BPTES. Oncogenic-Dbl-induced cells, but not uninduced cells, were capable of anchorage-independent growth. This too was completely blocked by BPTES treatment (Fig. 1i).
Rho GTPases signal to c-Jun to upregulate GLS expression. The results above show that oncogenic-Dbl signals to upregulate GLS at the transcript and protein level, and that cellular transformation driven by oncogenic-Dbl is dependent on GLS activity and an exogenous supply of glutamine. We next wanted to identify the signalling pathway downstream of the Rho GTPases that was responsible for regulating GLS expression. We first used phospho-specific antibodies to assess the effects of 0
50
100
Uninduced
Induced
0
20
40
60
80
100
120
0
Uninduced
Induced
α-KG
BPTES
α-KG
30 μM
40 μM
Glutaminase activity
nmol min⁻¹ per mg protein
Number of colonies
**

Figure 1 | Glutamine-dependent transformation by oncogenic-Dbl. (a) Western blot analysis showing timecourse of oncogenic-Dbl expression in an inducible MEF system, and downstream elevation of GLS levels. Cells were induced by plating in doxycycline-free growth medium, and samples were collected at time-points up to 72 h. (b) Glutaminase activity assay using mitochondria isolated from MEFs in which oncogenic-Dbl expression was either uninduced (+ Dox) or induced for 24 h (− Dox). Activity is expressed per mg of total cellular protein, and data presented are the mean ± s.d. of triplicate assays. (c) RT-PCR analysis of uninduced and induced (24 h) MEFs, showing relative levels of the GLS transcript. The data presented are the RQ values, with error bars marking RQ max and RQ min, from triplicate reactions. (d) Saturation density analysis showing the effect of glutamine withdrawal on oncogenic-Dbl inducible MEFs. Dishes of uninduced (+ Dox) and induced (− Dox) cells cultured in 4 mM glutamine, or in glutamine-free medium ± 2 mM dimethyl α-ketoglutarate, were fixed and then stained with crystal violet. (e) Cell death analysis for uninduced or induced cells after 6 days culture in 4.0 or 0.2 mM glutamine. Data presented are the mean ± s.d. of triplicate assays. (f) Cell proliferation assays showing the effect of glutamine depletion on proliferation of uninduced and induced MEFs over 6 days. Data presented are the mean ± s.d. of triplicate assays. (g) Saturation density analysis showing the effect of the GLS inhibitor BPTES on oncogenic-Dbl induced MEFs. Induced (− Dox) cells cultured in the absence or presence of BPTES (30 or 40 μM) ± 2 mM dimethyl α-ketoglutarate were fixed and then stained with crystal violet. (h) BPTES dose curves showing the effect of different BPTES concentrations on proliferation over 6 days of uninduced or induced MEFs. Fractional proliferation relative to untreated cells is shown. Assays were carried out in 10% FBS medium, and data presented are the mean ± s.d. of triplicate assays. (i) Anchorage-independent growth assay for uninduced (+ Dox) cells, and for induced (− Dox) cells cultured under increasing BPTES concentrations. Data presented are the mean ± s.d. of triplicate assays. Differences were analysed with Student’s t-test. *P<0.05, **P<0.01.
oncogenic-Dbl induction on key cellular signalling proteins. Here, and throughout the study, low-serum conditions (0.5% FBS) were used for signalling experiments to minimize ‘background’ cellular signalling activity. Western blot analysis of whole-cell lysates revealed an extremely potent activation of the MKK4/JNK-c-Jun signalling axis downstream of oncogenic-Dbl. Total c-Jun levels are also elevated, consistent with previous reports showing auto-regulation of JUN expression. The MAPK p38 is moderately activated, whereas ERK is only slightly activated on oncogenic-Dbl induction. ROCK activity, as read-out by MLC 2 phosphorylation, is activated on induction. Akt is slightly inhibited, and AMPK and mTORC1 activity (the latter read-out by 56K phosphorylation) are largely unaffected by oncogenic-Dbl induction. Relative densitometry data are the mean ± s.d. of triplicate blots.

