HHS Public Access

Author manuscript

Cancer Res. Author manuscript; available in PMC 2017 April 01.

Published in final edited form as:

Cancer Res. 2016 April 1; 76(7): 1825–1836. doi:10.1158/0008-5472.CAN-15-0847.

The ephrin-A1/EPHA2 signaling axis regulates glutamine metabolism in HER2-positive breast cancer

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Abstract

Dysregulation of receptor tyrosine kinases (RTKs) contributes to cellular transformation and cancer progression by disrupting key metabolic signaling pathways. The EPHA2 RTK is overexpressed in aggressive forms of breast cancer, including the HER2+ subtype, and correlates with poor prognosis. However, the role of EPHA2 in tumor metabolism remains unexplored. In this study, we used in vivo and in vitro models of HER2-overexpressing breast cancer to investigate the mechanisms by which EPHA2 ligand-independent signaling promotes tumorigenesis in the absence of its prototypic ligand, ephrin-A1. We demonstrate ephrin-A1 loss leads to upregulated glutamine metabolism and lipid accumulation that enhanced tumor growth. Global metabolic profiling of ephrin-A1-null, HER2-overexpressing mammary tumors revealed a significant increase in glutaminolysis, a critical metabolic pathway that generates intermediates for lipogenesis. Pharmacologic inhibition of glutaminase activity reduced tumor growth in both ephrin-A1-depleted and EPHA2-overexpressing tumor allografts in vivo. Mechanistically, we show that the enhanced proliferation and glutaminolysis in the absence of ephrin-A1 was attributed to increased RhoA-dependent glutaminase activity. EPHA2 depletion or pharmacologic inhibition of Rho, glutaminase, or fatty acid synthase abrogated the increased lipid content and proliferative effects of ephrin-A1 knockdown. Together, these findings highlight a novel,

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There are no conflicts of interest.

unsuspected connection between the EPHA2/ephrin-A1 signaling axis and tumor metabolism, and suggest potential new therapeutic targets in cancer subtypes exhibiting glutamine dependency.

Keywords

ephrin-A1; EPHA2; glutaminolysis; lipogenesis; breast cancer

Introduction

Receptor tyrosine kinases (RTKs) are key regulators of signal transduction pathways that promote cell growth, survival, and motility during malignant progression of solid tumors. Recent advances in the analysis of tumor genomes revealed that EPHA2 RTK is frequently overexpressed in aggressive human breast cancers and correlates with poor patient survival and resistance to therapeutic agents (1–4). EPHA2 belongs to the Eph family of RTKs, which contain distinct regions for ligand binding, receptor clustering, and signaling. Though receptor clustering and activation occur upon Eph receptor binding to their ligands, known as ephrins, Eph receptors can also be activated by other cell-surface receptors, such as EGFR and ERBB2 (3, 5). Thus, cumulative evidence in breast cancer supports two modes for EPHA2 signaling. In the ligand-dependent mode, EphA2 can engage in ligand-dependent forward signaling that suppresses tumor cell proliferation and invasiveness (6, 7). In the second mode, EphA2 can signal in a ligand-independent manner that promotes tumor malignancy—a mechanism highly dependent upon phosphorylation of S897 (6). This model, however, has not been directly tested in a transgenic mammary tumor model, and the mechanism by which ephrin-A1 exerts its tumor-suppressive role has yet to be fully elucidated.

Dysregulation of RTK signaling can result in aberrant metabolism through disruption of critical metabolic pathways. Recent studies in tumor metabolism have led to significant advances in our understanding of how tumor cells utilize metabolic switches to produce intermediates for cell growth and division (8, 9). One hallmark for proliferating tumor cells is high levels of glucose influx and subsequent aerobic glycolysis with lactate production, even in the presence of adequate oxygen [termed the “Warburg effect”; (10)]. While most cancers depend on a high rate of aerobic glycolysis, some cancer cells also display addiction to glutamine (11–13). Glutamine, an abundant amino acid, can be utilized for energetics in rapidly proliferating cells and is first catabolized to glutamate by the enzyme, glutaminase (GLS). Oxidative deamination by glutamate dehydrogenase in the mitochondrion, or the transamination of glutamate, then generates α-ketoglutarate [α-KG; (14)]. In this capacity, glutamine provides nitrogen for biosynthesis of nucleotides and amino acids, and serves as a mitochondrial substrate (11). α-KG can be further metabolized to citrate, which donates acetyl-CoA groups for lipid biosynthesis. This pathway is used for *de novo* lipogenesis under hypoxic conditions and is linked to tumor cell proliferation, motility, and survival (15). Although RTKs have been shown to regulate these metabolic pathways in tumors, the role of EPH RTKs in tumor metabolism has yet to be investigated.

In this study, we demonstrate a novel role for ephrin-A1 as a regulator of mammary tumor growth and tumor metabolism. Gene deletion of *ephrin-A1* (*Efna1*) increased the growth of endogenous mammary tumors in a mouse model of breast cancer driven by activated ErbB2 (MMTV-*NeuT*). RNAi-mediated silencing of ephrin-A1 increased lipid accumulation and cell proliferation in both mouse and human breast cancer models. Furthermore, metabolic profiling of tumors revealed increased glutaminolysis in ephrin-A1-null tumors. Mechanisticaly, we identified a signaling pathway involving EPHA2-RhoA-glutaminase that mediates ephrin-A1-dependent suppression of glutaminolysis and lipid accumulation in tumor cells. Collectively, these studies provide *in vivo* evidence for a tumor suppressive role of ephrin-A1 in breast cancer, and uncover a novel function of ephrin-A1 and EPHA2 in the regulation of tumor metabolism.

Material and Methods

Animal Models and *in vivo* studies

Animals were housed under pathogen-free conditions, and experiments were performed in accordance with AAALAC guidelines and with Vanderbilt University Institutional Animal Care and Use Committee approval. *Ephrin-A1*$^{-/-}$ mice were crossed with MMTV-*NeuT* mice. MMTV-*NeuT*-positive mice that were EphrinA1$^{+/+}$ or Ephrin-A1$^{-/-}$ were identified by PCR analysis of genomic DNA using the following primers: Ephrin-A1 forward primer (5′-CCCAACAAAAACAAACAGCCG-3′) and two allele specific reverse primers, WT (5′-GAGGTGGAGGAAGGGAAAAAGAC-3′) and KO (5′-TGGATG TGGAATGTGTGCGAGG-3′). The *NeuT* transgene was detected by PCR using the following primers: *NeuT* forward (5′-CATGGCCAGACAGTCTCCGT-3′) and reverse (5′-TGAGCTGTTTTGAGGCTGACA-3′). Allograft experiments are as described (3, 4) and in supplemental materials.

Analysis of human breast cancer tissue microarray and expression profiling datasets

Immunohistochemical staining was performed on a human Metastatic Breast Cancer Tissue Array from Cybrdi (Rockville, MD; Cat# CS08-10-001) for ephrin-A1 and phosphorylated EPHA2 (pS897). The scoring system is as follows: 0–10% + tumor epithelium = 0; 10–25% + tumor epithelium = 1; 25–50%+ tumor epithelium = 2; >50%+ tumor epithelium = 3. Samples were subdivided by scores of 0–1 as low/negative and scores of 2–3 as high. Statistical significance was determined using Chi Square Analysis.

