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An siRNA screen identifies the GNAS locus as a driver in 20q amplified breast cancer

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Abstract

Poor prognosis oestrogen receptor positive breast cancer is characterized by the presence of high-level focal amplifications. We utilized a focused siRNA screen in 14 breast cancer cell lines to define genes that were pathogenic in three genomic regions focally amplified in oestrogen receptor positive breast cancer, 8p11-12, 11q13, and 20q. Silencing the GNAS locus, that encodes the G protein alpha stimulatory subunit Gαs, specifically reduced the growth of 20q amplified breast cancer cell lines. Examination of a publically available shRNA data set confirmed GNAS silencing to be selective for 20q amplified cancer cell lines. Cell lines with 20q amplification were found to over-express specifically the extra long Gαs splice variant (XLαs). Over-expression of XLαs induced cAMP levels to a greater extent than Gαs, suggesting that amplification of the GNAS locus, and over-expression of the XLαs variant in particular, enhanced cAMP signalling. GNAS silencing in amplified cell lines reduced ERK1/2 phosphorylation, and conversely over-expression of exogenous XLαs in a non-amplified cell line increased MEK-ERK1/2 phosphorylation, identifying one potential down-stream consequence of enhanced cAMP signalling. Our data indicate that amplification of the *GNAS* locus may contribute to the pathogenesis of breast cancer, and highlight a previously unrecognized role for the GNAS XLαs variant in cancer.

Keywords

Breast Cancer; Amplification; GNAS; siRNA

Introduction

The presence of focal amplification characterizes poor prognosis, high proliferation oestrogen positive luminal B subtype breast cancer [1,2]. Within these amplifications, or

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amplicons, one or more genes are thought to act as oncogenes, oncogenic drivers, promoting the development of breast cancer. Amplified oncogenic drivers may represent potential therapeutic targets, as exemplified by amplification and over-expression of *HER2* (*ERBB2*) in 17q21 amplified breast cancer [3] which is associated with clinical benefit from drugs targeting HER2 such as the therapeutic antibody trastuzumab [4].

Amplifications of chromosome loci 8p11-12, 11q13, and 20q are frequent in poor prognosis luminal B subtype breast cancer [2]. Amplification of each individual locus is associated with poor prognosis, and identification of the drivers within these amplicons is likely an important first step towards improving the prognosis of these cancers. For each of these amplicons, potential driver oncogenes have been identified or proposed [2,5]. For 8p11-12 amplifications, both *ZNF703* and *FGFR1* have been identified as potential oncogenes [6-9]. For 11q, amplification of *CCND1* (Cyclin D1) has long been proposed as the driver oncogene [5]. Amplifications of 20q are frequently broad, and potential oncogenes include *AURKA* (Aurora kinase A) and *ZNF217* [10,11]. Which genes within the amplicons are required for ongoing growth and/or survival of established cancers is less clear.

Analysis of amplicon structures by array CGH, and more recently by massive parallel sequencing, has identified the complex genomic structure of many amplicons. Although amplicons at the genomic locus of 17q21 (*ERRB2)* are characterized by a single common region of high level amplification pointing to a dominant oncogene in that region, a minimally amplified region that is centred on *ERBB2 (HER2)*, other amplifications such as 8p11-12 and 20q do not share a single common region of amplification [12-14]. Similarly, distinct regions in the amplicon may be amplified only in a subset of amplified cancers [12-14]. This suggests that for the majority of amplifications in breast cancer, there are potentially multiple genes in each amplicon that may contribute to oncogenesis. Whole genome sequencing has subsequently revealed the disordered nature of many amplifications [15], adding further complexity to identifying potential oncogenes on the basis of genomic analysis.

RNA interference screens have the potential to identify key determinants of cancer cell line growth [16], as well as resistance to therapies [17]. We set out to interrogate a panel of breast cancer cell lines with an siRNA library targeting all genes within amplifications of 8p11-12, 11q13 and 20q, to identify novel amplicon drivers and genes relevant to the biology of breast cancer. We subsequently identify the *GNAS* locus as being a potential driver in the 20q amplification, and elucidate the molecular consequences of amplification of the *GNAS* locus.

Results

siRNA screen to identify drivers of breast cancer amplicons

We identified breast cancer cell lines with 8p11-12, 11q13 or 20q amplification by analysis of in-house and publically available array CGH data [18,19](www.cosmic.com). After optimization for siRNA screening, rejecting cell lines that did not transfect robustly, we identified eight cell lines with 8p11-12 amplification, eight with 11q13 amplification, and six with 20q amplification in a total of 14 cell lines (Figure 1 and Supplementary Tables 1

and 2). For each amplicon, we defined the amplicon boundaries as the genomic region that was amplified in at least 20% of cancers amplified at that locus (as discussed in Materials and Methods). Techniques such as GISTIC examine for the presence of a single driver in a region, focusing on minimally amplified areas and regions with the highest copy number ratio [20]. We used a wider definition to include the shoulders of amplicons that may also contain genes of potential clinical significance, as exemplified by co-amplification of *TOP2A* in approximately a third of *HER2* amplified cancers and the relationship with response to anthracycline chemotherapy [21].

An siRNA library was constructed targeting all genes in the amplified regions (284 genes, Supplementary Table 2). Each cell line was transfected with the siRNA library in triplicate, and the effect of siRNA on survival/growth expressed as a Z score (Figure 1). Individual screen replicas were highly reproducible (Figure 1b) and screens were only included in the analysis if they had a Z' factor >0.3 (Materials and Methods). We also transfected microRNA inhibitors (miRIDIAN microRNA hairpin Inhibitors) targeting all miRNA in the amplified regions (17 microRNAs), although none of the microRNA inhibitors had a detectable effect on cell line growth (data not shown).

For the panel of cell lines, genomic copy number was assessed by array CGH and gene expression by whole genome gene expression arrays, as published previously [16]. For each genomic region gene expression and copy number were significantly correlated (Pearson correlation coefficient $p<0.05$) for 50% (29/58) genes in 8p11-12, 55% (43/78) in 11q13, and 25% (36/148) in 20q (Figure 1c and Supplementary Table 2), indicating that in general, increased copy number was reflected in elevated mRNA expression. In contrast, siRNA Z scores did not in general correlate with copy number nor with gene expression levels, with only 5.6% of siRNA Z scores significantly correlated with copy number, and 4.9% siRNA Z scores correlated with gene expression (Figure 1c). This suggested, that overall, the majority of genes in amplicons do not function to promote ongoing proliferation in an amplification dependent manner.