Figure 2 | Oncogenic-Dbl potently activates the MKK4-JNK-c-Jun signalling axis in MEFs. Western blot analysis of whole-cell lysates of MEFs in which oncogenic-Dbl expression was either uninduced (+ Dox) or induced for 24 h (−Dox) under low-serum (0.5% FBS) conditions. The extent of activation of different signalling pathways was assessed using phospho-specific antibodies that recognize activated kinases or their downstream phosphorylation targets (left panels). Any changes in total protein levels were also assessed (right panels). (a) The MAPK signalling axis involving MKK4-JNK-c-Jun is potently activated downstream of oncogenic-Dbl. Total c-Jun levels are also elevated, consistent with previous reports showing auto-regulation of JUN expression. (b) The MAPK p38 is moderately activated, whereas ERK is only slightly activated on oncogenic-Dbl induction. (c) ROCK activity, as read-out by MLC 2 phosphorylation, is activated on induction. (d) Akt is slightly inhibited, and AMPK and mTORC1 activity (the latter read-out by 56K phosphorylation) are largely unaffected by oncogenic-Dbl induction. Relative densitometry data are the mean ± s.d. of triplicate blots.
Figure 3 | Oncogenic-Dbl signals to c-Jun to upregulate GLS in MEFs. (a) Western blot analysis of whole-cell lysates of uninduced (+ Dox) or induced for 48 h (– Dox) MEFs ± 10 μM selective inhibitors of ROCK (Y-27632), p38 (SB203580) or JNK (SP600125). Inhibition of JNK largely blocks the upregulation of GLS downstream of oncogenic-Dbl. (b) Western blot analysis showing that inhibition of mTORC1 by rapamycin has little effect on the upregulation of GLS downstream of oncogenic-Dbl. (c) Glutaminase activity assay using mitochondria isolated from uninduced (+ Dox) MEFs and from MEFs that had been induced for 24 h (– Dox) in the absence or presence of 10 μM SP600125 (JNK inhibitor). Activity is expressed per mg of total cellular protein and data presented are the mean ± s.d. of triplicate assays. (d) RT–PCR analysis of uninduced (+ Dox) and 24 h induced (– Dox) MEFs ± 10 μM SP600125, showing relative levels of the GLS transcript. The data presented are the RQ values, with error bars marking RQ max and RQ min, from triplicate reactions. (e) Western blot analysis showing that the AP-1 inhibitor SR11302 (10 μM) completely blocks GLS expression in oncogenic-Dbl-induced MEFs. (f) RT–PCR analysis showing that transient transfection of uninduced (+ Dox) MEFs with constitutively activated JNK/MKK fusion constructs leads to increased GLS mRNA abundance. The data presented are the RQ values, with error bars marking RQ max and RQ min, from triplicate reactions. (g) Western blot analysis of the samples from the previous panel, showing that ectopic expression of constitutively activated JNK fusion constructs in uninduced MEFs leads to increased phosphorylation of c-Jun and upregulated GLS protein levels. (h) RT–PCR analysis showing that transient transfection of uninduced (+ Dox) MEFs with constitutively activated JNK/MMK fusion constructs leads to increased GLS mRNA abundance. The data presented are the RQ values, with error bars marking RQ max and RQ min, from triplicate reactions. (i) Western blot analysis of the samples from the previous panel, showing that ectopic expression of JUN in uninduced MEFs leads to increased levels of phospho-c-Jun and upregulated GLS protein levels. Note that the ectopically expressed c-Jun contains a V5-tag, and consequently runs at a slightly higher molecular weight than endogenous c-Jun. Relative densitometry data are the mean ± s.d. of triplicate blots. Differences were analysed with Student’s t-test. *P < 0.05, **P < 0.01.
inhibitor of the AP-1 family of transcription factors to which c-Jun belongs. This resulted in the complete loss of GLS to undetectable levels (Fig. 3e).

The experiments above suggested that the signal to elevate GLS expression downstream of oncogenic-Dbl is transmitted via JNK-mediated phosphorylation of a Jun-family AP-1 transcription factor, most likely c-Jun, since it is highly expressed in these cells (Fig. 2a), in contrast to JunB and JunD (Supplementary Fig. 2c). To test whether JNK and c-Jun can upregulate GLS expression, we transiently transfected uninduced MEFs with constructs for expressing constitutively activated MKK/JNK fusion proteins or c-Jun. Uninduced cells were cultured for 48 h after transfection (in 10% FBS medium, as transfection reagents harmed viability of MEFs in 0.5% FBS medium), and then analysed by RT–PCR and western blot. Ectopic expression of JNK1, JNK2 or JNK3 fusion-constructs all led to elevated GLS transcript levels relative to vector-control cells (Fig. 3). Western blot analyses of whole-cell lysates showed that phospho-c-Jun and GLS were both elevated downstream of the JNK constructs (Fig. 3g). Similarly, ectopic expression of the JUN gene resulted in upregulated GLS transcript level (Fig. 3h), and elevated levels of both phospho-c-Jun and GLS (Fig. 3i).

Collectively, these results indicate that, downstream of oncogenic Rho GTPase signalling, JNK transmits a signal to increase transcription of the GLS gene by phosphorylating and activating the oncogenic transcription factor c-Jun. This prompted us to investigate the possible role of c-Jun in regulating GLS expression in human cancer cells.

c-Jun and GLS correlate in human breast cancer cell lines. Because the JUN proto-oncogene is overexpressed, and associated with aggressive behaviour, in a subset of human breast cancers, we probed a panel of 12 breast cancer cell lines for c-Jun and GLS. First, we obtained an estimate of the glutamine dependence of each cell line by assaying proliferation over 6 days in culture medium containing either 2.0 or 0.1 mM glutamine (Supplementary Fig. 3). We then collected cells at ~60% confluency from complete RPMI medium (10% FBS, 2 mM glutamine) and probed whole-cell lysates by western blot, with samples ordered by increasing glutamine dependence of the cell line, left to right. This revealed a very strong correlation ($R = 0.85$) between relative levels of c-Jun and GLS (Fig. 4a and Supplementary Fig. 4a). In particular, cell lines with high endogenous c-Jun levels (BT-549, Hs 578T, MDA-MB-231 and TSE) all showed highly elevated GLS levels. The level of c-Jun also correlated very strongly with the glutamine dependence of the cell lines ($R = 0.83$; see Supplementary Fig. 4b) and moderately strongly with their proliferation rates ($R = 0.63$; see Supplementary Fig. 4c). The abundance of activated c-Jun, as indicated by phosphorylation at residue S73 (a JNK target residue), similarly correlated very strongly with GLS (Fig. 4a), whereas the related Jun-family transcription factors, JunB and JunD, showed less correlation (Fig. 4b). Relative levels of glutamate dehydrogenase (GLUD1/2) showed little variation among the cell lines (Supplementary Fig. 5).

