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Abstract

Clinical data analysis reveals that the expression of the EphB4 receptor tyrosine kinase is significantly elevated in HER2-positive breast cancer and high levels of EphB4 strongly correlate with poor disease prognosis. However, the impact of EphB4 activation on HER2-positive breast cancer cells and the potential of EphB4 as a therapeutic target remain to be explored. Here, we show that EphB4 overexpression confers gain-of-function activities to HER2-positive breast cancer cells, rendering resistance to a HER2/EGFR inhibitor Lapatinib. Furthermore, using integrated transcriptomic and tyrosine phosphoproteomic analyses, followed by biochemical confirmation, we establish that EphB4 activation engages the SHP2/GAB1-MEK signaling cascade and downstream c-MYC activation, and thereby limits the overall drug responses to Lapatinib. Finally, we demonstrate that, in HER2-positive breast tumors, inhibition of EphB4 combined with Lapatinib is more effective than either alone. These findings provide new insights into the signaling networks dictating therapeutic response to Lapatinib as well as a rationale for co-targeting EphB4 in HER2-positive breast cancer.

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Running title: **EphB4 confers resistance to Lapatinib**

Key words: EphB4, HER2, breast cancer, Lapatinib, drug response

Abstract

Clinical data analysis reveals that the expression of the EphB4 receptor tyrosine kinase is significantly elevated in HER2-positive breast cancer and high levels of EphB4 strongly correlate with poor disease prognosis. However, the impact of EphB4 activation on HER2-positive breast cancer cells and the potential of EphB4 as a therapeutic target remain to be explored. Here, we show that EphB4 overexpression confers gain-of-function activities to HER2-positive breast cancer cells, rendering resistance to a HER2/EGFR inhibitor Lapatinib. Furthermore, using integrated transcriptomic and tyrosine phosphoproteomic analyses, followed by biochemical confirmation, we establish that EphB4 activation engages the SHP2/GAB1-MEK signaling cascade and downstream c-MYC activation, and thereby limits the overall drug responses to Lapatinib. Finally, we demonstrate that, in HER2-positive breast tumors, inhibition of EphB4 combined with Lapatinib is more effective than either alone. These findings provide new insights into the signaling networks dictating therapeutic response to Lapatinib as well as a rationale for co-targeting EphB4 in HER2-positive breast cancer.

1.Introduction

Amplification and/or overexpression of the receptor tyrosine kinase HER2 occur in approximately 20% of breast cancer [1-3]. Although both Trastuzumab and Lapatinib provide considerable clinical benefits for HER2-positive breast cancer patients, a large fraction of the diseases display primary resistance to these inhibitors [2-5]. Furthermore, tumors that are initially sensitive to these drugs go on to adapt to HER2-targeted inhibition or develop acquired resistance in patients with advanced diseases [2-5]. The Eph receptor tyrosine kinase EphB4 has been previously shown to promote mammary tumorigenesis and enhance metastatic burden in MMTV-*neuT* (an active mutant rat Her2/neu, ErbB-2) mice [6]. However, the impact of EphB4 activation on HER2-positive breast cancer and the potential of EphB4 as a therapeutic target still await further investigation.

Increasing evidence suggest that EphB4 may affect the growth, migration and invasion of cancer cells through differential activation of signal transduction pathways in different tissue types or genetic settings [7-12]. High levels of EphB4 are observed in a variety of human malignancies, including breast cancer [1,7-9,13-17]. Overexpression of EphB4 has been linked to malignant transformation and tumor progression through activation of the PI3K/AKT, MEK/ERK or RhoGTPase signaling pathways [8,11,12]. A more recent study reported EphB4 as an erythropoietin (Epo) receptor promoting rhEpo-induced tumor growth and progression via Src-Stat3 signaling in ovarian and breast cancers [18]. Other evidence, however, indicates that EphB4 may function as a tumor suppressor in EphrinB2 (an EphB4-preferred ligand)-dependent manner in breast cancer [10]. The opposing roles of EphB4 as reported in breast cancer, combined with the potential link of EphB4 to HER2-positive subtype of the disease [6], prompted us to investigate whether EphB4 activation confers gain-of-function to HER2-positive breast cancer and

whether EphB4 overexpression allows tumor cells to escape from HER2-targeted inhibition by Lapatinib.

2.Materials and methods

2.1. Cell culture and Reagents

All the cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, USA) and maintained in culture medium (HCC1954 and BT474 cells in RPMI-1640; SKBR3 cells in McCoy's 5A modified; MDA-MB-361 cells in Dulbecco's Modified Eagle's Medium) supplemented with 10% FBS and 1% penicillin/streptomycin. MCF10A were cultured in DMEM/F12 supplemented with 5% FBS, 20 ng/ml EGF, 10 μg/ml insulin, 500 ng/ml hydrocortisone, 100 ng/ml cholera toxin, and 1% penicillin/streptomycin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2. All cell lines were examined periodically for Mycoplasma infection by PCR. Lapatinib, MEK-162, MK-2206, Trastuzumab and JQ-1 were purchased from Medchemexpress (Shanghai, China) and BHG-712 was purchased from Selleck (USA). EphrinB2-Fc and Fc were purchased from Sino Biological (Beijing, China). Lipofectamine 3000 (Invitrogen, USA) was used to transfect siRNA oligos into the cells according to the manufacturer's guidelines. The siRNA reagents were purchased from GenePharma (Suzhou, China). siEphrinB2#1: CGAUUUCCAAAUCGAUAGUTT; siEphrinB2#2: GACAAGGACUGGUACUAUATT; siMYC: GCTTGTACCTGCAGGATCT.