Identification of potential amplicon drivers

To confirm the potential of the screen to identify drivers of growth/survival we supplemented the siRNA library with *ESR1* siRNA as an internal control. Oestrogen receptor (ER, ESR1) positive cell lines were substantially more sensitive to *ESR1* siRNA than ER negative cell lines (Median Z score ER positive −2.0, ER negative 0.1, $p<0.001$, Mann Whitney U Test) (Figure 2a). We similarly compared the siRNA Z scores for each gene between amplified cell lines and non-amplified cell lines, examining for genes with an absolute difference in median Z score >1 between amplified and non-amplified with the also difference being statistically significant by Mann Whitney U Test (p value <0.05). However, no siRNA were identified using these strict criteria as being selective between amplified and non-amplified cell lines.

We therefore examined with less strict criteria to identify potential drivers. We defined an siRNA as being one that significantly reduced survival with a Z score <−2 (occurring by chance in approximately 2.5% siRNA). We identified those siRNA that reduced survival in a greater proportion of amplified cell lines than non-amplified (Supplementary Table 3).

These criteria likely had high sensitivity in identifying potential drivers, but low specificity. Using these criteria we did identify a number of genes previously identified as potential amplicon drivers including *CCND1* on 11q13 and *ZNF217* and *AURKA* on 20q (Supplementary Table 3).

We selected 11 genes for revalidation and repeated siRNA transfection with an additional 6 cell lines making 20 cell lines in total (Supplementary Table 4). This revalidation identified siRNA against C11orf67, EYA2, and GNAS as having evidence of being amplicon selective (Figure 2b and Supplementary Table 4). The phosphatase EYA2 has previously been suggested to be a driver of 20q amplifications in ovarian cancer [22]. EYA2 silencing decreased the growth of the amplified MCF7 cell line with multiple different siRNA (Figure 2 c/d), suggesting that EYA2 may also be an oncogenic phosphatase in breast cancer.

GNAS locus is required for the growth of a subset of breast cancer cell lines

We noted that GNAS siRNA were amplicon selective in the screen, with GNAS siRNA reducing the survival of 50% of amplified cell lines, compared to 0% of non-amplified lines (Supplementary Table 3). GNAS siRNA had a significantly greater effect on amplified cell lines in the revalidation screen of 20 cell lines (Figure 2b and Supplementary Table 4). We examined publically available data to provide confirmatory evidence that the GNAS locus was a driver of 20q amplified cancers. GNAS locus expression was substantially higher in amplified cancer cell lines compared to non-amplified cancer cell lines in the Broad–Novartis Cancer Cell Line Encyclopaedia (Figure 2e, $P<0.001$ Student's T test) [23]. We next examined publically available pooled shRNA data from the Broad Institute Achilles project [24]. In this data set GNAS amplified cancers cell were more sensitive to GNAS shRNA than non-amplified cancers cell lines, both using the CCLE criteria of high level amplification (Figure 2f, $p=0.004$) and other criteria for amplification (Supplementary Figure 1). This therefore provided independent confirmation that the GNAS locus was a driver of 20q amplified cancers, not just 20q amplified breast cancer, and that amplification of the GNAS locus promoted cancer cell line growth.

Silencing GNAS with multiple different siRNA reduced the growth of amplified cell lines, confirming the effect to be on-target (Figure 3a/b), and induced G1 cell cycle arrest in SKBR3 cells (Figure 3c). GNAS is a complex locus that encodes multiple different transcripts including the G protein stimulatory alpha subunit (Gαs), a transcript variant that initiates from an alternative first exon encoding a variant stimulatory subunit with a distinct extra long N-terminus (XLαs), NESP55 and ALEX which are structurally unrelated proteins, and an antisense non-coding RNA transcript that may regulate the imprinting of this region in somatic tissues [25]. The GNAS siRNA used in this screen would target Gαs, XLαs, and NESP55, but not other GNAS locus RNA variants. To differentiate targeting of Gαs/XLαs from NESP55 we performed a rescue experiment with rat Gαs that differs from human Gαs in only a single amino acid. Of the individual siRNA targeting Human GNAS, siRNA-GNAS-B was predicted not to silence rat Gαs due to a divergent nucleotide sequence whereas the target sequence of siRNA-GNAS-A was identical between rat and human genes. Transfection of rat Gαs rescued Gαs expression following siGNAS B transfection but not following siGNAS A transfection (Figure 3d), and transfection of a rat

Gαs expression construct specifically rescued the effect of siRNA-GNAS-B, but not siRNA-GNAS-A, providing evidence that the siRNA effects were not due to targeting NESP55 (Figure 3d).

To assess whether Gαs or XLαs was most likely to contribute to the effects of amplification of the GNAS locus we examined relative expression of the two splice variants between amplified and non-amplified cancers. Overall, GNAS locus mRNA (with a probe that assesses both Gαs or XLαs) was over-expressed in amplified cell lines compared to non-amplified in whole genome gene expression arrays (Figure 3e). Interestingly, analysis by real-time PCR suggested that cancer cell lines specifically over-expressed the XLαs variant (Figure 3f). XLαs expression has previously only been documented in neuroendocrine tissues [25,26], and we therefore examined the potential consequences of GNAS XLαs in more detail.

Consequences of GNAS XLαs over-expression

To investigate this further we used lentiviral expression constructs to over-express Gαs and XLαs in the CAL120 cell line, which does not express XLαs (Figure 4a). We generated two independent stable pools for both variants, and an empty vector control (pLEX). Gαs is expressed at high levels in all the cancer cell lines, although over-expression of exogenous Gαs did increase levels approximately 3 fold (Figure 4a) Over-expression of XLαs, but not Gαs, significantly increased cAMP production (Figure 4b and Supplementary Figure 1). Over-expressed exogenous Gαs exhibited a cytoplasmic distribution similar to that of endogenous Gαs. In contrast, exogenous XLαs was membranous in distribution, and did not overlap with endogenous Gαs (Figure 4c). This concurs with recent observations for XLαs in neuroendocrine cell lines where XLαs has been shown to be constitutively active though escaping cytoplasmic redistribution [26,27].

We similarly generated stable over-expressing cell lines with MCF10A, a breast epithelial cell line (Supplementary Figure 2). MCF10A grow in an epithelial growth pattern, with cellular junctions expressing e-cadherin. Over-expression specifically of the XLαs variant, but not Gαs, resulted in loss of e-cadherin mediated cell junctions and organized cell-cell contact (Supplementary Figure 2). This data highlighted that XLαs has distinct biological functions compared to the ubiquitously expressed Gαs form.