The JNK1 and JNK2 proteins each exist as two splice variants, p46 (predominantly JNK1) and p54 (predominantly JNK2). Whereas p46 JNK1 is the primary kinase for activating c-Jun, endogenous p54 JNK2 can negatively regulate c-Jun stability. We found that p46 JNK is expressed and phosphorylated to a similar extent across the panel of breast cancer cell lines (Fig. 4c). In contrast, although p54 JNK is expressed in all cell lines, it only shows strong phosphorylation in ZR-75-1 and CAMA-1 (Fig. 4c). Given that p46 JNK is activated in all cell lines, and that phosphorylated c-Jun is proportional to total c-Jun across the panel (Fig. 4a), we conclude that differences in total c-Jun activity between the cell lines are due primarily to variations in c-Jun protein levels, rather than to differences in JNK activity.

**Figure 4 | c-Jun correlates strongly with GLS levels in human breast cancer cell lines.** Cells were collected at ~60% confluency from RPMI growth medium supplemented with 10% FBS, and whole-cell lysates prepared and analysed by western blot. Samples were ordered according to glutamine dependence, increasing from left to right (Supplementary Fig. 3). (a) Correlation between c-Jun/phospho-c-Jun and GLS levels. Quantification of GLS and c-Jun band intensities allowed a Pearson correlation coefficient of 0.85 to be determined (Supplementary Fig. 4a). (b) Other Jun-family members do not correlate strongly with GLS levels. (c) Under 10% FBS culture conditions, p46 JNK (lower band) is active in all of the breast cancer cell lines. Neither JNK nor phospho-JNK correlate with GLS levels. (d) Other reported regulators of GLS expression do not strongly correlate with GLS levels.
We also probed lysates for three previously reported regulators of GLS, namely c-Myc and STAT1 that upregulate GLS22,23,40, and the retinoblastoma protein (Rb), loss of which leads to elevated GLS levels through an unknown mechanism41 (Fig. 4d). None of these proteins correlated with GLS, although we note that all three are subject to post-translational regulation.

The GLS promoter directly binds c-Jun at a consensus motif. We next examined whether c-Jun binds directly to the GLS promoter. The promoter region for the human GLS gene was analysed to position −5,000 bp relative to the transcription start site (TSS), and a number of putative c-Jun binding sites (<15% dissimilarity to the consensus sequence) were identified using the PROMO42 resource (Supplementary Fig. 6). An earlier genome-wide survey of c-Jun binding sites found them to be located in close proximity to the TSS, and mammalian transcription factor binding sites in general are strongly enriched around −200 bp from the TSS53,43. We found a close match to the consensus c-Jun binding motif (TGA[G/C]TCA) at position −188 bp relative to the TSS of human GLS (5′-TGACTCC-3′) (Supplementary Figs 6 and 7a). A close match to the c-Jun consensus motif is also present at position −200 bp in the mouse GLS promoter (5′-TGAACCA-3′) (Supplementary Fig. 7b). Exact matches to the c-Jun consensus motif (5′-TGAGTCA-3′) were also found at position −2,211 bp for human GLS, and positions −2,469 and −1,545 bp for mouse GLS.

We then carried out chromatin immunoprecipitations (ChIPs) to test whether c-Jun binds directly to the GLS promoter. For these experiments, we used MDA-MB-231 breast cancer cells, which have high endogenous levels of both GLS and c-Jun (Fig. 4a). Briefly, cross-linked chromatin was digested to a length of ~150–900 bp, and an antibody against endogenous c-Jun was used to immunoprecipitate c-Jun/DNA complexes. A parallel immunoprecipitation was carried out using IgG, as a negative control. Protein-DNA cross-links were then reversed, and RT–PCR was performed using primers designed to amplify a 196-bp fragment centred on the putative c-Jun binding site at position −188 bp relative to the TSS (Supplementary Fig. 7a). This yielded a strong signal from the c-Jun ChIP relative to the IgG ChIP, indicating that c-Jun binds to this region of the GLS promoter (Fig. 5a). Similar results were obtained using two additional sets of primers to amplify slightly shorter fragments also centred on the predicted c-Jun binding site (Supplementary Fig. 8).

c-Jun regulates GLS expression and BPTES sensitivity. Since c-Jun directly binds the promoter of the GLS gene, and endogenous levels of c-Jun and GLS correlate strongly in human breast cancer cell lines, we determined whether inhibition of JNK, inhibition of AP-1 family transcription factors, or knockdowns of c-Jun, affected GLS levels in these cells. We first inhibited JNK using 15 μM SP600125 in the high-GLS cell lines MDA-MB-231 and TSE. This led to a sharp decrease in phospho-c-Jun levels, and GLS expression was strongly suppressed within 24 h and remained so through 72 h (Fig. 5b). We also directly inhibited AP-1 transcriptional activity in MDA-MB-231 cells using SR11320 (48h), and observed a dose-dependent suppression of GLS expression (Fig. 5c). Reciprocally, ectopic expression of a constitutively activated JNK1 fusion protein in MDA-MB-231 cells, under low serum (0.5% FBS) conditions, resulted in elevated c-Jun phosphorylation and elevated GLS levels (Fig. 5d).

In BT-549 cells, which are reported to be relatively drug-resistant44, treatment with 15 μM SP600125 caused only a very modest inhibition of c-Jun phosphorylation (Fig. 5e, left panel). Consistent with this, SP600125 treatment did not lead to decreased GLS levels in this cell line, and we therefore used siRNAs to knockdown JUN expression. Prolonged depletion of c-Jun in BT-549 cells (and in other cell lines with high endogenous c-Jun levels) could not be tolerated and resulted in cell death. Nevertheless, whole-cell lysates from BT-549 cells treated with JUN-targeted siRNAs and collected after 48 h repeatedly showed that GLS levels were lower in c-Jun-depleted cells (Fig. 5e, right panel). Quantification of band intensities confirmed that the degree of c-Jun depletion correlated with the degree of GLS depletion.