Survival analyses were performed as previously described (16). *EPHA2* and *EFNA1* gene expression data was downloaded from GEO (Affymetrix HGU133A, accession# GPL96, and HGU133 Plus 2.0, accession# GPL570) and recurrence-free survival information was analyzed using the online software package, Kaplan–Meier Plotter (http://kmplot.com). The packages ‘recurrence-free survival’ and ‘automatic cutoff’ were used to calculate and plot Kaplan–Meier survival curves for 936-patients with lymph node-positive breast cancer. Tumors were ranked according to gene expression values of *EPHA2* and *EFNA1*, scored as high or low. Statistical significance was determined by logrank *P*-value and hazard ratios with 95% confidence intervals are displayed.

Cell culture and in vitro studies

Human cell lines were purchased from the ATCC and maintained as described (3, 4). BT474 cell lines were authenticated by the ATCC cell authentication services utilizing short tandem repeat profiling. Other cell lines were used at low passage and were not authenticated. The mouse MMTV-*Neu* cell line was generated and provided by Rebecca Cook (Vanderbilt University). These cells were maintained in DMEM/F12 media supplemented with estrogen (5ng/ml), progesterone (5ng/ml), insulin (0.5μg/mL), EGF (5ng/ml), L-glutamine (2 mM), penicillin (100U/ml) and streptomycin (100U/ml) and 10% fetal bovine serum. siRNA-mediated knockdown, BrdU proliferation, 3D Matrigel culture, Rho activity assay, and western blot analysis are as described (39, 3, 4) and in supplemental materials.

Metabolite Assays

Intracellular glutamate concentrations were determined using the enzymatic assay, Glutamate Assay Kit (Sigma), according to the manufacturer's protocol. MCF-10A-HER2 or MMTV-*Neu* cells (10^6) were cultured in glutamine-free DMEM/F12 base media for 24 hours. Cells were stimulated with 5% serum, EGF (5ng/mL), and 2mMol of glutamine. Briefly, after treatment, cells were washed with PBS and collected with the provided glutamate assay buffer. Lysates were centrifuged and the supernatants were further centrifuged in 10kDa ultrafiltration spin columns. The concentrated samples were plated in 96-well plates with the supplied glutamate enzyme mix. Absorbance was measured at 450nm. Concentrations were determined from the standard curve. Statistical significance was determined using ANOVA analysis on GraphPad Prism software; a *P*-value <0.05 is considered significant. Lipid content was determined by Oil-Red-O staining as detailed in supplemental materials. Metabolomic profiling was performed in collaboration with Metabolon Inc, as detailed in supplemental materials.

Results

Ephrin-A1 inhibits mammary tumor growth in MMTV-*NeuT* transgenic mice and in human breast cancer cell lines

Ephrin-A1-null mice are viable, fertile, and do not exhibit overt phenotypes in a pathogen-free animal facility (17). Previous experimental data in breast cancer cell lines suggest a model in which the oncogenic role of EPHA2 is largely ligand-independent, whereas ephrin-A1, the prototypic ligand of EPHA2, transduces inhibitory signaling that blocks tumor cell proliferation and invasiveness (3, 6, 18). To test this hypothesis *in vivo* in a clinically relevant transgenic mammary tumor model, we crossed *ephrin-A1*-deficient mice with MMTV-*NeuT* transgenic animals (19) to determine whether loss of ephrin-A1 enhanced breast cancer tumorigenesis and progression. MMTV-*NeuT*/*Efna1*$^{-/-}$ mice developed larger tumors compared to MMTV-*NeuT*/*Efna1*$^{+/+}$ littermates (Figure 1A). PCNA staining revealed that ephrin-A1 null tumors had an elevated proliferative index compared to wild-type tumors (Figure 1B). However, vWF staining, an endothelial cell marker, was not significantly different (Supplementary Figure 1A), suggesting that tumor vasculature is not markedly affected by loss of ephrin-A1 in this model. These results suggest that Ephrin-A1 inhibits mammary tumor growth within its native environment, as genetic deletion of ephrin-A1 enhanced tumor growth in this aggressive breast cancer model.

To assess whether our data in this animal model is applicable to human disease, we tested whether loss of ephrin-A1 augmented growth in MCF-10A-HER2 cells, a human breast cell line overexpressing HER2. We used two independent shRNA sequences to stably knockdown ephrin-A1 in MCF-10A-HER2 cells and performed 3-dimensional (3D) spheroid growth assays. MCF-10A-HER2 cells treated with shEphrin-A1 developed significantly larger spheroids that displayed protrusive properties compared to control cells (Figure 1C). To directly evaluate proliferation, MCF-10A-HER2 cells were treated with siRNAs targeting ephrin-A1 or a non-targeting control and BrdU incorporation was measured. Knockdown of ephrin-A1 significantly enhanced proliferation (Figure 1D and Supplemental Figure 1B), which is accompanied by an increase in EPHA2 protein expression and phosphorylation of EPHA2 at Y588 and S897, markers of kinase activity and ligand-independent signaling, respectively. However, there were no discernable changes in the expression of EPHA4 or EPHB6, in which high expression levels were shown to correlate with poor patient survival in human breast cancer (2) (Figure 1 D).

To test whether ephrin-A1-induced growth suppression requires EPHA2, we silenced EPHA2 and ephrin-A1 simultaneously. Knockdown of EPHA2 in MCF-10A-HER2 cells inhibited the elevated cell proliferation in ephrin-A1-deficient cells (Figure 1E, Supplementary Figure 1B), suggesting that EPHA2 is the receptor for ephrin-A1 that links this ligand to tumor suppression in breast cancer. To investigate whether EPHA2 kinase activity or phosphorylation of S897 is required for ligand-independent signaling, we expressed wild-type EPHA2, EPHA2^{S897A}, or EPHA2^{D739N} (kinase dead) in MCF-10A-HER2 cells and evaluated spheroid growth. We found that both S897 phosphorylation and kinase activity are required for the EPHA2-dependent increase in cell growth (Figure 1F), suggesting that EPHA2 receptor forward signaling is important in this process.

To complement loss-of-function studies, we determined whether overexpression of ephrin-A1 could inhibit growth and proliferation of breast cancer cells *in vivo*. MMTV-*Neu* cells overexpressing ephrin-A1 or control LacZ protein were transplanted into mammary fat pads of recipient mice and tumor volume was measured. Overexpression of ephrin-A1 significantly reduced tumor volume relative to controls (Figure 2A). Analyses of PCNA expression in these tumor allografts revealed that tumor cell proliferation was significantly lower in MMTV-*Neu* tumors expressing ephrin-A1 compared with that of MMTV-*Neu* tumors expressing the control LacZ protein (Figure 2B). We next evaluated the effect of ephrin-A1 overexpression in the HER2-dependent human breast cancer cell line, BT474. Overexpression of ephrin-A1 by either an adenoviral delivery or a lentiviral doxycycline-inducible system reduced EPHA2 expression, spheroid size and BrdU incorporation compared to control cells expressing GFP (Figure 2, C–E). Collectively, our findings support the model in which ephrin-A1 acts as a molecular switch to modify EPHA2 receptor signaling output, such that loss of ephrin-A1 enhances tumor cell growth.