Signalling consequences of XLαs over-expression

We examined the signalling consequences of GNAS silencing in cancer cell lines, examining two amplified cell lines that showed reduced proliferation with GNAS siRNA (SKBR3 and ZR75.1, Figure 3) and three non-amplified cell lines that were not sensitive to GNAS siRNA (JIMT1, CAL51 and CAL120). Silencing GNAS, with an siRNA SMARTpool that targets both Gαs and XLαs, reduced ERK1/2 phosphorylation and ERK1/2 total levels, specifically in *GNAS* amplified XLαs expressing cell lines SKBR3 and ZR75.1 (Figure 5). The same effect was also seen with multiple different siRNA targeting GNAS in SKBR3 cells (Supplementary Figure 1). G protein signalling via cAMP and Protein Kinase A has been shown to potentially activate SRC [28], yet we found no consistent effect on SRC family Tyr416 phosphorylation that correlates with SRC kinase activity

(Figure 5). Finally, we noted that one of the amplified XLαs expressing cell lines was *HER2* amplified and dependent (SKBR3). No difference in HER2 autophosphorylation was observed suggesting the effect of GNAS silencing was not mediated by modulating HER2 activity (Figure 5).

To extend these observations we examined signalling in the stable XLαs over-expressing CAL120 cell lines (Figure 6). XLαs over-expression increased the level of phosphorylation of C-RAF, MEK, and ERK1/2 (Figure 6a), with no change in PI3K-AKT-mTOR phosphorylation (Figure 6b), giving the consistent opposite effect of silencing with siGNAS in amplified cell lines (Figure 5). cAMP stimulates the guanidine exchange factor EPAC1 (RAPGEF3) for the small G protein RAP1 [29], and thereby stimulates MAPK pathway RAF, MEK, and ERK1/2 signalling. We utilised the EPAC specific cAMP analogue 8CPT-2Me-cAMP [30] to examine whether EPAC activation stimulated MAP MEK-ERK signalling in CAL120. Activation of EPAC1 with 8CPT-2Me-cAMP specifically increased ERK1/2 phosphorylation, to a substantially greater degree than activation of adenylate cyclase with forskolin, suggesting that stimulation of EPAC1 could enhance MAPK pathway phosphorylation in CAL120 cells (Figure 6c). Together this data suggested that expression of XLαs in breast cancers through increased cAMP production potentiates MAPK pathway signalling to promote breast cancer growth.

Discussion

We report a comprehensive analysis of three focal amplifications of poor prognosis luminal B type breast cancer. We identify the GNAS locus as a novel amplicon selective driver in 20q amplified breast cancer, and confirm the selectivity in an independent publically available shRNA data set. We subsequently elucidate the molecular mechanisms of GNAS over-expression, and show that amplified cancers over-express the GNAS XLαs splice variant and that over-expression of the GNAS XLαs splice variant enhances cAMP production. The potential consequences and mediators of enhanced cAMP production are likely multiple, although we show in part that enhanced cAMP may promote breast cancer growth through potentiation of MAPK signalling.

The data from our screen suggests that this study, as initially conceived, was underpowered to detect amplicon drivers. Whereas the majority of ER positive cell lines show some degree of dependence on ER (ESR1, Figure 2a), we were not able to identify such strong effects in the amplicon screen. There are a number of potential explanations for this observation. This may reflect substantial heterogeneity in the amplicon drivers, the oncogenes driving proliferation in the same amplicon may differ between individual cancers, or that the oncogene drivers are the same in all cancers but secondary resistance mechanisms blunt the consequences of silencing the gene. For example, silencing of GNAS with siRNA did not reduce the survival of all amplified cell lines likely reflective of co-activation of alternative drivers in these cell lines. In addition, cooperation between different amplicon drivers may remain unmasked by silencing a single gene, and amplicon drivers may be required for initiation of breast cancer but not ongoing proliferation of established breast cancer. For example, we noted that *ZNF703*, and *FGFR1*, were not included on the list of potential 8p drivers despite strong evidence that these are drivers of 8p amplification [6-9]. A substantial

increase in the number of cancer cell lines would be required to tackle the issue of power in similar future screens. This may require combining cell lines of different tissue types, although this will assume the driver is the same in all tissues, or the generation of new cell lines or primary xenografts.

A substantial body of evidence links G protein mediated cAMP signalling to modulation of MAPK pathway activity, both stimulating and inhibiting depending on the cellular context [31], acting through classical signalling mediated by protein kinase A and through the cAMP sensing guanine nucleotide exchange factors EPAC1 and EPAC2 (also known as RAPGEF3 and RAPGEF4) [31,32]. Protein kinase A can modulate MAPK signalling through Protein kinase A mediated phosphorylation of SRC [33], inhibitory phosphorylation of C-RAF [34], and phosphorylation of the scaffold protein KSR-1 to promote signalling along the RAF-MEK-ERK1/2 cascade [35]. Separately, binding of cAMP to the EPACs stimulates the small GTP binding protein RAP1, which subsequently activates RAF and downstream MEK-ERK1/2 signalling [31,32]. In this manuscript we demonstrate that over-expression of XLαs stimulates cAMP production, and this promotes MAPK pathway activity. Stimulation of EPAC1 results in increased ERK1/2 phosphorylation (Figure 6c), although we have not directly addressed the mechanisms through which XLαs and subsequent cAMP expression (Figure 4b) promotes MAPK pathway activation.

Our study provides evidence that XLαs expression contributes to the development of breast cancer, through membrane localization (Figure 4) that may exaggerate cAMP production [26]. XLαs expression has previously been demonstrated in neuroendocrine tissues, although we demonstrate expression both in breast cancer cell lines (Figure 3f) and breast cancers (Figure 6d). Analysis of cDNA from 18 high grade ER positive breast cancers, demonstrated substantial expression of XLαs in a subset of non-amplified breast cancers suggesting a possible role for XLαs outside the context of gene amplification (Figure 6d), although this would require future validation. XLαs expression did not transform MCF10A cells, as assessed by the inability to grow in soft agar (data not shown), suggesting that expression of GNAS-XLαs alone is not oncogenic, but may cooperate with other oncogenic drivers to facilitate pathogenesis through potentiation of cAMP and MAPK pathway signalling.

Activating *GNAS* mutations are frequent in neuroendocrine malignancies of the pituitary and adrenal gland [36], and have also been recently described in epithelial malignancies including ovarian and pancreas cancers [37,38]. GNAS has been reported to be over-expressed in breast cancer when amplified [37], and our data extends this observation providing functional confirmation that the GNAS locus is a driver in 20q amplified breast cancer. Our observations further extend the role of the *GNAS* locus in epithelial cancers and identifying a novel role for the GNAS XLαs variant.