The results above indicate that the relationship between c-Jun and GLS levels in breast cancer cells is not only correlative but is also causative. We therefore examined whether cell lines with high c-Jun levels are more sensitive to the GLS inhibitor BPTES. Proliferation assays were carried out for all 12 breast cancer cell lines under a range of BPTES concentrations. Defining highly sensitive cell lines as those with an IC₅₀ < 10 μM BPTES, none of the eight cell lines with low c-Jun levels were highly sensitive to BPTES, whereas three of the four cell lines with high c-Jun levels were highly sensitive (Supplementary Table 1). The one exception was the drug-resistant cell line BT-549. Representative dose curves for a highly sensitive cell line (MDA-MB-231, IC₅₀ = 1.8 μM), a moderately resistant cell line (T-47D, IC₅₀ = 20 μM), and a highly resistant cell line (CAMA-1, IC₅₀ = n/a) are shown (Fig. 5f). The much greater BPTES sensitivity of the high c-Jun cell lines, Hs 578T, MDA-MB-231 and TSE, relative to the low c-Jun lines, is illustrated by the response to 2 μM BPTES (Fig. 5g and Supplementary Fig. 9).

Inhibition of JNK by SP600125 led to a sharp decrease in GLS levels in MDA-MB-231 (and other) breast cancer cells (see above). We therefore carried out proliferation assays to compare BPTES sensitivity of MDA-MB-231 cells cultured in the absence or presence of 15 μM SP600125. Treatment with the JNK inhibitor sharply decreases the proliferation rate relative to untreated cells. However, the slow proliferation that still occurs in the presence of SP600125 is markedly desensitized to BPTES, with the IC₅₀ for BPTES shifting ~7-fold from 1.8 to 12 μM (Fig. 5h).

c-Jun is sufficient to elevate GLS in breast cancer cells. Using the MDA-MB-468 breast cancer cell line, which exhibits relatively low endogenous levels of c-Jun and moderate levels of GLS (Fig. 4a), we generated derivative cell lines that stably overexpress JUN-V5 or carry the empty plasmid vector. We then collected vector-control cells and JUN-overexpressing cells at ~60% confluency from RPMI medium (10% FBS, 2 mM glutamine), and probed whole-cell lysates for GLS, c-Jun and V5-tag by western blot (Fig. 6a). This showed that GLS levels are elevated in the JUN-overexpressing cells relative to the vector-control cells. Mitochondria isolated from the two cell lines were assayed for glutaminase activity, which was markedly higher in the JUN-overexpressing cells (Fig. 6b). Moreover, RT–PCR analysis confirmed that GLS transcript levels were upregulated in JUN-overexpressing cells relative to vector-control cells (Fig. 6c). We made equivalent derivative cell lines of the MCF7 parental cell line, which has very low endogenous levels of both c-Jun and GLS (Fig. 4a), and similarly found that GLS expression was upregulated in JUN-overexpressing cells (Supplementary Fig. 10a).

The level of c-Myc did not vary between the vector-control and JUN-overexpressing cells (Fig. 6d), and treatment with the c-Myc inhibitor 10058-F4 at concentrations up to 60 μM (48h) had little effect on GLS levels in the derivative cell lines (Fig. 6e and Supplementary Fig. 10b). In contrast, treatment with the AP-1 inhibitor SR11320 (1–10 μM) caused a dose-dependent
Figure 5 | Inhibition of c-Jun suppresses GLS expression and BPTES sensitivity in human breast cancer cell lines. (a) ChIP analysis showing that c-Jun binds to the GLS promoter. Complexes containing c-Jun were immunoprecipitated from cross-linked, digested, chromatin isolated from MDA-MB-231 cells. A parallel immunoprecipitation using rabbit IgG was carried out as a negative control. Following reversal of cross-links and purification of DNA, RT–PCR was run using primers designed to amplify a 196-bp fragment centred on the putative c-Jun binding site at position – 188 bp relative to the TSS. The data presented are the RQ values, with error bars marking RQ max and RQ min, from triplicate reactions. (b) Western blot analysis showing that treatment of MDA-MB-231 or TSE cells with the JNK inhibitor SP600125 (15 μM) leads to decreased phosphorylation of c-Jun, and decreased GLS levels. (c) Treatment of MDA-MB-231 cells with the AP-1 inhibitor SR11302 (1–10 μM) for 48 h results in a dose-dependent decrease in GLS. (d) Western blot analysis showing that transient transfection of MDA-MB-231 cells with a constitutively activated JNK fusion construct results in increased c-Jun phosphorylation and upregulated GLS levels. Cells were collected 48 h after transfection. (e) Western blot analysis showing that in the drug-resistant breast cancer cell line BT-549, treatment with 15 μM SP600125 has little effect on c-Jun phosphorylation and does not lead to decreased GLS levels (left panels). However, knockdown of JUN expression using siRNAs leads to decreased GLS levels. Relative band intensities are indicated. (f) Representative BPTES dose curves showing the effect of BPTES on the proliferation of breast cancer cell lines over 6 days. Curves were fitted using SigmaPlot, with data from triplicate assays. (g) Sensitivity of breast cancer cell lines to GLS inhibition, as indicated by inhibition of proliferation over 6 days by 2 μM BPTES. Of the high-c-Jun lines, only the drug-resistant BT-549 cells were not highly sensitive to BPTES. None of the low-c-Jun lines were highly sensitive. Data presented are the mean ± s.d. of triplicate assays. (h) BPTES dose curves for MDA-MB-231 cells ± 15 μM SP600125, showing that inhibition of JNK desensitizes cells to GLS inhibition (the IC50 for BPTES shifts from 1.8 to 12 μM). Curves were fitted using SigmaPlot, with data from triplicate assays. Relative densitometry data are the mean ± s.d. of triplicate blots.
suppression of GLS in both cell lines, an effect that was especially pronounced in the JUN-overexpressing cells (Fig. 6f).