Low ephrin-A1 or high EPHA2 expression is associated with poor survival in lymph node positive breast cancer patients

We previously reported that *EPHA2* overexpression is linked to poor prognosis in breast cancer patients (2, 4). To investigate whether high EPHA2 expression correlates with ligand-

independent signaling, we analyzed the level of phospho-S897 EPHA2, a marker for ligand-independent activation (6), and ephrin-A1 expression in adjacent sections of tumor samples in a human breast cancer tissue microarray (TMA). As shown in Figures 3A and 3B, 75% of pS897-EPHA2-positive samples were negative for ephrin-A1 in primary tumors, and 74% of pS897-EPHA2 positive lymph node metastases were negative for ephrin-A1 (n= 20 and 27 for primary and metastatic tumors, respectively; $p<0.05$), suggesting that high levels of ligand-independent EPHA2 signaling in human breast cancer are associated with low levels of ephrin-A1 expression. To investigate whether the expression level of *ephrin-A1* is associated with clinical outcomes, we mined datasets from the NCBI Gene Expression Omnibus [GEO; (16); Figure 3, C&D]. We found no correlation between ephrin ligand expression and clinical outcome in overall breast cancer samples. However, high *EFNA1* expression was associated with increased probability of 20-year recurrence-free survival in lymph node-positive breast cancer patients (n=936, $p<0.05$), whereas high *EPHA2* expression is associated with decreased survival ($p<0.01$). Collectively, these data support a tumor-suppressive role for ephrin-A1 in metastatic malignant human breast cancer.

Ephrin-A1 regulates lipid accumulation in breast cancer cells

Reprogramming of energy metabolism is a hallmark of cancer that confers growth and survival advantages to tumor cells (8, 9). We discovered that knockdown of ephrin-A1 leads to accumulation of vacuole-like structures in MCF10A-HER2 cells (Figure 4A, arrowhead). To investigate whether these vacuole-like structures represented lipid droplets, we stained cells with the lipid soluble dye, Oil-Red-O. As shown in Figure 4B, the Oil-Red-O positive area was significantly increased in ephrin-A1 knockdown cells compared to control cells. As fatty acid synthase (FASN) is a key enzyme for *de novo* fatty acid synthesis, we tested whether inhibition of FASN could rescue increased lipid deposits in ephrin-A1-deficient cells. Orlistat, an irreversible inhibitor of FASN (20, 21), suppressed lipid accumulation in ephrin-A1 knockdown cells (Figure 4C) and inhibited the proliferative phenotype observed in *ephrin-A1*-deficient MCF-10A-HER2 cells (Figure 4D).

Since EPHA2 is the primary receptor for ephrin-A1 (Figure 1E), we reasoned that overexpression of EPHA2 should produce a similar lipid accumulation phenotype as ephrin-A1 knockdown cells. Indeed, overexpression of wild-type EPHA2, but not $EPHA2^{S897A}$ or $EPHA2^{D739N}$ mutants, enhanced lipid content in MCF-10A-HER2 cells (Figure 4E, Supplementary Figure 2A). In contrast, overexpression of ephrin-A1 significantly reduced lipid deposits in BT474 cells, which have high endogenous levels of lipids (Figure 4F). Orlistat treatment inhibited proliferation and lipid accumulation in BT474 cells, and EPHA2 silencing also reduced lipid content (Figure 4, G and H, and Supplementary Figure 2B). Collectively, Orlistat-induced inhibition of lipid content and a reduction in tumor cell proliferation suggests a link between increased lipogenesis and tumor cell growth upon loss of ephrin-A1 or overexpression of EPHA2.

Ephrin-A1 regulates glutamine metabolism through modulation of glutaminase activity

To globally assess tumor metabolite profiles, tumors from MMTV-*NeuT*/*Efna1*$^{+/+}$ and MMTV-*NeuT*/*Efna1*$^{-/-}$ were analyzed by mass spectrometry. We observed no consistent pattern of metabolite profile differences related to glucose metabolism. However, there were

marked increases in metabolites associated with glutamine metabolism in MMTV-*NeuT*/*Efna1*$^{-/-}$ tumors relative to wild-type control tumors (Figure 5, A and B, Supplementary Figure 3), suggesting that ephrin-A1 may regulate glutaminolysis.

A decrease in glutamine coupled with an increase in glutamate (Figure 5B) in ephrin-A1-null tumors suggests enhanced glutamine consumption by glutaminase (GLS), a key rate-limiting enzyme in glutaminolysis. To directly test whether ephrin-A1 regulates the activity of GLS, MMTV-*Neu* cells treated with shEphrin-A1 or shControl were glutamine-starved, stimulated by fresh glutamine, and intracellular glutamate was measured. Figure 5C shows that intracellular glutamate steadily accumulated after addition of fresh glutamine in the media; however, there were higher glutamate levels in ephrin-A1 knockdown cells relative to control cells, and this increase was blocked by the treatment of GLS inhibitors, 968 or BPTES (Figure 5D). Because loss of ephrin-A1 promoted growth in both *in vitro* and *in vivo* models and enhanced GLS activity, we sought to determine whether GLS plays a direct role in tumor cell proliferation. Ephrin-A1 knockdown and control MMTV-*Neu* cells were treated with glutaminase inhibitors, 968 or BPTES, and BrdU incorporation was assessed. As expected, knockdown of ephrin-A1 enhanced proliferation in MMTV-*Neu* cells, and 968 or BPTES was able to inhibit the elevated BrdU incorporation in ephrin-A1-deficient cells (Figure 5E). To test the effects of GLS inhibition on tumor growth *in vivo*, shEphrin-A1 and shControl MMTV-*Neu* cells were transplanted into contralateral mammary fat pads of recipient mice and treated with either BPTES (10mg/kg) or vehicle control every other day by intraperitoneal (IP) injection. Ephrin-A1 knockdown tumors were significantly larger than the shControl tumors (Figure 5F). BPTES treatment significantly reduced the volume of ephrin-A1 knockdown tumors, whereas BPTES appeared to have no significant effect on shControl tumors at the indicated dose (Figure 5F).

To complement ephrin-A1 knockdown studies, we tested the effects of EPHA2 overexpression on GLS activity and tumor volume. We reasoned, since ephrin-A1 is the prototypic ligand of EPHA2 receptor and loss of ephrin-A1 can augment EPHA2 activity, that EPHA2 overexpression should have a similar effect on GLS activity and tumor cell growth as ephrin-A1 deficiency. Indeed, we observed that MMTV-*Neu* cells overexpressing EPHA2 had enhanced GLS activity compared to control cells (Figure 5G) and this increase could be blocked by GLS inhibitors, 968 or BPTES (Figure 5H). EPHA2 overexpression also enhanced BrdU incorporation, which was inhibited by 968 or BPTES (Figure 5I). Furthermore, EPHA2 overexpression enhanced tumor growth *in vivo*, and BPTES significantly decreased tumor volume to a level similar as control tumors (Figure 5J). Together, these data show that inhibition of GLS activity can impair proliferation and tumor growth, suggesting that enhanced glutaminolysis is, at least in part, the mechanism for the enhanced tumor growth in EphA2 overexpressing or ephrin-A1-deficient tumors.