2.2. Plasmids and stable cell line generation

To construct pLKO.1 puro (addgene #8453) or pLKO-tet-on shRNAs (kindly provided by Dr. Jean Zhao at Dana-Farber Cancer Institute, USA) targeting human EphB4, SHP2, GAB1, and STAT3, oligonucleotides synthesis were performed by Sangon Biotech (Shanghai, China) Co. Ltd.

EphB4 shRNA#1: CAATGGGAGAGAAGCAGAATA

EphB4 shRNA#2: CACCACCAAACTCAATCATTT

SHP2 shRNA#1: TATACCCTTAACCAGTTTAAT

SHP2 shRNA#2: AGATGTCATTGAGCTTAAATA

GAB1 shRNA#1: AGTTAACACACTCGTAGTATT

GAB1 shRNA#2: GTTACGCAGTGGCCGTTTAAC

STAT3 shRNA#1: CTCAGAGGATCCCGGAAATTT

STAT3 shRNA#2: GGCGTCCAGTTCACTACTAAA

To construct pWzl-EphB4 and pBABE-puro HER2, human EphB4 or HER2 were amplified and cloned into pWzl-blast (kindly provided by Dr. Jean Zhao at Dana-Farber Cancer Institute, USA) or pBABE-puro (addgene #1764).

2.3. Western blot analysis

Cells were lysed in RIPA lysis buffer supplemented with phosphatase (1 mM NaF, 1 mM Na_3VO_4, 1 mM $C_3H_7Na_2O_6P$, and 10 mM $Na_4O_7P_2$), and protease inhibitors (Roche, USA). The immunoblotting was conducted in accordance with the standard procedures previously described [19]. The blots were probed with the following antibodies: anti-EphB4 (Cell signaling technology, CST#14960, USA), anti-pHER2 Y1221/1222(CST#2243), anti-HER2 (Abcam, ab134182, USA), anti-cleaved PARP (CST#5625), anti-pAKT S473 (CST#4060), anti-AKT (H-136) (Santa Cruz Biotechnology, sc8312, USA), anti-Vinculin (Sigma Aldrich, V9131, USA), anti-phosphotyrosine antibody (4G10) (Millipore#05-1050, Germany), anti-pERK1/2 (CST#4370), anti-ERK1/2 (CST#9102), anti-pGAB1 Y659 (CST#12745), anti-GAB1 (CST#3232), anti-SHP2 Y542 (CST#3751), anti-SHP2 Y580 (CST#5431), anti-SHP2 (CST#3397), anti-c-Myc (CST#5605), anti-pSTAT3 Y705 (CST#9145) and anti-GST Tag (Proteintech#666001-2-Ig). For the immunoblotting assay with SH2 superbinder-based phosphotyrosine, standard manual was performed as previously described [20].

2.4. Immunoprecipitation of Phosphotyrosine-containing proteins

Cells were lysed in TEB lysis buffer (50mM Tris pH 7.5, 150 mM NaCl, 1.0 % Nonidet P-40, 10% glycerol, 0.5mM phenylmethylsulfonyl fluoride) supplemented with phosphatase inhibitor (1mM NaF, 1mM Na_3VO_4, 1mM $C_3H_7Na_2O_6P$, and 10mM $Na_4O_7P_2$) and protease inhibitor (Roche, USA), and the debris was cleared by centrifugation. Cell lysate was then incubated with GST-SH2 superbinder [20] at 4˚C overnight, and followed by the addition of Glutathione sepharose (GE Healthcare, USA) for an additional 1 hour. The beads were then washed three times with ice-cold TEB buffer, and eluted with 2XSDS loading buffer. The eluted pTyr proteins were subjected to Western blot analysis.

2.5. Clonogenic survival assay

The breast cancer cells were trypsinized and seeded in 12-well plates. Fresh medium was replaced every 3 days. At the end point, cells were washed once with PBS then fixed with fixation solution and subsequently stained with 0.5% crystal violet (Sigma) and dissolved with 10% glacial acetic acid as previously described [19]. The optical density (OD) was measured at 590 nm by an xMark Microplate Spectrophotometer (Bio-Rad, USA).

2.6. Cell viability assay

The breast cancer cells were seeded in 96-well plates, indicated inhibitors were added in the next day for another 72 hours. The Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Japan) was carried out according to the manufacturer's suggestions as previously described [19]. The optical density (OD) was measured at 450nm by an xMark Microplate Spectrophotometer (Bio-Rad). The IC50s (half maximal inhibitory concentration) were calculated from the sigmoidal dose-response curves utilizing Prism.

2.7. 3-dimensional spheroid assay

The 3D cell culture experiments were carried out in 96-well plates precoated with 50% Matrigel (BD Biosciences, USA) plus 50% medium without serum. Breast cancer cells were trypsinized then seeded at a density of 3000 cells per well. Cells were grown in respective culture medium supplemented with 2% Matrigel and 2% FBS that was replaced every 3 days. Quantification was conducted according to the surface area of the spheroid using ImageJ software or the 3D structure integrity based on the resemblance to images shown along with the figures. Over 100 colonies were scored in each condition.