Overexpression of JUN sensitizes cancer cells to BPTES. As described above, MDA-MB-231 cells treated with the JNK inhibitor SP600125 become desensitized to the GLS inhibitor BPTES (Fig. 5h). To complement this result, we carried out proliferation assays to determine whether overexpression of the JUN proto-oncogene is sufficient to increase the BPTES sensitivity of MDA-MB-468 cells. Although vector-control cells showed some response to BPTES treatment, overexpression of JUN greatly sensitized cells to BPTES treatment (Fig. 6g). Supplementation of the medium with 1 mM dimethyl α-KG completely rescued cells from ≤12 μM BPTES, and partially rescued cells from higher BPTES concentrations (Fig. 6g). Although the parental and vector-control MDA-MB-468 cell lines

Figure 6 | c-Jun increases GLS expression and BPTES sensitivity in breast cancer cells. (a) Western blot analysis of GLS and c-Jun levels in whole-cell lysates of MDA-MB-468 cells, stably carrying either pCDNA3.1 or the JUN-V5 expression vector pCDNA3.1-JUN. Relative band intensities are indicated for GLS. (b) Glutaminase activity assay using mitochondria isolated from the derivative MDA-MB-468 cell lines. Activity is expressed per mg of total cellular protein, and data presented are the mean ± s.d. of triplicate reactions. (c) RT-PCR analysis of the derivative MDA-MB-468 cell lines, showing relative abundance of the GLS transcript. The data presented are the RQ values, with error bars marking RQ max and RQ min, from triplicate reactions. (d) Western blot analysis showing c-Myc levels in the derivative cell lines. (e) Western blot analysis showing that treatment of vector-control, or JUN-overexpressing, MDA-MB-468 cells with the c-Myc inhibitor 10058-F4 at concentrations up to 60 μM for 48 h has little effect on GLS levels. Relative band intensities are indicated for GLS. (f) Western blot analysis showing that treatment with the AP-1 inhibitor SR11302 (1-10 μM) for 48 h leads to a dose-dependent decrease in GLS levels. The effect is especially pronounced in JUN-overexpressing cells. (g) Cell proliferation assays for MDA-MB-468 cells stably carrying pCDNA3.1 or the JUN expression vector. Cells were seeded in 12-well dishes at a density of 1 × 10^4 cells per well, cultured for 6 days in the absence or presence of 9 μM (left panel), 12 μM (middle panel) or 15 μM (right panel) BPTES ± 1 mM dimethyl α-KG. Inhibition of proliferation by BPTES treatment is plotted as a percentage. The data presented are the mean ± s.d. of triplicate blots. Differences were analysed with Student’s t-test. *P<0.05, **P<0.01.
showed little dependence on the GLS reaction, both are moderately glutamine-dependent (Supplementary Figs 3 and 11). This reflects the importance of an exogenous glutamine supply for other metabolic processes that do not involve the GLS reaction, such as nucleotide biosynthesis and protein synthesis. Glutamine dependence of the JUN-overexpressing derivative cell line was unchanged from that of the parental cells (Supplementary Fig. 11).

c-Jun regulated transcript levels in invasive breast cancer.
To establish further the relationship between c-Jun activity and GLS expression in breast cancer, we used the cBioportal suite of tools (www.cbioportal.org) to analyse data from The Cancer Genome Atlas (TCGA) Breast Invasive Carcinoma (TCGA, provisional) data set (959 samples). In many cancers, upregulation of c-Jun occurs at the post-transcriptional and post-translational levels, but not at the transcriptional level (see Discussion). Consequently, JUN mRNA levels are rarely upregulated, even though c-Jun protein levels can be highly elevated. Consistent with these findings, TCGA data showed that JUN mRNA was elevated (z score >2.0) in only 3% of invasive breast carcinoma samples. We therefore determined whether the expression levels of some validated c-Jun transcriptional targets correlate with those of GLS. We selected only confirmed direct targets of c-Jun (and not v-Jun) that contain a c-Jun binding site within the promoter region35,47–49, and we eliminated from the list those c-Jun target genes that are frequently altered at the genomic level within the data set (for example, CCND1, amplified in 16% of samples, and TP53, mutated in 31% of samples). Using the resulting list of c-Jun targets (Supplementary Table 2), we found that transcripts that are upregulated by c-Jun almost invariably correlate positively with GLS mRNA levels, whereas those targets that are transcriptionally repressed by c-Jun exhibit a negative correlation with the GLS transcript (Fig. 7; see also Supplementary Figs 12 and 13).

Discussion
In proliferating cells, the TCA cycle serves as a major source of biosynthetic precursors. Metabolic intermediates that are lost to anabolic pathways must be rapidly replenished, and the most abundant carbon sources for these anaplerotic reactions are glucose and glutamine. Indeed, anaplerosis is a key function underlying the upregulated consumption of these nutrients by cancer cells10,11,50. An important route for the delivery of glutamine-derived carbon into the TCA cycle begins with the hydrolysis of glutamine to glutamate, a reaction catalysed by glutaminase. The TCA cycle intermediate α-ketoglutarate can then be generated through the action of glutamate dehydrogenase, or through transaminase activities.