We next tested whether ephrin-A1 also regulates GLS activity in the human cell line MCF-10A-HER2. Similar to MMTV-*Neu* cells, knockdown of ephrin-A1 in MCF-10A-HER2 cells enhanced GLS activity compared to control cells (Figure 6A), and inhibition of GLS rescued the pro-proliferative phenotype induced by ephrin-A1 knockdown (Figure 6B). We reasoned, if ephrin-A1 regulates cell proliferation through modulation of GLS, supplementing with downstream metabolites such as α-ketogluterate (αKG) should rescue

the proliferative phenotype induced by the GLS inhibitor. We observed that addition of dimethyl-αKG (DM-αKG), the soluble form of αKG, restored proliferation inhibited by 968 in ephrin-A1 knockdown cells (Figure 6B). Additionally, overexpression of wild-type EPHA2, but not S897A and D739N mutants, enhanced glutamine metabolism (Supplementary Figure 4D), suggesting that both EphA2 kinase activity and phosphorylation of S897 are required for EphA2-dependent regulation of glutamine metabolism.

Glutaminase exists in two isoforms, GLS1 and GLS2, or kidney-type isoform and liver-type isoform, respectively (22). To determine which isoform regulates glutamine levels in ephrin-A1 knockdown cells, we silenced these distinct enzymes with siRNAs. Knockdown of GLS1 rescued the elevated glutaminase activity in *ephrin-A1*-deficient cells (Figure 6C–D, Supplementary Figure 4A–4C), suggesting that ephrin-A1 regulates glutaminolysis through inhibition of GLS1. It remains to be determined if GLS2 is also important in ephrin-A1-dependent glutaminolysis (Figure 6C and 6E).

Regulation of glutaminase activity by ephrin-A1 is mediated through RhoA GTPase

GLS, the biologic target of compound 968, was previously shown to be regulated by Rho family GTPases (23). Inhibition of GLS or aminotransferase (AT) has been shown to suppress Rho GTPase-induced transformation and inhibit tumor growth in breast adenocarcinoma xenografts, respectively (23, 24). Since overexpression of EPHA2 receptor can activate RhoA activity (3, 25, 26), we tested whether the GLS activity induced by ephrin-A1 depletion could be mediated through RhoA signaling. RNAi-mediated silencing of ephrin-A1 increased the levels of active GTP-bound RhoA, relative to non-targeting siRNA controls (Figure 7A). CT04, a cell permeable Rho inhibitor, inhibited RhoA-GTP levels and decreased GLS activity in ephrin-A1 knockdown cells (Figure 7, B and C). Furthermore, inhibition of RhoA, GLS, or FASN decreased elevated Oil-Red-O staining and cellular proliferation induced by knockdown of ephrin-A1 (Figure 7, D and E), suggesting that inhibition of tumor cell growth by ephrin-A1 is mediated through RhoA and glutaminase.

Discussion

RTK signaling is critical to cell growth and survival in normal epithelial cells. Dysregulation of RTKs by mutations, amplification, or overexpression can increase kinase activity, leading to oncogenic transformation and malignant progression. Yet, recent studies have discovered dual roles for Eph receptors in both promoting and inhibiting tumor initiation and metastatic progression (7, 27–30) based on *in vitro* and allograft studies using mammary epithelial and cancer cell lines. Ephrin-A1 overexpressing xenograft models and intratumoral delivery of Ad-ephrinA1-Fc both reduced tumor volume (31). In breast cancer cell lines, ligand-induced EPHA2 signaling inhibits proliferation, whereas ligand-independent crosstalk between EPHA2 and other oncogene pathways results in tumor promotion (3, 4). These data suggest ephrin-A1 can act as a molecular switch in breast cancers, such that loss of ligand-dependent signaling switches EPHA2 to function as a tumor promoter. Herein, we provide functional evidence in a transgenic MMTV-*NeuT* mouse model that genetic deletion of *Ephrin-A1* enhances tumor cell proliferation and mammary tumor growth. The role of ephrin-A1 in

suppression of tumor malignancy is also supported by human breast cancer data in which lower *ephrin-A1* (*EFNA1*) gene expression is associated with poor survival in lymph node-positive patients, thus demonstrating clinical relevance.

Our previous studies in the 4T1 model showed knockdown of ephrin-A1 had no effect on tumor volume, but resulted in decreased tumor angiogenesis and lung metastasis (32). Consistent with our previous studies, orthotopic transplantation of wild-type 4T1 tumors into ephrin-A1-deficient hosts resulted in marked decrease of vWF staining in tumor sections (data not shown), suggesting that host ephrin-A1 deficiency affects tumor neovascularization. However, global knockout of ephrin-A1 in our MMTV-*NeuT* model displayed no significant changes in tumor vascular density. Lack of significant changes in tumor vessels in this model could be due to an inhibitory effect of ephrin-A1 on tumor cells that override the angiogenesis promoting effects.

Studies in this report unequivocally demonstrate a role of ephrin-A1 in regulating cell proliferation in the HER2/Neu model. The differential effects of ephrin-A1 deficiency on tumor growth between the 4T1 and the HER2/Neu models may at least be in part due to the differences in breast cancer subtype, as 4T1 cells are mesenchymal without normal cell-cell contacts. Thus, even if ephrin-A1 was expressed in these cells, it may not bind to EPHA2 on neighboring cells to inhibit its function, whereas ephrin-A1 in MMTV-*Neu* cells may interact with EPHA2 on adjacent tumor cells more effectively to exert its inhibitory role. It is currently unknown whether ephrin-A1 affects metastasis in this model. However, in view of the potential role of ephrin-A1 in angiogenesis and metastasis, inhibiting EPHA2 receptor, rather than focusing on overexpressing ephrin-A1, may improve future targeting strategies.

Metabolic reprogramming is a hallmark of cancer that confers growth and survival (33). While RTKs are known to regulate tumor metabolism through modulation of signaling pathways such as Akt and mTOR, the connection between Eph RTKs and tumor metabolism remained unexplored. The discovery that ephrin-A1 regulates lipid and glutamine metabolism suggests ephrin-A1 is capable of inhibiting tumor growth by modulation of key metabolic enzymes. One rate-limiting enzyme for neoplastic lipogenesis, FASN, has been shown to be induced by RTKs via the SREBP1 transcription factor and promotes proliferation in breast cancer cells (34, 35). Alternatively, both glucose and glutamine can be metabolized to provide citrate that supports acetyl-coA production for *de novo* lipid biosynthesis (36, 37). We did not observe significant changes in FASN or SREBP1 expression in human breast cancer cells. Interestingly, our global metabolic study revealed glutaminolysis, but not glycolysis, is increased in ephrin-A1-null tumors compared to wild-type tumors. These findings are consistent with recent reports that during hypoxia, glutaminolysis is the predominant pathway for *de novo* lipogenesis (15). Thus, our data support a model in which EPHA2 ligand-independent signaling through down-regulation of ligand levels, EPHA2 overexpression, and/or failed engagement of endogenous ligand-receptor on adjacent cells, promotes tumor cell growth and progression by elevating glutaminolysis.