Materials and Methods

Cell lines and antibodies

Cell lines were obtained from ATCC or Asterand, and maintained in phenol red free DMEM or RPMI with 10% FBS (PAA gold) and 2mM L-glutamine (Sigma-Aldrich, Dorset, UK). For all cell lines serum was Charcoal-Dextran stripped and supplemented with 1nM

estradiol (Sigma). All cell lines were banked in multiple aliquots on receipt to reduce risk of phenotypic drift, and identity confirmed by STR profiling with the PowerPlex 1.2 System (Promega) and arrayCGH profiling. Antibodies used were phosphorylated AKT-Ser473 (4058), AKT-Thr308 (2965), AKT (4691), phosphorylated mTOR (2971/2894), mTOR (2983), phosphorylated cRAF (9421), cRAF (9422) phosphorylated MEK (9121), MEK (9122), phosphorylated ERK1/2-Thr202/Tyr204 (4370), phosphorylated RSK-Thr359/Ser363 (9344) (all Cell Signalling Technology, Danvers, MA), β-actin (sc-1616) GNAS (sc-383), HA tag (sc-7392) (Santa-Cruz Biotechnology, Santa Cruz, CA). Compounds used were Forskolin (Sigma F6886), IBMX (Sigma I5879), and 8CPT-2Me-cAMP (Tocris Bioscience).

siRNA library and screening

The screening library consisted of Dharmacon siGENOME SMARTpools targeting all genes within the wider regions of amplification, supplemented with non-targeting siRNA Pool#1 (siCON, D-001206-13), PLK1 siGenome SMARTpool (siPLK1, M-003290-01) as a positive control, and ESR1 siGenome SMARTpool (M-003401-04). Screening was performed in 96 well plates essentially as described previously [16,39]. Briefly, cells were reverse transfected in 96 well plates at a final siRNA concentration of 50nM, and 5 doubling times post transfection the proportion of surviving cells was assessed with Cell Titre-Glo cell viability assay (Promega, Madison, WI). Individual plates were median normalised before combination, and the effect of each siRNA was expressed as a Z score, with the standard deviation estimated from the median absolute deviation. The robustness of the screen was assessed by the siCON and siPLK1 control wells, with the screen rejected unless the Z′ factor was > 0.3. Screen data were analysed in Microsoft Excel and R software package. miRIDIAN microRNA Hairpin Inhibitors (Dharmacon) were transfected in parallel for all microRNA to a final concentration of 5μM and analysed separately as surviving fraction compared to median level of miRIDIAN Negative Control #1 and #2.

For revalidation of results with individual siRNA, cell lines were similarly transfected, with results expressed as a surviving fraction relative to the growth of siCON control wells.

Expression vectors

cDNA encoding GNAS-Gαs and GNAS-XLαs [40] was cloned into the pLEX-MCS lentiviral expression vector (OpenBiosystems). Virus was generated as previously described [41], before infection of cells and selection with puromycin of independent stable pools.

cAMP assessment

For cAMP measurements, cells were washed once with PBS and incubated for 15 min at 37°C with IBMX buffer (RPMI medium supplemented with 35mM HEPES pH7.4, 2mM IBMX, 0.1% BSA) (Mariot et al 2011). Cells were then washed twice on cold PBS and the cell number assessed with a Beckman Coulter Counter to standardise. cAMP concentrations were measured using R&D systems cAMP parameter assay kit (KGE002B) as per manufacturer instructions. Measurements were standardised to total protein content of the samples.

Array CGH and gene expression

Cell line array CGH and whole genome gene expression data (Ilumina ref 6 V2) were analysed as reported previously [16]. Amplicon boundaries were assessed using previously published array CGH data [42,43], including all regions amplified in at least 20% of breast cancers with amplification of the genomic locus, defined as 8p 35.1Mb-43.1Mb, 11q 67.8Mb-78.4Mb, 20q 45.0-62.9Mb (as per genome reference GRCh37/hg19). Amplification defined as a log2 ratio >0.45 as validated in previous publications with the array CGH platform [42,43]. All genes within defined regions were included in the siRNA screening library, along with all microRNA.

Western blotting and FACS

Indicated cell lines where grown on 10cm plates, treated as indicated, and lysed in NP40 lysis buffer. Western blots were carried out with precast TA or Bis-Tris gels (Invitrogen) as previously described. FACS analysis was performed as previously described [41].

Immunofluorescence

Cells grown on coverslips were fixed with 4% paraformaldehyde, before incubation with primary antibodies against GNAS (1:100, sc-383), HA (1:100, sc-7392,), E-cadherin (1:500, Abcam Ab1416), and Actin-488 (1:1000, Invitrogen A12379), and corresponding secondary Alexa-444 or Alexa-555 conjugates antibodies, with DAPI nuclear stain. Cells were visualized with a Leica Confocal microscope.

Quantitative PCR

cDNA was synthesised from RNA using Superscript III and random hexamers (Invitrogen). Quantitative PCR was performed by absolute quantification with TAQMAN chemistry on an ABI Prism 7900T System (Applied Biosystems) with GNAS (Hs00255603_m1), EYA2 (Hs00193347_m1), and control genes MRPL19 (Hs00608519_m1) and TFRC (Hs00951083_m1). GNAS-XLαs specific gene expression was assessed by SYBR green chemistry (QuantiTect SYBRgreen, Qiagen) with the oligos forward 5′-TCGACAAACAACTCCAGGAC-3′ and 5′-GCAGGATCCTCATCTGCTTC-3′ reverse spanning the exon 1-2 boundary of NM_080425.2.

Analysis of publically available data sets

Publically available copy number data assessed with Affymetrix Genome-Wide Human SNP Array 6.0, and gene expression arrays assessed with the GeneChip Human Genome U133 Plus 2.0 Array, were from 947 cancer cell lines [23]. Cancer cell lines were defined as being amplified with a copy number ratio >1.0 as defined by Barrentina et al for high-level amplification using Segmented copy-number profiles 2012-09-29.seg, and GNAS mRNA expression was RMA-normalised data from probe 2778_at 2012-10-18.res http://www.broadinstitute.org/ccle/home. shRNA targeting GNAS were identified from the publically available pooled shRNA data [24]. The mean log fold changes in abundance of five shRNA targeting the GNAS locus (TRCN0000083413m_st, TRCN0000083414m_st, TRCN0000083415m_st, TRCN0000083416m_st, TRCN0000083417m_st) was calculated

as the survival effect of GNAS shRNA, and compared between cancers with high-level amplification and those without.