In this study, we used an inducible MEF system for hyper-activating Rho GTPase signalling, which we previously linked to increased mitochondrial GLS activity in NIH-3T3 cells20, and also to upregulated glutamine-mediated anaplerosis for oxidative TCA cycle flux in MEFs51. Ectopic expression of oncogenic-Dbl in NIH-3T3 cells leads to elevated levels of the GLS transcript (Supplementary Fig. 14a), but the lower background level of GLS in MEFs relative to NIH-3T3 cells (Supplementary Fig. 14b) allows for a much clearer read-out, by RT–PCR and especially by western blot, of changes in GLS expression. By selectively inhibiting signalling pathways activated downstream of the Rho GTPases in the inducible system, we have now discovered that the c-Jun N-terminal kinase (JNK) and the oncogenic transcription factor c-Jun are critical for upregulating GLS at the transcript and protein levels. We also found that in human breast cancer cell lines, c-Jun levels correlate strongly with GLS levels and with sensitivity to the GLS inhibitor BPTES. Moreover, we determined that c-Jun directly binds the promoter of the GLS gene and increases its expression. Importantly, this not only leads to elevated mitochondrial glutaminase activity, but also causes cancer cells to become more dependent on glutamine-mediated anaplerosis.

The c-Jun transcription factor is the cellular homologue of v-Jun, the transforming oncoprotein of avian sarcoma virus 17 (refs 52,53). A key role for c-Jun in both healthy and neoplastic tissue is to drive cell cycle progression, and fibroblasts derived from JUN-null mouse embryos exhibit a severe defect in proliferation54. The c-Jun protein is phosphorylated at the M/G1 transition55, and loss of c-Jun or expression of mutants defective for JNK-catalysed phosphorylation leads to a G2/M cell cycle block56. One transcriptional target of c-Jun is the CCND1 gene, which encodes the G1 to S phase regulator cyclin D1 (ref. 57). c-Jun also binds to a variant AP-1 site in the TP53 gene promoter, leading to suppression of p53 and of the p53-regulated cyclin-dependent kinase inhibitor p21 (ref. 54). The net effect is that c-Jun increases the activity of G1 cyclin-dependent kinase complexes.

When taken together, these findings provide a biological rationale for the positive regulation of GLS by c-Jun that we describe. In order for sustained proliferation to occur, signalling to the cell cycle machinery must be coordinated with reprogramming of cellular metabolism to support biomass accumulation. By simultaneously driving cell cycle progression and upregulating GLS expression, c-Jun promotes cell proliferation and also activates TCA cycle anaplerosis to replenish metabolites that have been directed to biosynthetic pathways (Fig. 8). Notably, two other reported regulators of GLS expression are involved in cell cycle control. The Rb protein prevents G1 to S phase progression58, and loss of this tumour suppressor leads to upregulation of GLS41. Similarly, the oncogenic transcription factor c-Myc promotes G1 to S phase transition, and is a positive regulator of GLS expression22,23. Mirroring the ability of v-Jun to cause avian sarcoma, the JUN gene is highly amplified and overexpressed in aggressive human sarcomas59, and in an analysis of copy number alterations across 3,131 diverse cancer samples, JUN was found to be significantly
amplified across the entire data set. In many cancers, protein levels of c-Jun are highly upregulated even in the absence of JUN amplifications or elevated transcription of the JUN proto-oncogene. This can be due to both translational activation and increased protein stability. For example, c-Jun is highly elevated in melanoma cells as a result of increased translation following loss of mi-R125b, which binds to the coding region of the JUN transcript and suppresses translation in normal melanocytes. In breast cancer cells, c-Jun is also upregulated at the protein level but not at the transcriptional level. One mechanism for this involves downregulation of the tumour suppressor COP1 E3 ubiquitin ligase, which targets c-Jun for degradation. Indeed, among the cell lines used in the present study, BT-549, Hs 578T and MDA-MB-231 are reported to have low COP1 levels, whereas MCF7 and T47D have elevated COP1 (ref. 37).

For transcriptional activity, c-Jun and related transcription factors must form a dimeric complex, which is known as AP-1 and consists of Jun–Jun or Jun–Fos dimers. Jun-family members (c-Jun, JunB and JunD) can dimerize with themselves or with Fos proteins, whereas Fos-family members (c-Fos, Fra-1, Fra-2 and FosB) must heterodimerize with a Jun protein. This raises some questions concerning the fine-tuning of GLS regulation. Of the AP-1 transcription factors, c-Jun is the most potent transcriptional activator. However, Jun-Fos heterodimers are more stable and efficient at driving transcriptional activation than Jun–Jun dimers. Importantly, the promoter specificity of dimers containing c-Jun differs according to the dimerization partner, and the relative abundances of potential partners vary between cell types. Consequently, the Jun- and Fos-family members that are expressed within a given cell likely influence the regulation of GLS expression by c-Jun, and it is possible that JunB or JunD might substitute for c-Jun in some contexts. High levels of c-Jun are associated with aggressive, invasive and metastatic behaviour in breast cancer. Ectopic overexpression of JUN in the MCF7 cell line leads to increased invasion and tumorigenicity, whereas a dominant-negative variant of c-Jun causes cell cycle arrest and suppression of tumorigenicity in vivo. Several studies have found that c-Jun is specifically elevated in triple-negative breast cancer (TNBC) cells and MYC expression is also reported to be upregulated in TNBC. This could explain why TNBC cell lines have higher GLS levels and greater sensitivity to the GLS inhibitor CB-839 (a BPTES derivative) than receptor-positive breast cancer cells.