2.8. Quantitative RT-PCR analysis

Total RNA was extracted from cultured cells using the Nucleozol Reagent (Macherey-Nagel, Germany), 0.5 μg of the total RNA was subjected to a reverse transcriptase reaction according to the manufacturer's guidelines of a high-capacity cDNA PrimeScript™ RT master mix (Takara, Japan). For the quantitative real-time PCR, the complementary DNAs (cDNAs) were analyzed by a SYBR Green PCR Master Mix (Applied Biosystems, USA) on an StepOnePlus Real-time PCR system (Applied Biosystems). The primer synthesis and DNA sequencing were conducted by Sangon Biotech (Shanghai, China). The relative expression of cDNA was normalized to *β-actin* expression and was calculated by the 2ΔΔCt method. All used primers were listed as follows:

EphrinB2-Forward: TGTGCCAGACAAGAGCCATG

EphrinB2-Reverse: AGCTTCTAGTTCTGGACGTCTTG

c-MYC-Forward: AGGGTCAAGTTGGACAGTGTCA

c-MYC-Reverse: TGGTCGATTTTCGGTTGTTG

ODC1-Forward: AGCCATCGTGAAGACCCTTG

ODC1-Reverse: TGCATAGATAATCCTCTCTGGAGGC

LDHA-Forward: ATGTTGCTGGTGCCTCTCTGAAG

LDHA-Reverse: GCCCAGGATGTGCAGCCT

STAT3-Forward: AAGGACATCAGCGGTAAGAC

STAT3-Reverse: GAGATAGACCAGTGGAGACAC

2.9 Flow cytometry analysis

Apoptosis was analyzed with Annexin V-FITC Apoptosis Detection Kit (Dojindo Molecular Technologies, Japan) according to manufacturer's instructions. Briefly, cultured cells were trypsinized with 0.25% trypsin without EDTA, and then stained with Annexin V-FITC and Propidium iodide (PI) solution. Stained cells were subjected to flow cytometry analysis on BD FACS Aria II (BD Biosciences, USA).

2.10. RNA sequencing

The RNA-Seq experiments were performed by Shanghai Biotechnology Corporation (China). The extracted total RNAs were sequenced on the Illumina HiseqX10 platform using the standard paired-end protocol. Approximately 40 million 75-bp paired reads were acquired for each sample. RNA-Seq data were analyzed using GSEA software provided by the Broad Institute, Cambridge, MA, USA, as previously described [21]. A false discovery rate (FDR q value) of < 0.25 and a nominal P value of < 0.05 were considered statistically significant.

2.11. Tyrosine phosphoproteomics

Detailed methodology is described in Supplementary method.

2.12. In vivo Xenograft Mouse Study

Eight-week-old female NOD-SCID mice were purchased from Beijing Vital River Laboratory Animal Technology. The HCC1954 TetO-EphB4 shRNA cells

($5x10^6$ per injection) in PBS/Matrigel mixture were injected orthotopically into the mammary fat pads. Drug treatment started when average tumor size reached about 100 mm^3. Lapatinib was dissolved in 0.5% methylcellulose/0.5% Tween 80 and administered once daily via oral gavage at 100 mg/kg. Doxycycline was given in drinking water (5% dextrose), refreshed every 3 days. The tumor volumes were measured every other day with a digital caliper according to the following formula: tumor volume= (length × $width^2$)/2. All animal experiments were carried out in accordance with the approval of the Animal Research Committee of Dalian Medical University.

2.13. Clinical data analysis

The gene expression data were downloaded from TCGA or other published database (http://www.ncbi.nlm.nih.gov/geo/). GSE25055, GSE25065 and GSE250660 were for Hatzis_Breast [22]. Kaplan–Meier survival analyses for disease outcomes were performed using the online database (www.kmplot.com) [23] or other published database (http://www.ncbi.nlm.nih.gov/geo/): Desmedt_breast (GSE7390) [24] and Kao_breast (GSE20685) [25].

2.14. Statistics

Statistical analysis was performed by two-tailed unpaired Student's *t* test for two group comparisons, and by one-way ANOVA followed by Tukey's multiple comparison tests wherever appropriate. The survival analysis was performed by the Kaplan–Meier method and compared statistically using the log-rank test. *P*-value < 0.05 was considered as statistical significance.

3. Results

3.1. Expression of EphB4 is significantly elevated in HER2-positive breast cancer, strongly correlated with the poor disease outcomes

It has been reported that the receptor tyrosine kinase EphB4 is overexpressed in a variety of human cancers including breast cancer [1,7-9,13-17]. Analysis of the two breast cancer databases [1,22] reveals that EphB4 mRNA is preferentially elevated in basal-like and HER2-enriched breast carcinoma compared to luminal A and B tumors (**Fig. 1A** and **B**). To gain insights into the potential relevance of EphB4 overexpression in breast cancer, we asked whether EphB4 expression correlates with disease prognosis. By analyzing gene expression data across three independent cohorts [23-25], we found that EphB4 expression was inversely associated with overall survival and metastasis-free survival (**Fig. 1C** and **D**). Specifically, higher expression levels of EphB4 were strongly associated with poor prognosis in HER2-positive and basal-like subtypes (**Fig. 1E** and **Supplementary Fig. 1A** and **B**), thus implicating a potential oncogenic role of EphB4 in these diseases.