Statistical analysis

All statistical tests were performed with GraphPad Prism version 5.0 or Microsoft Excel. Unless stated otherwise, p values were two tailed and considered significant if $p<0.05$. Error bars represent SEM of three experiments

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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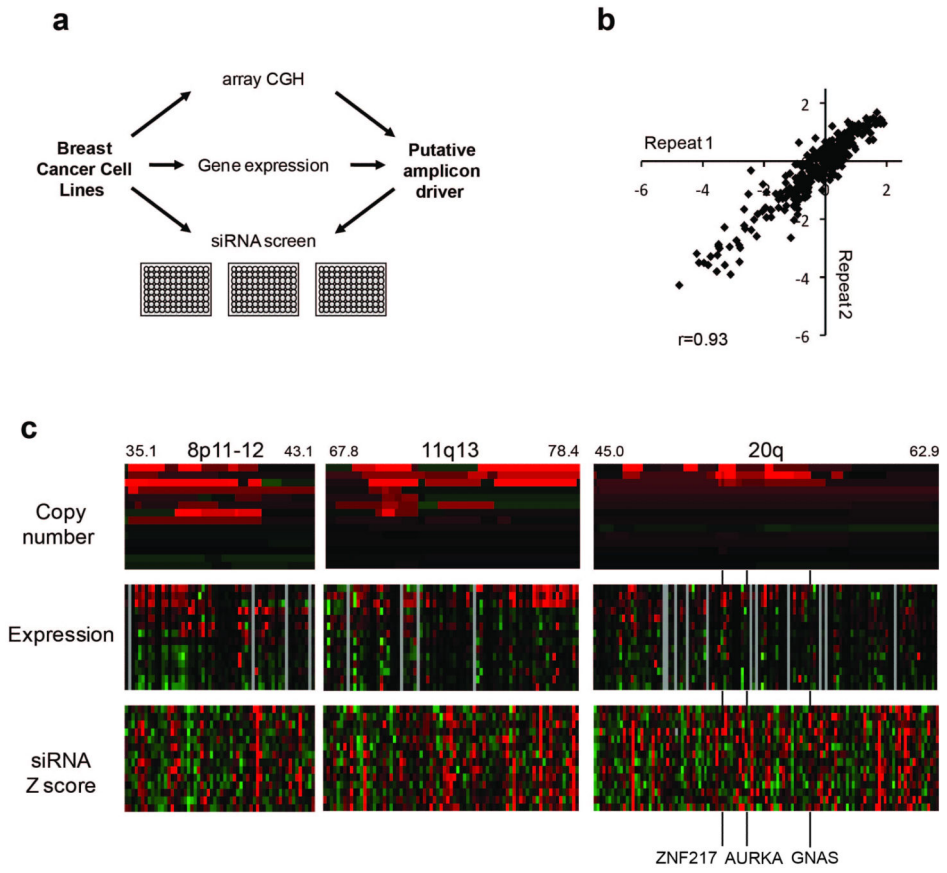

Figure 1. High-throughput siRNA amplicon driver screen

(a) Schematic of siRNA screen. Breast cancer cell lines were selected for the presence of amplifications in 8p11-12, 11q13, and 20q, and subject to array CGH profiling and gene expression analysis. The cell lines were reverse transfected in 96 well plates with siRNA targeting all genes in amplified regions, along with microRNA inhibitors. Survival was assessed after 4-6 days and expressed as a Z score with negative Z scores indicating loss of viability. Data were combined to identify genes that were selectively lethal to amplified cell lines.

(b) Correlation of two repeat siRNA screen in MCF7 cells, demonstrating high levels of reproducibility. Pearson correlation coefficient r=0.93, p<0.0001.

(c) Heat maps of genomic copy number as assessed by array CGH (top, amplified red, deleted green), gene expression analysis (Middle, over-expressed red underexpressed green), and siRNA Z score (bottom, decreased survival red). Each amplicon is displayed separately with each row representing a different cell lines, and each column the gene targeted by siRNA in order of genomic position. The gemonic position of ZNF217, AURKA and GNAS is shown.

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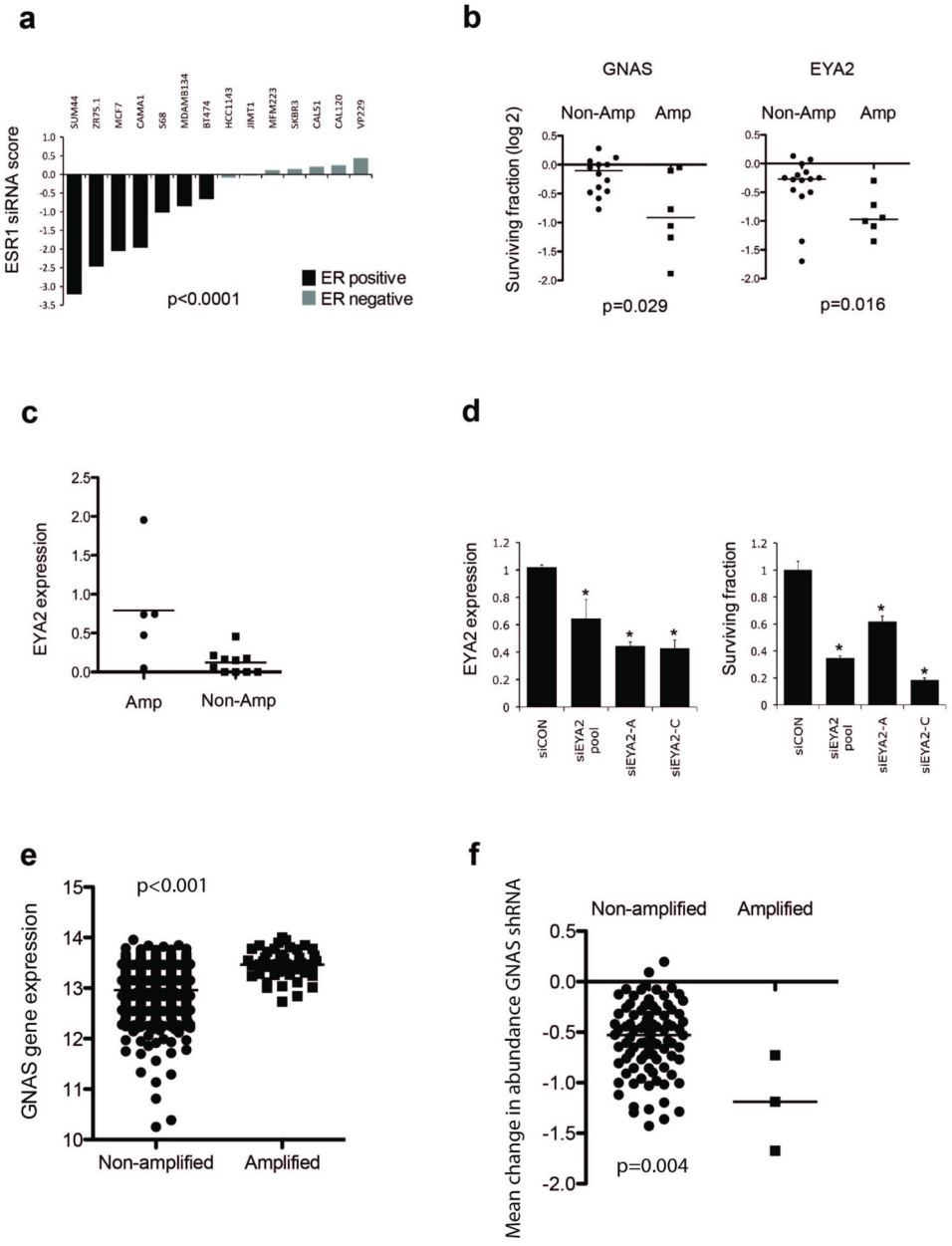