In conclusion, we have identified a JNK/c-Jun-dependent signalling pathway that is responsible for upregulating GLS levels during cellular transformation. Moreover, we show that the c-Jun transcription factor is an important regulator of GLS expression in breast cancer, and can drive cellular dependence on the glutaminase reaction, thus conferring sensitivity to the GLS inhibitor BPTES. Our findings link a key player in cell cycle progression with a metabolic pathway that supports cell proliferation, and illustrate that re-programming of cellular metabolism is intricately connected with oncogenic transformation. Furthermore, our work reveals a novel function for the c-Jun oncoprotein in cancer, and suggests why certain types of cancer such as triple-negative breast cancer might be more susceptible to glutaminase-targeted therapy.

Methods

Isogenic inducible MEK system for oncogenic-Dbl expression. The inducible MEK line used in this study was generated as described previously. Briefly, the gene encoding oncogenic-Dbl was sub-cloned into vector pTRE-HA (Clontech). Parental MEFs containing the transcriptional transactivator TTA (Clontech) were then co-transfected with the resulting pTRE-HA-onco-Dbl vector along with vector pMET-puro, in a 20:1 ratio. Following puromycin selection (~3 weeks), colonies were screened for inducible expression of oncogenic-Dbl. Cells were maintained at 37 °C, 5% CO2 atmosphere, in DMEM medium containing 4 mM glucose (Gibco) and supplemented with 10% (v/v) tetracycline-free FBS (Gibco) and 0.6 μg mL⁻¹ doxycycline. Expression of oncogenic-Dbl was induced by re-plating cells in doxycycline-free medium (10% FBS). Residual doxycycline was removed by replacing the medium (with the appropriate concentration of FBS, and small-molecule inhibitor where relevant) after 5 h. Uninduced control samples were treated in the same way: re-plating followed by media change at 5 h, but with 0.6 μg mL⁻¹ doxycycline present at all times. For glutamine-withdrawal experiments, glutamine-free DMEM medium (Gibco) was supplemented with dialysed FBS (Gibco). Growth medium containing other concentrations of glutamine was prepared by mixing appropriate volumes of DMEM (4 mM glucose) and glutamine-free DMEM. DNA constructs. DNA primers were synthesized by Integrated DNA Technologies. Plasmid pCDNA3.1-V5-His TOPO was purchased from Life Technologies. The JUN gene was PCR amplified from plasmid pMEGIC-c-Jun, which was a gift from Alexander Dent (Addgene plasmid # 40348), using primers JUN_AMP_F (5′-TTATGGATCCATGACTGCAAAGATGGAAACGACC-3′) and JUN_AMP_R (5′-TTATGATATCAAATGTTTGCAACTGCTGCGTTAGC-3′), which added a BamH1 and EcoRV site, respectively. The PCR product was purified (QiAquick PCR purification kit, Qiagen), digested with BamH1 and EcoRV (New England Biolabs), re-purified and ligated (T4 DNA ligase, New England Biolabs) into pre-digested and purified pCDNA3.1-V5-His TOPO to obtain an expression vector for JUN-V5, pCDNA3.1-JUN. The construct was verified by sequencing. The plasmids pCDNA3 Flag M KK7B2Mkk3a2 (Addgene plasmid # 19726), pCDNA3 Flag M KK7B2Mkk3a2 (Addgene plasmid # 19727) and pCDNA3 Flag M KK7B2Mkk3a2 (Addgene plasmid # 19729) were all gifts from Roger Davis.
Antibodies and reagents. Antibodies recognizing the following proteins were purchased from Cell Signaling Technology: HA-tag (cat. no. 3724) used at 1:5,000, β-glycerophosphate (50 mM solution in DMSO) and doxycycline (5 h), growth medium was replaced with fresh medium containing appropriate supplements. Growth medium was subsequently replaced at 48 h intervals. At day 8, growth medium was removed and 1.5 ml 3.7% formaldehyde solution in H2O was added to 30 min. The formaldehyde solution was removed, and 1.5 ml crystal violet solution was added for 20 min at room temperature. This was then removed, and dishes washed four times with 3 ml H2O and allowed to dry before imaging.

Mitochondrial isolation. Mitochondria were isolated using the Qproteome mitochondrial isolation kit (QIAogen), following the manufacturer’s instructions. Briefly, cells were scraped into 1 ml of ice-coldPBS containing 1% (w/v) aprotinin and insoluble debris was pelleted by centrifugation and removed. The supernatant was washed in PBS, and the mitochondrial pellet was resuspended and kindly provided by Dr Scott Urlich, Ithaca College. ROCK inhibitor Y-27632 (10 mM solution in DMSO), the INK inhibitor SP600125 (50 mM solution in DMSO) and G418 were purchased from Calbiochem. All other reagents were purchased from Sigma-Aldrich unless otherwise stated.

Real-time PCR analysis. Total RNA was extracted from cells using the RNAeasy mini kit (QIAGEN), and cDNAs were prepared using the SuperScript III first-strand synthesis system (Life Technologies). RT–PCR was carried out using the 7,500 fast real-time PCR system (Applied Biosystems), with cDNA as a template and GLS primers GFS-L (5'-TCGTAACCAGCAATGTTCCAG-3') and GFS-R (5'-TCTGATCGACCAGATCCTGCA-3') or anti-mouse IgG, HRP-linked (cat. no. 7076), or Santa Cruz Biotechnology donkey anti-goat IgG-HRP (cat. no. sc-2020). BPTES was used at the final concentration in TBST. They were then washed in TBST, and protein concentration was determined by Bradford assay (Bio-Rad), and lysates proteins denatured by boiling for 5 min in reducing SDS-sample buffer. Lysate protein (15 μg total protein/lane) were then resolved on Novex 4–20% Tricine glycerol mini or midi protein gels (Life Technologies), and transferred to polyvinylidine difluoride membranes (PerkinElmer). Membranes were blocked in 5% bovine serum albumin in tris-buffered saline and tween 20 (TBST) for 1 h at room temperature, and probed overnight at 4 °C in primary antibody solution (manufacturer recommended concentration) in TBST. The membranes were washed in TBST, and incubated in TBST solution containing 25% (v/v) non-fat dry milk powder and appropriate secondary antibody at the manufacturer’s recommended concentration for 1 h. Finally, the membranes were washed in TBST, and bands imaged using Western Lightning Plus-ECL (PerkinElmer) and HyBlot E2 autoradiography film (Denville Scientific Inc.).