Our findings that higher levels of EphB4 expression significantly correlate with poor prognosis in HER2-positive breast cancer (**Fig. 1E**), together with the previous report that transgenic overexpression of EphB4 expedited the tumorigenesis and metastatic potential of the mouse mammary cancer model transgenic for *neuT* [6], prompted us to further investigate the potential oncogenic role of EphB4 in HER2-positive breast cancer. It has been demonstrated that the presence of endogenous oncogenic PIK3CA mutations renders HER2-positive breast cancer cells less responsive to HER2 blockade [26]. We found that EphB4 knockdown in HCC1954 (HER2 amplification, PIK3CA H1047R) and MDA-MB-361 (HER2 amplification, PIK3CA E545K) cells markedly reduced the proliferation of cells cultured in 2D monolayer and the growth of spheroids cultured in 3D on Matrigel (**Supplementary Fig. 2A-C**), a condition that closely mimics tumor microenvironment [27-29]. Conversely, overexpression of EphB4 resulted in significant clonogenic survival in 2D cultures and conferred a remarkable growth advantage in the 3D cultures of HER2-amplified breast cancer cell lines BT474 and SKBR3 as well

as MCF10A/HER2, a normal human mammary epithelial cell line with ectopic overexpression of HER2 (**Supplementary Fig. 2D-F**). Together, these results suggest that EphB4 confers gain-of-function activities to HER2-positive breast cancer.

It has been previously shown that EphrinB2-dependent EphB4 signaling exhibits a tumor suppressive role in breast cancer cells including MCF7, MDA-MB-435, and MDA-MB-231 [10]. Notably however, none of these cell lines belong to the HER2-overexpressing subtype. To further investigate whether the HER2- positive breast cancer cell lines examined in our study may depend on EphrinB-mediated EphB4 signaling, we first performed shRNA knockdown of EphrinB2 and measured the cell viability. Indeed, EphrinB2 knockdown had no discernable impact on the growth of HER2-overexpressing breast cancer cells HCC1954 and MDA-MD-361 (**Supplementary Fig. 3A-D**). Meanwhile, we also failed to observe an effect of EphinB2-Fc on the growth of these cancer cells (**Supplementary Fig. 4**). Together, these results argue against the presence of EhrinB2-mediated tumor suppressor signaling in HER2-positive breast cancer.

3.2. EphB4 activation confers resistance to Lapatinib

We next investigated whether EphB4 could affect the response of HER2-overexpressing breast cancer cells to Lapatinib, a dual HER2/EGFR tyrosine kinase inhibitor. Notably single-agent Lapatinib treatment strongly inhibited HER2 phosphorylation but had little impact on the levels of cleaved PARP, an indicator of apoptosis, in HCC1954 cells (**Fig. 2A**). In contrast, knockdown of EphB4 sensitized cells to Lapatinib as determined by the cell viability assay and the 3D spheroid assay (**Fig. 2B** and **C**). In line with this, combinatorial EphB4 knockdown and Lapatinib induced apoptosis (**Fig. 2A** and **Supplementary Fig. 5A**). Furthermore, BHG712 [30,31], a kinase inhibitor of

EphB4, enhanced the cytotoxic effect of Lapatinib in the 2D culture and 3D spheroid models of HCC1954 cells (**Fig. 2D-F** and **Supplementary Fig. 5B**). Conversely, EphB4 activation by ectopic overexpression of EphB4 rendered HER2-positive BT474 and HER2-overexrpressing MCF10A (MCF10A/HER2) cells insensitive to Lapatinib (**Fig. 2G-I, Supplementary Fig. 5C and 6A-B**). In addition to Lapatinib, inhibition of EphB4 also sensitized HCC1954 cells to Trastuzumab, a humanized monoclonal antibody targeting HER2 (**Supplementary Fig. 7)** Together, these results suggest for the first time that EphB4 activation allows HER2-positive breast cancer cells to escape from HER2-targeted inhibition by Lapatinib.

3.3. EphB4 engages KRAS/MEK signaling in HER2-positive breast cancer cells

To investigate the mechanism underlying the oncogenic role of EphB4 in HER2-positive breast cancer, we performed RNA sequencing (RNA-Seq) analysis. Specifically, we subjected RNAs isolated from HCC1954 with or without EphB4 shRNA knockdown, and parallelly BT474 cells with or without ectopic overexpression of EphB4, to transcriptional profiling by RNA-Seq. Subsequent gene set enrichment analyses (GSEA) of both RNA-Seq data sets pointed to a highly significant association between EphB4 expression and KRAS-associated gene signatures (**Fig. 3A** and **B**). KRAS regulates numerous cellular processes by activating signaling pathways such as MEK/ERK mitogen-activated protein kinase and PI3K-AKT pathways [32,33]. To identify which specific effector pathway downstream of KRAS is associated with the effect of EphB4 on HER2-positive breast cancer cells, we further analyzed the *in vivo* signaling by western blot analysis. We found that knockdown of EphB4 led to significantly decreased ERK phosphorylation (**Fig. 3C**). Conversely, ectopic overexpression of EphB4 remarkably induced ERK phosphorylation (**Fig. 3D**). Notably, the levels of phosphorylated AKT remained intact

regardless of whether EphB4 is silenced or ectopically overexpressed (**Fig. 3C** and **D**). These results suggest that the signaling of MEK/ERK, rather than AKT, is most likely associated with EphB4 activation.