Figure 2. Identification of potential drivers from siRNA screen

(a) siRNA screen Z scores for ESR1 siRNA included as a positive control. siRNA Z score divides cell lines into two groups according to oestrogen receptor (ER, ESR1) expression, p<0.001 Mann Whitney U test.

(b) Revalidation results for siRNA targeting GNAS (left) and EYA2 (right). Displayed are surviving fractions with siRNA SMARTpool targeting indicated genes. Comparison between amplified and non-amplified cancers with Mann-Whitney U Test.

(c) *EYA2* expression in amplified and non-amplified cell lines assessed by quantitative RT-PCR relative to *MRPL19* control gene expression, p=0.004 Mann Whitney U test.

(d) Multiple siRNA targeting EYA2 decrease survival of amplified MCF7 cell line. *Left.* *EYA2* expression assessed by quantitative RT-PCR in MCF7 cells transfected 72 hours earlier with control non targetting siRNA (siCON), pool siRNA and two individual siRNA targeting EYA2 (siEYA2 A and C). *Right* Relative survival of MCF7 cells transfected 96 hours earlier with indicated siRNA. * $p<0.05$ siRNA EYA2 versus siCON, Student's T test.
(e) Publically available gene expression data from 947 cancer cell lines [23]. GNAS expression is higher in cancer cell lines with high-level GNAS amplification ($p<0.001$, Student's T test).
(f) Publically available pooled shRNA data from an independent set of cell lines. Displayed is the mean log fold change in abundance of shRNA targeting GNAS [24]. Cancer cell lines with high-level amplification of the GNAS locus are more sensitive to GNAS shRNA ($p=0.004$, Student's T test).

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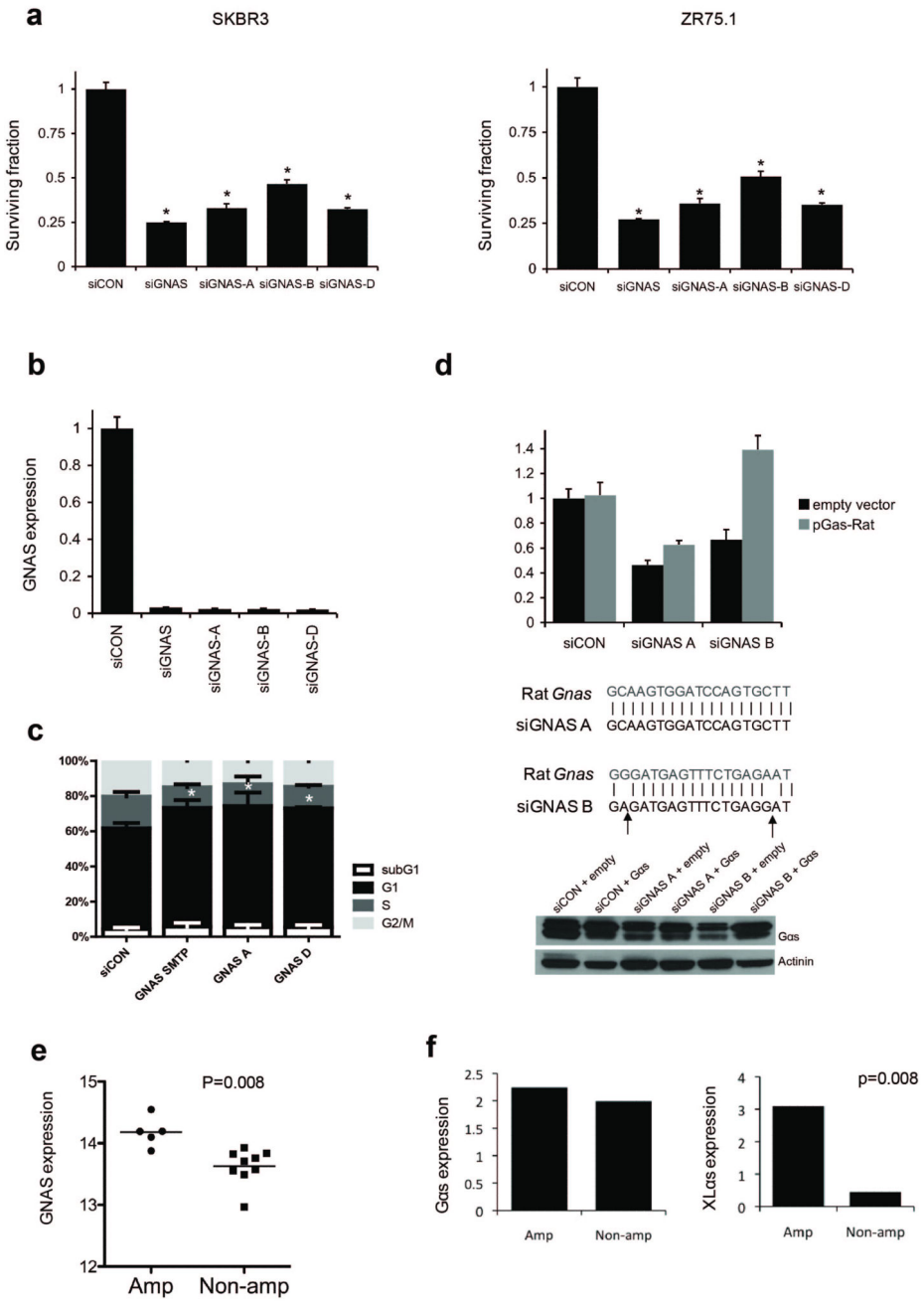

Figure 3. GNAS locus expression promotes the growth of cell lines with 20q amplification

(a) Amplified cell lines SKBR3 (*left*) and ZR75.1 (*right*) were transfected with siCON, pooled siRNA targeting GNAS (siGNAS), and individual siRNA targeting GNAS (A,B,D), with relative survival assessed after 4 and 5 days growth respectively. * $p<0.05$ siRNA GNAS versus siCON, Student's T test.