While most cancers depend on a high rate of aerobic glycolysis, some cancers also display glutamine addiction (11, 12), including breast (38). Glutamine metabolism has been shown

to be regulated by a number of signaling pathways including Rho family GTPases. Compound 968, a small molecular inhibitor of glutaminase, suppressed oncogenic transformation induced by Rho GTPases (23). We found that this pathway is repressed by ephrin-A1. First, EPHA2 RTK is known to regulate RhoA activity (3, 25). Second, RhoA-GTP levels are substantially elevated in ephrin-A1 knockdown cells. Third, inhibition of Rho activity significantly decreased intracellular glutamate levels in ephrin-A1 knockdown cells. Finally, inhibition of Rho, GLS, or FASN suppressed proliferation induced by depletion of ephrin-A1. These results demonstrate that ephrin-A1 regulates glutaminolysis and support a model in which ephrin-A1 inhibition of glutaminase activity is, at least in part, through Eph receptor-dependent activation of RhoA GTPases (Figure 7F).

Although our work emphasizes the role of ephrin-A1 in glutamine metabolism and lipid biogenesis, other glutamine metabolism pathways may also be important in ephrin-A1-induced growth inhibition. For example, glutamine contributes to *de novo* synthesis of the major cellular antioxidant glutathione (GSH), nucleic acids, and certain amino acids. Indeed, we observed increases in GSH, nucleosides, and amino acid levels in ephrin-A1-null tumors relative to control wild-type tumors (Supplementary Figure 3). Accordingly, future investigations will reveal whether these branches of glutamine metabolism are also critical in ephrin-A1-mediated inhibition of cell proliferation.

The identification that ephrin-A1 and EPHA2 are linked to tumor metabolism opens up exciting new questions in tumor biology. Although ephrin-A1 inhibits both glutamine metabolism and accumulation of lipids, it is unclear whether increased lipids are due to augmented glutaminolysis in ephrin-A1 knockdown cells, or occur as an independent event. In addition to Rho GTPase, glutamine metabolism is also regulated by other signaling molecules relevant to breast cancer, such as c-Myc and PKC-delta (12). Future studies will focus on whether ephrin-A1/EPHA2 regulation of glutaminolysis is also modulated by c-Myc or PKC and whether these molecules operate in the same linear pathway or in different, parallel pathways as Rho. Furthermore, the results presented in this study have significant translational potential. Ephrin-A1 may be used as a biomarker, as decreased ephrin-A1 expression may predict poor clinical outcome in metastatic breast cancer. Finally, glutamine addiction in breast cancer is associated with elevated EPHA2 receptor levels in subtypes of disease that are refractory to current therapies, such as drug-resistant HER2-positive tumors. Since EPHA2 kinase activity is required for its regulation of glutamine metabolism, selective EPHA2 kinase inhibitors (39, 40) and/or inhibitors of key metabolic enzymes may provide more effective cancer therapeutics in these difficult to treat subtypes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial Support: Department of Veterans Affairs through a VA Merit Award 5I01BX000134 (to J. Chen), NIH grants R01 CA177681, R01 CA95004 (J. Chen) and R01 CA148934 (D. Brantley-Sieders), T32 HL007751, F-31 CA180407 (V. Youngblood) and T32 CA009592 (L. Kim and D. Edwards). This work was also supported in part by the NCI Cancer Center Support Grant # P30 CA068485 utilizing the Translational Pathology Shared Resources.

We would like to thank Drs. Jay A. Berzofsky (NCI, Bethesda, MA) and Wenqiang Song (Vanderbilt University, Nashville, TN) for providing MMTV-*NeuT* breeder mice and mutant EPHA2 constructs, respectively. We also thank Drs. Rebecca Cook and Joshua Fessel (Vanderbilt University, Nashville, TN) for scientific discussion. This work was supported by Department of Veterans Affairs through a VA Merit Award 5I01BX000134 (to J. Chen), NIH grants R01 CA177681, R01 CA95004 (J. Chen) and R01 CA148934 (D. Brantley-Sieders), T32 HL007751 and F-31 CA180407 (V. Youngblood), and T32 CA009592 (L. Kim and D. Edwards). This work was also supported in part by the NCI Cancer Center Support Grant # P30 CA068485 utilizing the Translational Pathology Shared Resources.

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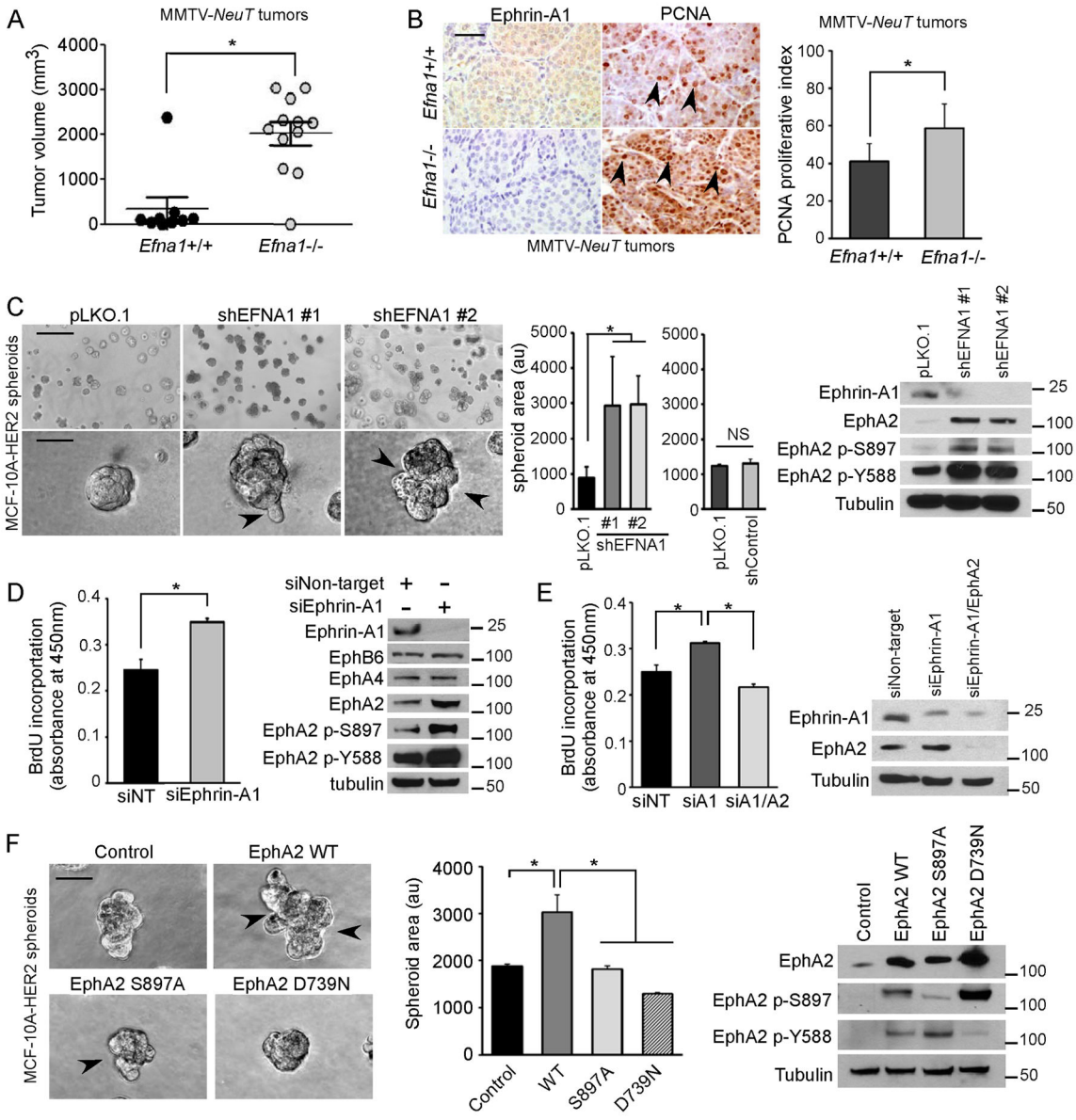