We extended these findings into investigate whether blockade of effectors downstream of KRAS signaling, ERK or AKT, may abrogate the gain-of-function activity of EphB4 on HER2-positive breast cancer cells. Indeed, while single-agent MEK inhibitor MEK126 or Lapatinib alone had little inhibitory effect, the combination treatment nearly abolished the 3D spheroid structures (**Fig. 3E, middle panels**). In contrast, combined use of AKT inhibitor MK2206 and Lapatinib had only moderate effect on the growth of 3D spheroids (**Fig. 3E, bottom panels**). Consistently, compound Lapatinib/MEK162 fully suppressed phosphorylated ERK signals and induced strong cleaved PARP signals (an indicator of apoptosis), whereas compound Lapatinib/MK2206 induced only moderate levels of apoptosis (**Fig. 3F**). It is worth noting that while the GSEA analysis identified an enrichment of IL6-JAK-Stat3 signature in BT474 cells with EphB4 overexpression, subsequent 3D spheroid assays by using Stat3 shRNA knockdown failed to establish a causal relationship (**Supplementary Fig. 8A-E**). Collectively, these findings suggest that EphB4 activation confers oncogenic activities to HER2-positive breast cancer through activation of KRAS downstream effector MEK/ERK rather than AKT, and that blocking MEK/ERK activation may sensitize EphB4-overexpressing cells to HER2 blockade by Lapatinib.

3.4. EphB4 activation allows HER2-positive breast cancer cells to escape from Lapatinib through engaging the SHP2/GAB1-MEK signaling

To gain insights into how EphB4 mediates MEK/ERK activation in HER2-positive breast cancer cells, we conducted quantitative tyrosine phosphoproteomics using our newly developed SH2 superbinder [34-36]. Interestingly, tyrosine phosphoproteomics analysis identified markedly

increased intensity of phosphorylation at Tyr542 and 580 in Src homology-2 (SH2) domain-containing phosphatase 2 (SHP2) in EphB4-overexpressing BT474 cells when compared to the control cells (**Fig. 4A** and **Supplementary Table 1**). We validated these specific tyrosine phosphorylation events in SHP2 by western blot analysis (**Fig. 4B** and **Supplementary Fig. 9A**), suggesting the mass spectrometry data generated by our SH2 superbinder-based approach could faithfully recapitulate tyrosine phosphorylation *in vivo*. It has been previously shown that phosphorylation of SHP2 at Tyr542 and 580 is critically important for SHP2-mediated full activation of the MEK/ERK signaling cascade [37-40]. In line with this, knockdown of SHP2 in EphB4-overexpressing BT474 cells led to significantly reduced ERK phosphorylation (**Supplementary Fig. 9B**). These results for the first time brought our attention that EphB4 activation may engage the SHP2-MEK signaling axis in HER2-positive breast cancer.

Several lines of evidence indicate that SHP2 in complex with phosphorylated GRB2-associated binder protein 1 (GAB1) at Y627 and Y659 relieves the auto-inhibition of SHP2, promoting the activation of MEK/ERK signaling [41-44]. Indeed, our tyrosine phosphoproteomics analysis also identified a discernable increase in GAB1 phosphorylation at Y659 in EphB4-overexpressing BT474 cells when compared to the control cells (**Fig. 4A** and **Supplementary Table 1**). Subsequent western blot analysis validated the increase of this site-specific GAB1 phosphorylation (**Fig. 4B** and **Supplementary Fig. 9A**). Although to a lesser extent than the effect of SHP2 silencing (**Supplementary Fig. 9B**), knockdown of GAB1 in EphB4-overexpressing BT474 cells led to a decrease in phosphorylated ERK1/2 signals (**Supplementary Fig. 9C**). Notably, blockade of HER2 activity by Lapatinib alone did not cause noticeable changes in SHP2 or GAB1 phosphorylation in EphB4-overexpressing BT474 cells (**Fig. 4B**). In contrast,

knockdown of either SHP2 or GAB1 combined with Lapatinib nearly abolished the ERK phosphorylation induced by EphB4 (**Supplementary Fig. 9B** and **C**). These data strongly suggest that EphB4 activation may confer resistance to Lapatinib through engaging the SHP2/GAB1-MEK signaling cascade. We further looked into this possibility by conducting 3D spheroid assay. While overexpression of EphB4 allowed BT474 cells to survive on Matrigel in the presence of Lapatinib, concomitant knockdown of either GAB1 or SHP2 rendered the sensitivity of these EphB4-overexpressing cells to Lapatinib (**Fig. 4C**). In addition, as EphrinB2 knockdown had little impact on the phosphorylation levels of SHP2, GAB1 or ERK in EphB4-overexpressing BT474 cells (**Supplementary Fig. 10A and B**), it is less likely that EphrinB2 is involved in EphB4-mediated signal transduction in HER2-positive breast cancer. Together, these data suggest that EphB4 activation allows HER2-positive breast cancer cells to escape from Lapatinib through engaging the SHP2/GAB1-MEK signaling.