(b) *GNAS* expression assessed by quantitative RT-PCR in SKBR3 cells transfected 72 hours earlier with indicated siRNA.

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(c) Cell cycle analysis by propidium iodide FACS in SKBR3 cells transfected 72 hours earlier with indicated siRNA. Displayed mean of three independent experiments. All GNAS siRNA increased the G1 fraction as compared to siCON, * $p<0.05$ Student's T Test.
(d) Over-expression of rat Gnas (pGαs-Rat, α stimulatory subunit) rescues effect of GNAS siRNA. SKBR3 cells were transfected simultaneously with rat Gnas expression vector, or empty vector, and siRNA GNAS-A or siRNA GNAS-B. Rat Gnas rescues the effect only of the mismatched GNAS siRNA.
(e) *GNAS* is overexpressed in whole genome gene expression arrays in amplified cell lines, $p=0.008$ Mann Whitney U test.
(f) Analysis of *GNAS* locus transcripts in amplified versus non-amplified cell lines by quantitative PCR. Expression of XLαs (extra long α stimulatory subunit) is significantly raised in amplified cell lines ($p=0.008$ Mann Whitney U test), although Gαs expression is similar (p=NS).

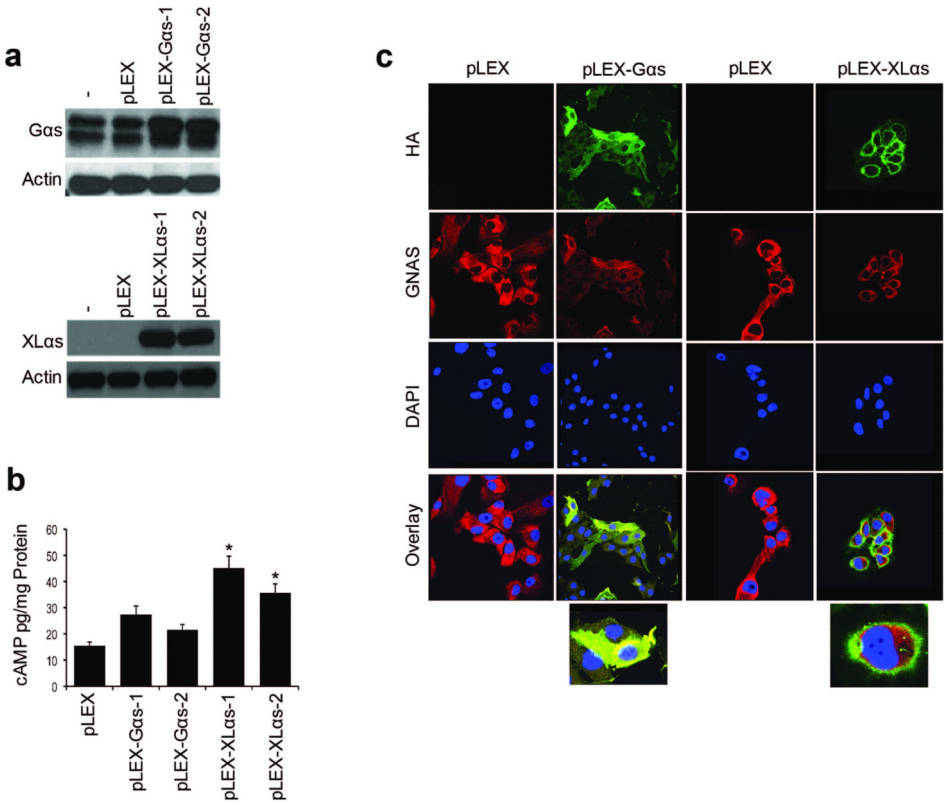

Figure 4. Analysis of over-expression exogenous XLαs

(a) CAL120 cells were infected with lentiviral pLEX empty vector, pLEX-Gαs, or pLEX-XLαs expression constructs, and two independent stable pools were selected for each GNAS expression vector. Western blots of stable pools, and CAL120 parental cells (−), probed for Gαs and XLαs.

(b) CAL120 stable pools described in (a) were treated for 15 minutes with 2mM IBMX (phosphodiesterase inhibitor) prior to assessment of cAMP levels. * $p<0.05$ compared with pLEX levels, Student's T test.

(c) Localization of GNAS variants assessed by immunofluoresence with antibodies against HA tag, GNAS (endogenous GNAS and both exogenous variants), and DAPI nuclear stain, in indicated cell lines pools. Images were taken at 40x magnification. Insert below magnified overlay to illustrate localization.