Figure 1. Loss of ephrin-A1 enhances breast cancer growth

(A) Tumor volume from 22-week old MMTV-*NeuT*/*Efna1*$^{+/+}$ and MMTV-*NeuT*/*Efna1*$^{-/-}$ mice. **P*<0.05, Student's t-test. (B) Immunohistochemistry of ephrin-A1 and PCNA in MMTV-*NeuT* tumors. Arrowhead indicates PCNA+ nuclei. **P*<0.05, Student's t-test. Scale bar 200μm. (C) 3D-spheroids expressing shRNAs targeting ephrin-A1 or pLKO.1 vector control at day 8. Average spheroid area is presented as arbitrary units (au) ± SEM. **P*<0.05, one-way ANOVA. Arrowheads indicate protrusions in the spheroids. Scale bars (top) 500μm; (bottom) 100μm. (D) BrdU incorporation assay in MCF-10A-HER2 cells transfected with control siNT or ephrin-A1 siRNA. Data expressed as mean ± SEM. **P*<0.05, one-way ANOVA. (E) BrdU incorporation assay in MCF-10A-HER2 cells transfected with control (siNT), siRNA targeting ephrin-A1 (siA1) or ephrin-A1 and EPHA2 (siA1/A2). Data expressed as mean ± SEM. **P*<0.05, one-way ANOVA. (F) 3D-spheroids

expressing vector control, wild-type EPHA2, EPHA2^{S897A}, or EPHA2^{D739N} (kinase dead) at day 8. Average spheroid area is presented as arbitrary units (au) ± SEM. *P<0.05, one-way ANOVA, Scale bar 100μm. *In vitro* data are representative of 2–5 biological replicates.

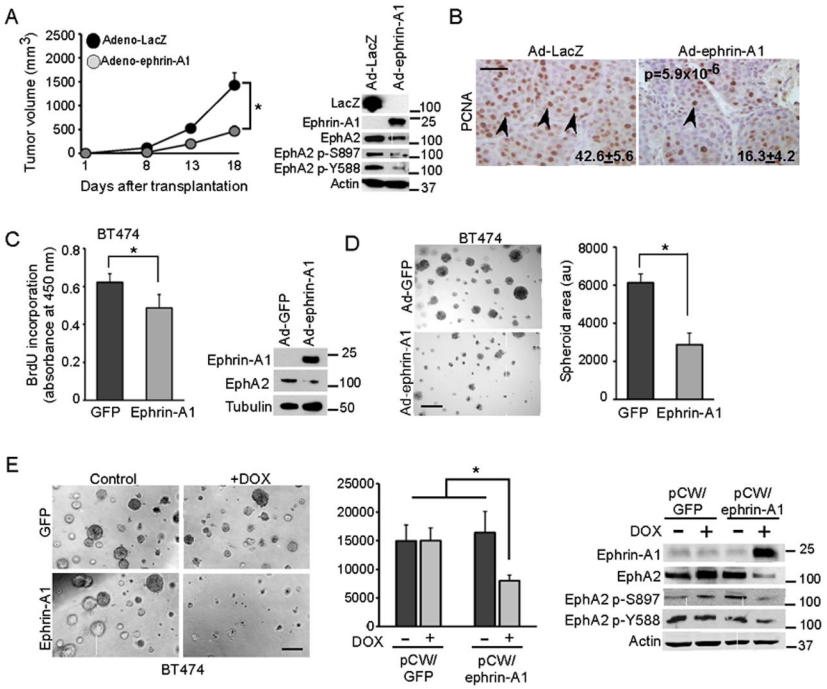

Figure 2. Overexpression of ephrin-A1 inhibits breast cancer growth

(A) MMTV-*Neu* cells (10^6) expressing LacZ or ephrin-A1 were injected into mammary fat pads of mice. Tumor volume was recorded over a time course. Data are presented as average tumor volume ± SEM; n=6 per group. *P<0.05, two-way ANOVA repeated measures. (B) Immunohistochemistry of PCNA in tumor sections. Arrowhead indicates PCNA+ nuclei. *P<0.05, Student's t-test. Scale bar 200μm. (C) BrdU incorporation in BT474 cells expressing GFP or ephrin-A1. Data expressed as mean ± SEM. *P<0.05, t-test. (D) 3D-spheroids with BT474 cells expressing GFP control or ephrin-A1 at day 8. Average spheroid area is presented as arbitrary units (au) ± SEM. *P<0.05, Student's t-test. Scale bar 500μm (E) 3D-spheroids with BT474 cells expressing inducible GFP or EFNA1 in the presence of PBS or 1 μg/mL doxycycline (DOX) at day 12. Average spheroid area is presented as arbitrary units (au) ± SEM. *P<0.05, one-way ANOVA. Scale bar 500μm. *In vitro* experiments represent 2–4 biological replicates.

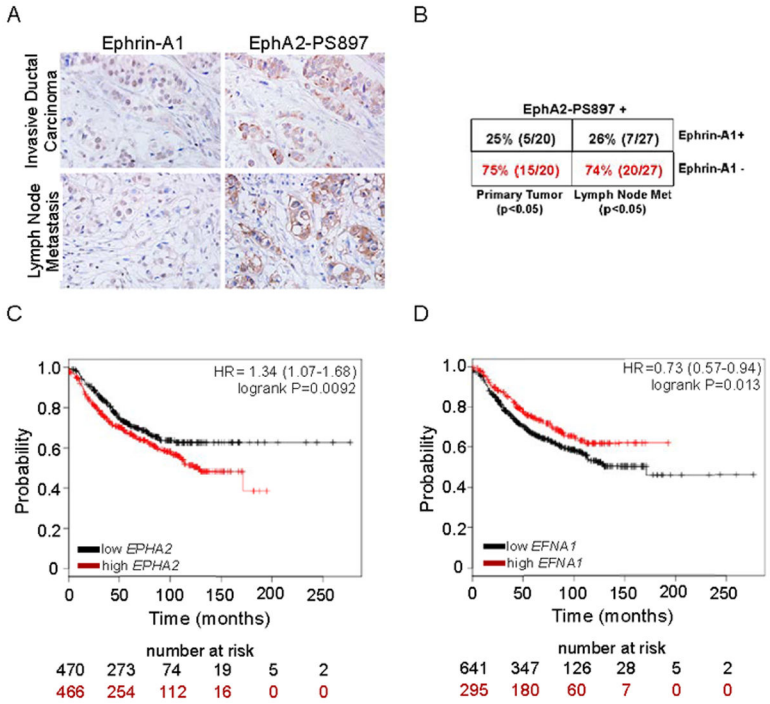

Figure 3. Low ephrin-A1 expression is linked to poor prognosis in lymph node positive breast cancer patients

(A) Immunohistochemistry of human breast cancer tissue microarrays for ephrin-A1 and EPHA2 phospho-S897 (pS897). (B) The percentage of tumors staining positive for EPHA2-phospho-S897 in ephrin-A1-positive and ephrin-A1 negative samples are summarized (n= 20 and n=27 for primary and metastatic tumors). *P<0.05, Chi Square test. (C,D) Kaplan-Meier analysis from NCBI GEO datasets of recurrence-free survival for 936-patients with lymph node-positive breast cancers. Tumors were ranked according to gene expression values of *EPHA2* and *EFNA1* scored as high (red) or low (black). Hazard ratios with a 95% confidence interval are displayed and statistical significance (P<0.05) was determined by log rank test (16).