3.5. Inactivation of the EphB4-SHP2-MEK signaling axis attenuates c-MYC activation

Previous work has shown that SHP2-mediated activation of MEK/ERK signaling induces the expression of *c-MYC* [45]. We extended this finding into examine whether EphB4-mediated MEK/ERK signaling also involves c-MYC activation. Indeed, our RNA-Seq analysis revealed an association of EphB4 expression and MYC-targeted gene signature (**Fig. 5A**). Consistently, EphB4 knockdown led to reduced c-MYC expression at both mRNA and protein levels, and concordantly reduced expression of two well-described MYC targets, *ODC1* and *LDHA* [46,47] (**Fig. 5B-D**). Furthermore, siRNA-mediated knockdown of c-MYC sensitized HCC1954 cells to Lapatinib treatment (**Supplementary Fig. 11**). Concordantly, JQ-1 [48], a BET bromodomain inhibitor that suppresses MYC transcription, combined with Lapatinib induced

strong apoptotic signals and abrogated the integrity of the 3D spheroids of HCC1954 cells (**Fig. 5E** and **F**). Conversely, ectopic expression of EphB4 led to dramatically increased expression of *c-MYC* mRNA and protein (**Fig. 5G** and **H**). The combination treatment by Lapatinib and JQ1 strongly induced apoptotic signals and disrupted the 3D spheroid growth of EphB4-overexpressing BT474 cells (**Fig. 5I** and **J**). Together, these results suggest that therapeutically targeting c-MYC may enhance the anti-tumor effect of Lapatinib in HER2-positive breast cancer with EphB4 overexpression.

3.6. Targeting EphB4-MEK signaling improves the tumor response to Lapatinib *in vivo*

To extend these *in vitro* findings, we generated a HCC1954 cell line expressing EphB4 shRNA in which knockdown of EphB4 expression is under the control of a tetracycline-inducible promoter (TetO), designated as HCC1954 TetO-EphB4 shRNA. Prior to the *in vivo* study, we first assessed the response of this cell line model to drug treatments *in vitro*. When compared to single-agent treatments, combined use of Lapatinib and doxycycline resulted in a substantial decrease in ERK phosphorylation and, to a lesser extent, c-MYC protein abundance (**Supplementary Fig. 12A**). Strikingly, the drug combination completely suppressed cell proliferation and induced apoptosis (**Supplementary Fig. 12A** and **B**). Furthermore, we found that ectopic expression of c-MYC T58A, a more stable form of c-MYC [49], at least in part, rescued the growth inhibitory effect caused by doxycycline-induced silencing of EphB4 (**Fig. 5K** and **L**). Together, these data suggest that inhibition of the EphB4-mediated ERK-MYC signaling cascade may augment the response to lapatinib.

We next established the orthotopic xenograft model using the HCC1954 TetO-EphB4 shRNA cell line and subjected the tumor-bearing mice to drug

treatment *in vivo*. Compared to single-agent or vehicle treatments, combined use of Doxycycline and Lapatinib significantly inhibited the tumor growth (**Fig. 6A**). While Lapatinib sufficiently blocked HER2 signaling and Doxycycline attenuated EphB4 expression, these single-agent treatments only moderately reduced ERK phosphorylation (**Fig. 6B**). Consistent with the results obtained in the *in vitro* studies, Doxycycline-induced EphB4 silencing combined with Lapatinib led to remarkably diminished phosphorylated ERK1/2 signals and further decreased c-MYC protein abundance (**Fig. 6B**). Meanwhile, the combination treatment elicited strong apoptotic signals *in vivo* (**Fig. 6B**), explaining at least in part for the strong therapeutic efficacy exerted by combined blockade of HER2 and EphB4.

4. Discussion

Resistance to HER2-targeted therapies remains a big challenge in the treatment of HER2-positive breast cancer patients. Accumulating research studies have reported diverse molecular mechanisms of resistance to HER2-targeted therapies [2-5], including dysregulation of mitogenic signaling by constitutively activated oncogenic proteins. We and others have previously shown that compensatory activation of MEK-ERK signaling confers resistance to HER2-targeted agents in HER2-positive breast tumor models carrying oncogenic *PIK3CA* mutations [50-54]. In the current study, we demonstrate that overexpression of EphB4 renders the escape of HER2-positive breast cancer cells from Lapatinib treatment through engaging the SHP2/GAB1-MEK-MYC signaling cascade.

Eph receptors belong to the largest family of receptor tyrosine kinases, among which Eph receptor A2 (EphA2) is the most extensively studied [55-59]. EphA2 has been shown to cooperate with HER2 to promote malignant transformation and metastatic progression in a genetic mouse model of breast cancer [55]. Similarly, EphB4 transgene expression has been shown to

expedite mammary tumorigenesis and enhance metastatic burden in *neuT* transgenic mice [6]. While our findings highlight a more broad investigation of the potential involvement of additional Eph RTKs in HER2-targeted therapies, further thorough studies may be required to investigate whether EphB4 also confers resistance to other HER2-targeted agents, *e.g.* the newly FDA approved HER kinase inhibitor Neratinib. Meanwhile, as the expression levels of EphB4 are also elevated in basal-like breast cancer and higher levels of EphB4 expression are significantly associated with the poor prognosis of the disease (**Fig. 1** and **Supplementary Fig. S1**), future studies with focus on uncovering potential oncogenic activities of EphB4 in the basal-like subtype would be necessary to expand the scope of the current study.