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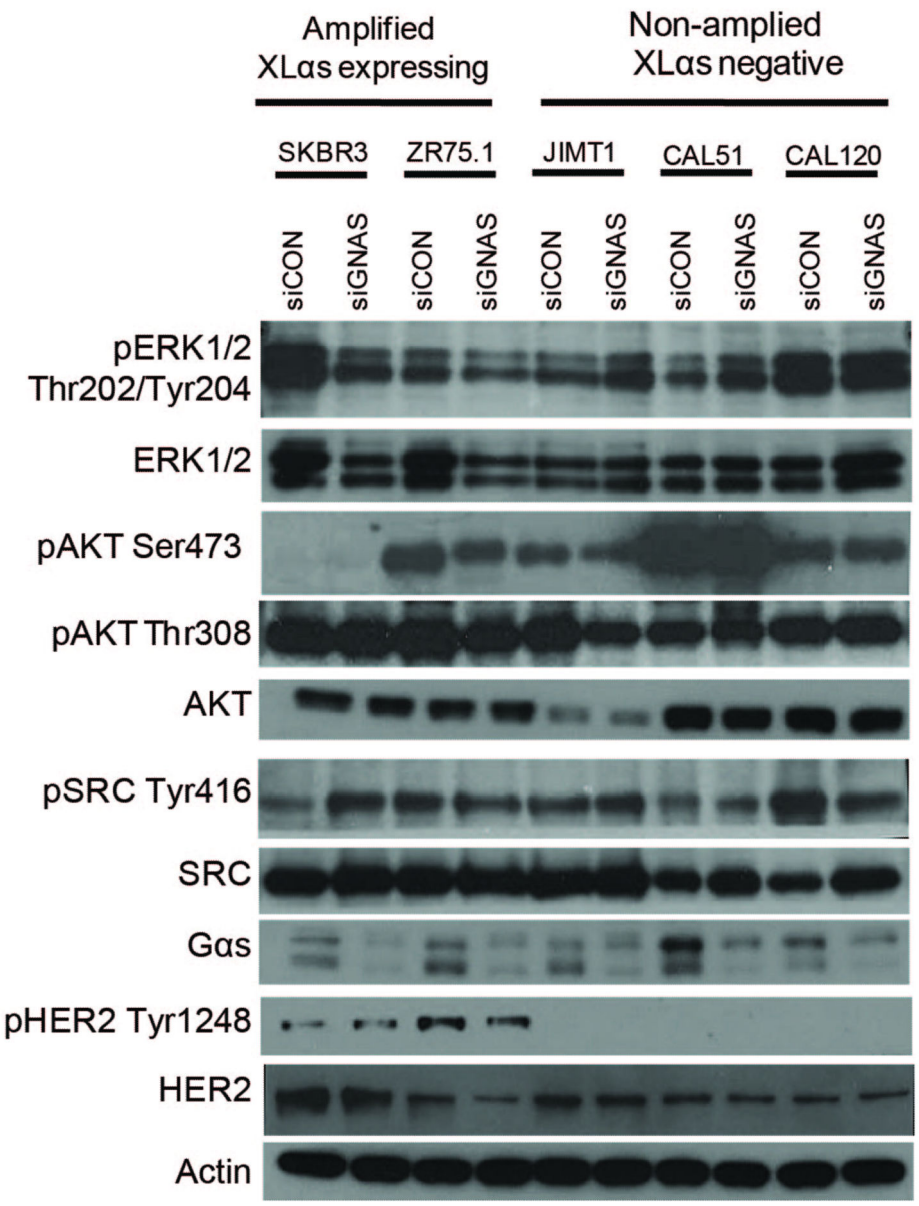

Figure 5. MAPK signalling is decreased by GNAS siRNA specifically in amplified cell lines

Indicated cell lines were transfected with siCON or siGNAS SMARTpool, and lysates made 72 hours post transfection. Lystates were probed with indicated phosphospecific antibodies and corresponding total antibodies. Probing for GNAS confirmed silencing in all cell lines.

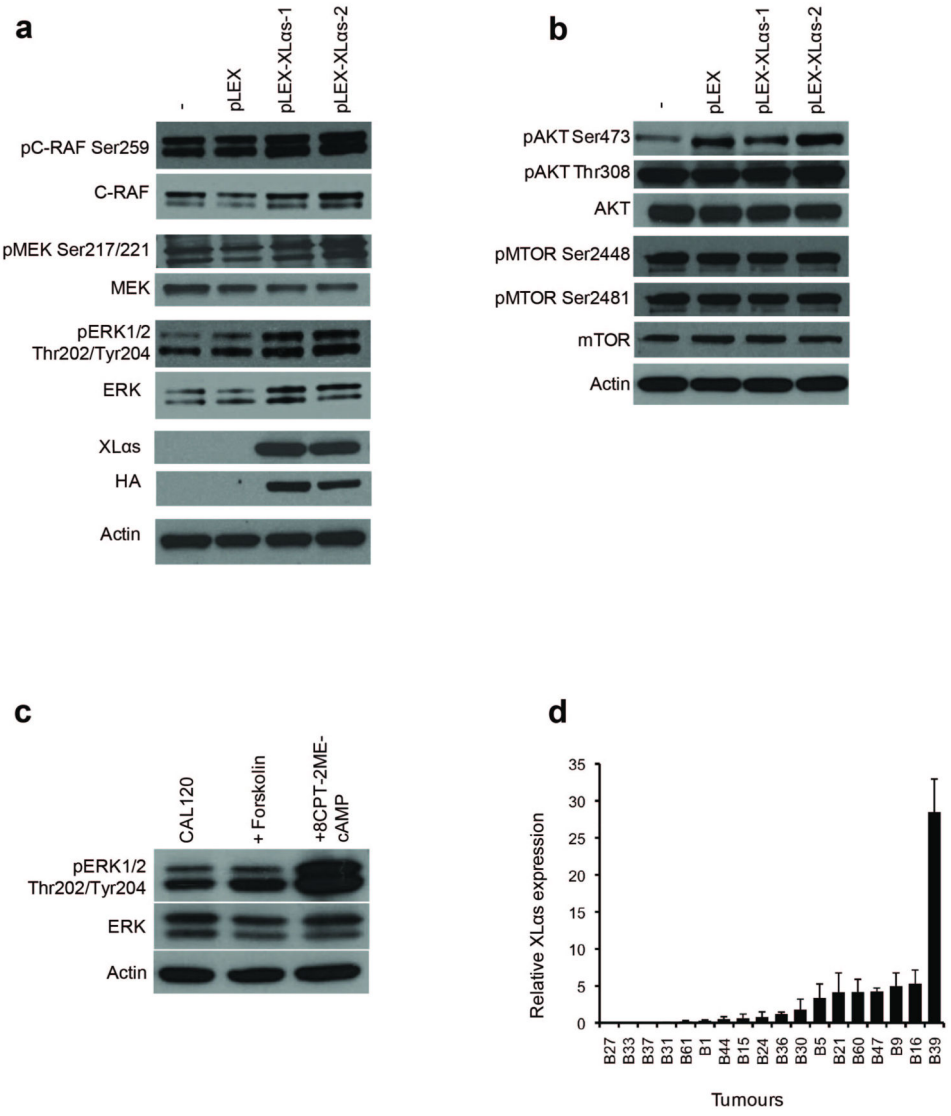

Figure 6. Signaling consequences of exogenous XLαs expression

(a) and (b) Lysates from indicated lentiviral pools, probed with indicated antibodies in MAPK signaling (a) and PI3K-ATK-mTOR signalling (b) pathways.

(c) CAL120 cells were treated with 100μM forskolin (adenylate cyclase activator), 50μM 8CPT-2Me-cAMP (selective EPAC activator) or control, lysates made after 30 minutes and probed with indicated antibodies.

(d) Assessment of XLαs expression in a small series of high grade ER positive breast cancers (described in supplementary table 5). XLαs expression was assessed relative to the weighted mean of control genes *MRPL19* and *TFRC*, and normalized to the median expression level. XLαs is also expressed in a subset of non-amplified breast cancers.