MEF saturation density analysis. DMEM medium supplemented with 10% FBS ± 0.6 μg/ml -1 doxycyline was added to 60 mm dishes (3 ml per dish), and dishes were then seeded with 1 × 10^5 MEFs per dish. Following cell attachment (5 h), growth medium was replaced with fresh medium containing appropriate supplements. Growth medium was subsequently replaced at 48 h intervals. At day 8, growth medium was removed and 1.5 ml 3.7% formaldehyde solution in H2O was added for 30 min. The formaldehyde solution was removed, and 1.5 ml crystal violet solution was added for 20 min at room temperature. This was then removed, and dishes washed four times with 3 ml H2O and allowed to dry before imaging.

Epiphone analysis. Whole-cell lysates were prepared in lysis buffer (50 mM HEPES pH 8.0, 150 mM NaCl, 1 mM Na2VO4, 25 mM NaF, 1% (v/v) Triton X-100, 1 mM MgCl₂, 50 mM β-glycerophosphate, 30 μg/ml -1 leupeptin, 5 μg/ml -1 aprotinin) and insoluble debris was pelleted by centrifugation and removed. Protein concentration was determined by Bradford assay (Bio-Rad), and lysates proteins denatured by boiling for 5 min in reducing SDS-sample buffer. Lysate protein (15 μg total protein/lane) were then resolved on Novex 4–20% Tricine glycerol mini or midi protein gels (Life Technologies), and transferred to polyvinylidine difluoride membranes (PerkinElmer). Membranes were blocked in 5% bovine serum albumin in tris-buffered saline and tween 20 (TBST) for 1 h at room temperature, and probed overnight at 4 °C in primary antibody solution (manufacturer recommended concentration) in TBST. The membranes were washed in TBST, and incubated in TBST solution containing 25% (v/v) non-fat dry milk powder and appropriate secondary antibody at the manufacturer’s recommended concentration for 1 h. Finally, the membranes were washed in TBST, and bands imaged using Western Lightning Plus-ECL (PerkinElmer) and HyBlot E2 autoradiography film (Denville Scientific Inc.).

MFE anchorage-independent growth assay. Uninduced or onconogenic-Dbl-induced MEFs were seeded at a density of 8,000 cells/ml in medium containing 0.3% (v/v) agarose, onto underlayers of medium containing 0.6% agarose, in six-well plates. Two layers of medium were supplemented with appropriate concentrations of doxycyline (uninduced cells) and BPTES. Cultures were fed every 3 days, and the total number of colonies was counted after 15 days.

Cell proliferation assays. Culture medium supplemented with glutamine or with inhibitors at the described concentrations was added to 12-well plates (1 ml per well), and dishes were seeded with cells at day 0 as follows (numbers optimized to avoid overcrowding of the assay): MDA-MB-231, MDA-MB-435, MDA-MB-468, BT-549: 1 × 10^4 cells per well. HS 578T, MCF7, T-47D, CAMA-1, SK-BR-3, BT-474: 1.5 × 10^4 cells per well. ZR-75-1: 2 × 10^5 cells per well. Following cell attachment, growth medium was replaced at 12 h and subsequently every 48 h. At day 6, cells were trypsinized, suspended in an appropriate medium supplemented with 10% FBS, and a hemocytometer was used to determine the total number of cells per well. All cell proliferation assays were carried out in triplicate, and the mean and s.d. calculated.

Stable cell lines. Transfection of breast cancer cell lines with plasmid DNA constructs (pcDNA3.1 or pCDNA3-I) were carried out using Lipofectamine 2000 transfection reagent (Life Technologies), following the manufacturer’s instructions. At 48 h after transfection, cells were placed under G418 selection by supplementing the growth medium with G418 (500 μg/ml) for MCF7, and BT-474. Media was changed every 3 days for 2–3 weeks, until isolated colonies (~2 mm diameter) were apparent on the plate. At this point, individual clones were transferred to 12-well dishes and expanded in
250 μg ml⁻¹ G418 for further analysis. Appropriate clones were screened for c-Jun-V5 by western blot, and V5-positive clones were maintained in 250 μg ml⁻¹ G418.

**TCGA data.** The Cancer Genome Atlas (TCGA) Breast Invasive Carcinoma (TCGA, provisional) data set was accessed, all data were analysed, and correlation plots prepared using the cBioportal⁴⁵,⁴⁶ suite of tools (www.cibioportal.org).

**Statistical analyses.** All differences were analysed with Student’s t-test. A P value <0.05 was considered to be significant and marked (*), and a P value below 0.01 was considered to be highly significant and marked (**).
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Author contributions
M.J.L. conceived and designed the study, carried out experiments, analysed data and drafted the manuscript. K.S.G. prepared cDNAs, carried out RT-PCR experiments, carried out anchorage-independent growth assays and analysed data. J.W.E. and K.F.W. provided conceptual advice, supplied reagents and contributed to the manuscript text. R.A.C. helped conceive and design the study, provided conceptual advice and contributed to the manuscript text. All authors read and approved the final manuscript.

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