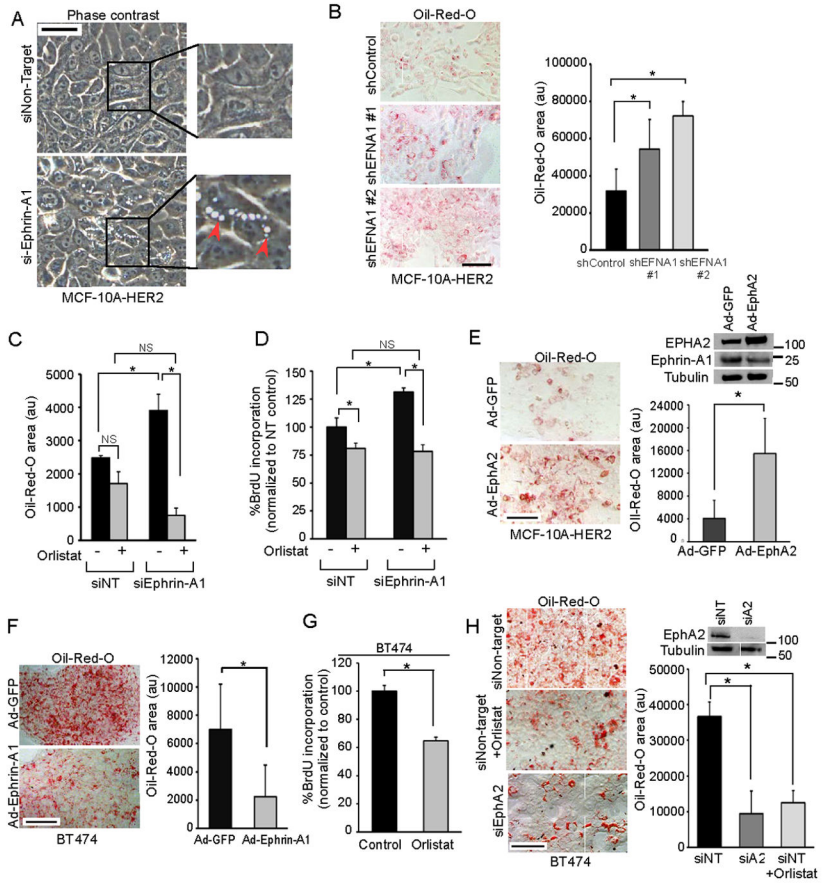

Figure 4. Ephrin-A1 regulates lipid accumulation in breast cancer cells

(A) Phase contrast microscopy images of MCF-10A-HER2 cells transfected with non-targeting or ephrin-A1 siRNAs. Scale bar 50μm. Arrows (red) show vacuole-like structures. (B) Oil-Red-O staining in control and ephrin-A1 shRNA knockdown cells. Data are presented as average Oil-Red-O area in arbitrary units (au). **P*<0.05, one-way ANOVA. Scale bar 100μm. (C) Oil-Red-O staining in siNon-Targeting (siNT) and siEphrin-A1 MCF-10A-HER2 cells treated with DMSO or Orlistat (20μM) for 24 hrs. **P*<0.05, one-way ANOVA. (D) BrdU incorporation in siNT and siEphrin-A1 MCF-10A-HER2 cells treated with DMSO or Orlistat (20μM) for 18 hrs. Data normalized to siNT control. **P*<0.05, one-way ANOVA. (E) Oil-Red-O staining in cells overexpressing EPHA2 or control GFP. **P*<0.05, Student's t-test. Scale bar 100μm. (F) Oil-Red-O staining in BT474 cells expressing GFP or ephrin-A1. **P*<0.05, Student's t-test. Scale bar 200μm. (G) BrdU incorporation in BT474 cells treated with Control (DMSO) or Orlistat (20μM). Data normalized to the siNT control. **P*<0.05 by Student's t-test. (H) Oil-red-O staining in BT474 cells treated with Control (DMSO) or Orlistat (20μM) for 24 hrs or transfected with non-targeting siRNA or EPHA2 siRNA. **P*<0.05, one-way ANOVA. Scale bar 100μm. All experiments represent 2–5 biological replicates. Error bars represent SEM. NS, not significant.