We report for the first time that EphB4 activation engages the SHP2-MEK signaling cascade in HER2-positive breast cancer. Indeed, SHP2 has been previously shown to promote HER2-induced signaling and transformation by increasing the RAS activity [45,60,61]. In the current study, we took integrated approaches to investigate the oncogenic activity of EphB4 in HER2-positive breast cancer. First, using RNA-Seq transcriptional profiling followed by western blot analysis, we identified the impact of EphB4 activation on the effector signaling downstream of KRAS, specifically ERK, rather than AKT. Second, to examine the phosphotyrosine profiles affected by EphB4 activation, we performed SH2 superbinder-based tyrosine phosphoproteomics analysis, and identified an increase in phosphorylation of SHP2 Tyr542 and Tyr 580 in association with EphB4 activation. As these two SHP2 phosphorylation events are known to be critical for full activation of MEK/ERK signaling [37-40], the findings from our integrated molecular analyses, together with subsequent functional characterization data, provide strong evidence for the role of EphB4 in activation of the SHP2- MEK signaling axis in HER2-positive breast cancer.

Accumulating evidence indicates that GAB1 phosphorylation at Tyr 627 and Tyr 659 recruits and activates SHP2, leading to MEK/ERK activation

[41-44]. Our finding that EphB4 overexpression enhances phosphorylation of GAB1 at Tyr 659 thus supports the notion that EphB4 regulates GAB1-SHP2 mediated MEK/ERK activation. Furthermore, we demonstrate the growth dependence of HER2-positive breast cancer cells on EphB4-activated SHP2/GAB1-MEK signaling cascade. In addition, our findings also establish a causal relationship between EphB4-mediated signaling and c-MYC activity. It has been shown that SHP2-mediated activation of MEK/ERK signaling promotes breast cancer progression via activation of c-MYC [45]. We extend this finding to show that EphB4 activation induces c-MYC activity and that pharmacological inhibition of c-MYC by JQ1 sensitizes EphB4-overexpressing breast cancer cells to Lapatinib. Collectively, our data uncover the complex rewiring of the oncogenic signaling networks conferred by EphB4 activation in the context of HER2-positive breast cancer.

While our work has established for the first time a functional link between EphB4 activation and the SHP2/GAB1/MEK-1/c-MYC signaling axis, in-depth characterization of EphB4-SHP2 signaling network activity at systems-levels has been lacking. The interaction of SH2 domain containing protein with phosphorylated tyrosine residues is a key feature of signaling initiation events and thus have a substantial effect on signal transduction of RTK activation. However, as these binding events are typically of relatively low affinity and occur rapidly, it would be difficult to capture these events by routine coimmunoprecipitation assays with potential loss of protein complex stability during incubations. Indeed, this may be the case in our study as we failed to observe phosphorylation events of Grb2 in the tyrosine phosphoproteomic profile of EphB4 activating cells. Nevertheless, we cannot rule out the possibility that Grb2 is not involved in EphB4 activation-mediated signaling. On the other hand, among all RTKs, the dynamic interaction of EGFR with the SHP2-GAB1-MEK1 signaling axis has been most extensively studied and interestingly, it has been recently shown that EGFR-activated Src family

kinases maintain GAB1-SHP2 complexes distal from EGFR[43], a regulatory mechanism uniquely used by EGFR (but not c-Met) to remotely control the duration of the signaling cascade. It will be interesting to investigate whether EphB4 activation would adopt a mechanism of signaling initiation similar to that of EGFR activation. To further understand how EphB4 activation regulates the phosphorylation dynamics of SHP2-mediated signaling, we propose to determine the extent to which EphB4 activation is perturbed by using allosteric inhibitors of SHP2 (*e.g.* SHP099) in our future quantitative phosphoproteomics studies.

In summary, we propose the model based on our understanding to explain the regulation of SHP2/GAB1-MEK signaling cascade activated by EphB4 overexpression and how inhibition of EphB4 or its downstream effectors may sensitize HER2-positive breast cancer cells to Lapatinib (**Fig. 6C**). Altogether, our study provides new insights into the signaling networks dictating therapeutic response to Lapatinib as well as a rationale for targeting EphB4 in HER2-positive breast cancer.

Author contributions

H.C., M.L., M.Y., P.L., and J.D. conceived and designed the project. J.D., X.W., G.H. and N.Z designed and performed most of the experiments, computational analysis for gene signature and statistical analysis. Y.Y. and J.D. carried out the pTyr enrichment experiments, MS analysis and analyzed the proteomics data under the supervision of M.Y., J.D., J.Y., and C.L. conducted the animal experiments; K.W., Y.Z. and M.W. assisted with the essential experiments. H.C. and J.D. wrote the manuscript with input from P.L. and M.Y.

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Conflict of interest

The authors declare that they have no conflict of interest.

Reference

1. Comprehensive molecular portraits of human breast tumours. *Nature* **490**, 61-70 (2012).
2. Arteaga, C.L., Sliwkowski, M.X., Osborne, C.K., Perez, E.A., Puglisi, F. & Gianni, L. Treatment of HER2-positive breast cancer: current status and future perspectives. *Nature reviews. Clinical oncology* **9**, 16-32 (2011).
3. Baselga, J. & Swain, S.M. Novel anticancer targets: revisiting ERBB2 and discovering ERBB3. *Nature reviews. Cancer* **9**, 463-475 (2009).
4. Lan, K.H., Lu, C.H. & Yu, D. Mechanisms of trastuzumab resistance and their clinical implications. *Annals of the New York Academy of Sciences* **1059**, 70-75 (2005).
5. Piccart, M. Circumventing de novo and acquired resistance to trastuzumab: new hope for the care of ErbB2-positive breast cancer. *Clinical breast cancer* **8 Suppl 3**, S100-113 (2008).
6. Munarini, N., Jager, R., Abderhalden, S., Zuercher, G., Rohrbach, V., Loercher, S., Pfanner-Meyer, B., Andres, A.C. & Ziemiecki, A. Altered mammary epithelial development, pattern formation and involution in transgenic mice expressing the EphB4 receptor tyrosine kinase. *Journal of cell science* **115**, 25-37 (2002).
7. Kumar, S.R., Singh, J., Xia, G., Krasnoperov, V., Hassanieh, L., Ley, E.J., Scehnet, J., Kumar, N.G., Hawes, D., Press, M.F., Weaver, F.A. & Gill, P.S. Receptor tyrosine kinase EphB4 is a survival factor in breast cancer. *The American journal of pathology* **169**, 279-293 (2006).
8. Merchant, A.A., Jorapur, A., McManus, A., Liu, R., Krasnoperov, V., Chaudhry,