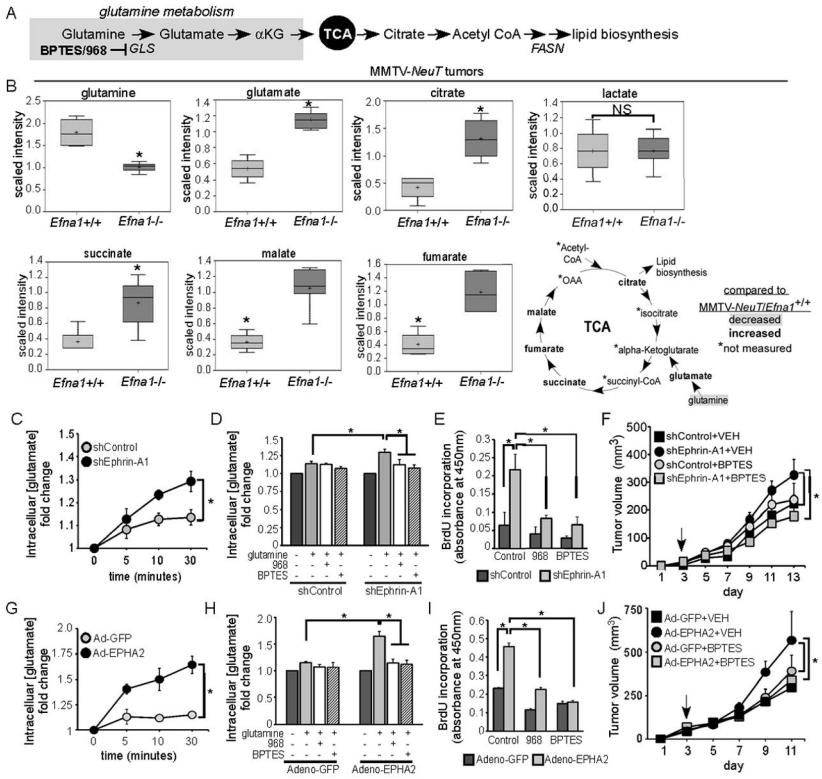

Figure 5. Loss of ephrin-A1 augments glutamine metabolism

(A) A schematic diagram of glutamine metabolism pathway. (B) LC/GC-MS derived relative metabolites abundance from MMTV-*NeuT*/*Efna1*$^{+/+}$ and MMTV-*NeuT*/*Efna1*$^{-/-}$ tumors. TCA schematic depicts relative changes (increase, bolded; decrease, shaded) from tumors. **P*<0.05, Welsh's two sample t-test. (C,G) Glutaminase activity was measured by intracellular glutamate concentrations upon 2mM glutamine and 5 ng/ml EGF stimulation in MMTV-*Neu* cells over a time course. **P*<0.05, two-way ANOVA repeated measures/ randomized block. (D,H) Intracellular glutamate was measured upon addition of 2mM glutamine and 5ng/ml EGF. Cells were treated with 968 (10μM) or BPTES (10μM) and were normalized to baseline. **P*<0.05, one-way ANOVA. (E,I) BrdU incorporation in MMTV-*Neu* cells treated with vehicle Control, 968 (10μM) or BPTES (10μM). **P*<0.05, one-way ANOVA. (F) shControl or shEphrin-A1 MMTV-*Neu* cells were transplanted into mammary fat pads of mice. BPTES (10mg/kg) or vehicle (VEH) was delivered by IP injection every other day starting on day 3 (arrow). *n*=8 per group. **P* <0.05, two-way ANOVA repeated measures. (J) MMTV-*Neu* cells overexpressing GFP or EPHA2 were transplanted into mammary fat pads of mice. BPTES (10mg/kg) or VEH was delivered by IP injection every other day starting on day 3 (arrow). *n*=5 per group. **P* <0.05, two-way ANOVA repeated measures. All *in vitro* experiments represent 2–4 biological replicates. Data are expressed as mean ± SEM.

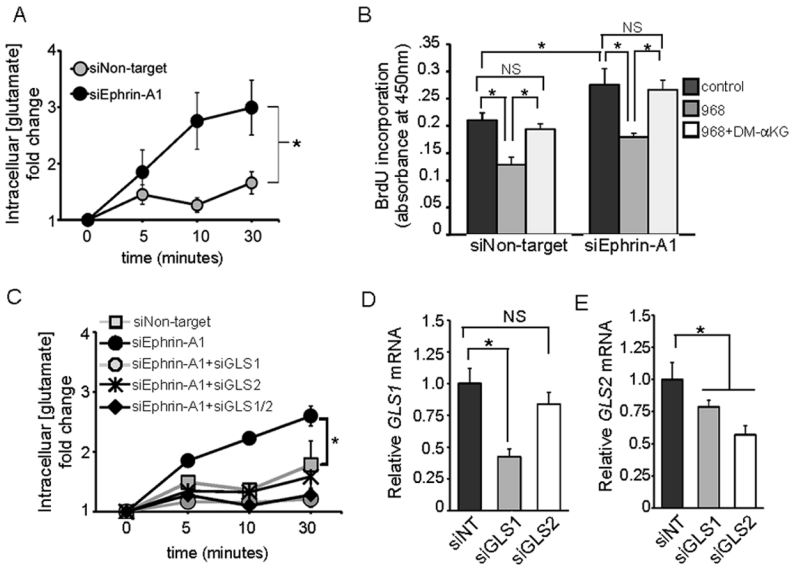

Figure 6. Knockdown of either GSL1 or GSL2 restored glutaminase activity in ephrin-A1-difficient cells

(A) Glutaminase activity was measured by intracellular glutamate concentrations upon 2 mM glutamine and 5 ng/ml EGF stimulation in MCF-10A-HER2 cells over a time course; expressed as fold change ± SEM. **P*<0.05, two-way ANOVA repeated measures/ randomized block. (B) BrdU incorporation in MCF-10A-HER2 cells after 968 or 968+DMαKG treatment. **P*<0.05, one-way ANOVA. (C) Intracellular glutamate in siNon-target, siEphrin-A1, siEphrin-A1+siGLS1, siEphrin-A1-siGLS2 and siEphrin-A1+siGLS1+siGLS2 MCF-10A-HER2 cells upon 2 mM glutamine and 5 ng/ml EGF stimulation. **P*<0.05, two-way ANOVA repeated measures/randomized block. (D,E) Relative mRNA levels of *GLS1* and *GLS2* were measured by real-time qRT-PCR to confirm knockdown. **P*<0.05, one-way ANOVA. All experiments represent 2–5 biological replicates. Error bars represent SEM. NS, not significant.

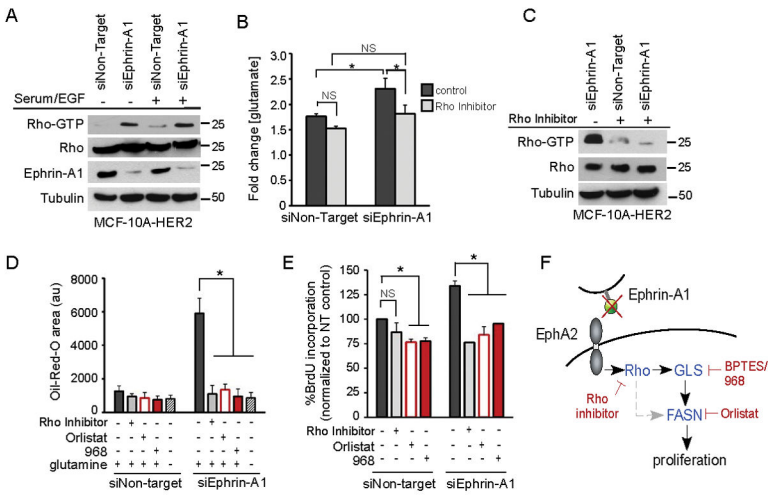

Figure 7. Ephrin-A1 regulates glutamine metabolism in a Rho-dependent manner

(A) Rhotekin-GST effector pull-down activity assay in MCF-10A-HER2 cells. (B) Glutaminase activity assay in the presence or absence of Rho inhibitor CT04 (1μg/mL) for 18 hours. Data expressed as the fold change normalized to siNT control ± SEM. **P*<0.05, one-way ANOVA. (C) Rho-GTP levels were measured by Rhotekin-GST effector pull-down assay. Shown are representative western blots with indicated antibodies. (D) Oil-red-O staining in MCF-10A-HER2 cells with treatments of Rho inhibitor CT04 (1μg/mL), glutaminase inhibitor 968 (10uM), FASN inhibitor Orlistat (20uM), or glutamine (glut) withdrawal for 24 hours. Data presented as average Oil-Red-O area and expressed in arbitrary units (au). **P*<0.05, one-way ANOVA. (E) BrdU incorporation in MCF-10A-HER2 cells after treatment with CT04 Rho inhibitor (1μg/mL), 968 (10uM), or Orlistat (20uM) for 18 hours. Data are normalized to the siNT control; error bars represent SEM. **P*<0.05, one-way ANOVA. NS, not significant. (F) A working model of ephrin-A1 regulation of tumor metabolism in breast cancer cells.