P., Singh, M., Harton, L., Agajanian, M., Kim, M., Triche, T.J., Jr., Druker, B.J., Tyner, J.W. & Gill, P.S. EPHB4 is a therapeutic target in AML and promotes leukemia cell survival via AKT. *Blood advances* **1**, 1635-1644 (2017).

9. Mertens-Walker, I., Fernandini, B.C., Maharaj, M.S., Rockstroh, A., Nelson, C.C., Herington, A.C. & Stephenson, S.A. The tumour-promoting receptor tyrosine kinase, EphB4, regulates expression of integrin-beta8 in prostate cancer cells. *BMC cancer* **15**, 164 (2015).
10. Noren, N.K., Foos, G., Hauser, C.A. & Pasquale, E.B. The EphB4 receptor suppresses breast cancer cell tumorigenicity through an Abl-Crk pathway. *Nature cell biology* **8**, 815-825 (2006).
11. Xiao, Z., Carrasco, R., Kinneer, K., Sabol, D., Jallal, B., Coats, S. & Tice, D.A. EphB4 promotes or suppresses Ras/MEK/ERK pathway in a context-dependent manner: Implications for EphB4 as a cancer target. *Cancer biology & therapy* **13**, 630-637 (2012).
12. Yang, N.-Y., Pasquale, E.B., Owen, L.B. & Ethell, I.M. The EphB4 Receptor-tyrosine Kinase Promotes the Migration of Melanoma Cells through Rho-mediated Actin Cytoskeleton Reorganization. *Journal of Biological Chemistry* **281**, 32574-32586 (2006).
13. Alam, S.M., Fujimoto, J., Jahan, I., Sato, E. & Tamaya, T. Overexpression of ephrinB2 and EphB4 in tumor advancement of uterine endometrial cancers. *Annals of oncology : official journal of the European Society for Medical Oncology* **18**, 485-490 (2007).
14. Ferguson, B.D., Liu, R., Rolle, C.E., Tan, Y.H., Krasnoperov, V., Kanteti, R., Tretiakova, M.S., Cervantes, G.M., Hasina, R., Hseu, R.D., Iafrate, A.J., Karrison, T., Ferguson, M.K., Husain, A.N., Faoro, L., Vokes, E.E., Gill, P.S. & Salgia, R. The EphB4 receptor tyrosine kinase promotes lung cancer growth: a potential novel therapeutic target. *PloS one* **8**, e67668 (2013).
15. Hasina, R., Mollberg, N., Kawada, I., Mutreja, K., Kanade, G., Yala, S., Surati, M., Liu, R., Li, X., Zhou, Y., Ferguson, B.D., Nallasura, V., Cohen, K.S., Hyjek, E., Mueller, J., Kanteti, R., El Hashani, E., Kane, D., Shimada, Y., Lingen, M.W., Husain, A.N., Posner, M.C., Waxman, I., Villaflor, V.M., Ferguson, M.K., Varticovski, L., Vokes, E.E., Gill, P. & Salgia, R. Critical role for the receptor tyrosine kinase EPHB4 in esophageal cancers. *Cancer research* **73**, 184-194 (2013).
16. Yin, J., Cui, Y., Li, L., Ji, J. & Jiang, W.G. Overexpression of EPHB4 Is Associated with Poor Survival of Patients with Gastric Cancer. *Anticancer research* **37**, 4489-4497 (2017).
17. Sinha, U.K., Mazhar, K., Chinn, S.B., Dhillon, V.K., Liu, L., Masood, R., Rice, D.H. & Gill, P.S. The association between elevated EphB4 expression, smoking status, and advanced-stage disease in patients with head and neck squamous cell carcinoma. *Archives of otolaryngology--head & neck surgery* **132**, 1053-1059 (2006).
18. Pradeep, S., Huang, J., Mora, E.M., Nick, A.M., Cho, M.S., Wu, S.Y., Noh, K., Pecot, C.V., Rupaimoole, R., Stein, M.A., Brock, S., Wen, Y., Xiong, C.,

Gharpure, K., Hansen, J.M., Nagaraja, A.S., Previs, R.A., Vivas-Mejia, P., Han, H.D., Hu, W., Mangala, L.S., Zand, B., Stagg, L.J., Ladbury, J.E., Ozpolat, B., Alpay, S.N., Nishimura, M., Stone, R.L., Matsuo, K., Armaiz-Pena, G.N., Dalton, H.J., Danes, C., Goodman, B., Rodriguez-Aguayo, C., Kruger, C., Schneider, A., Haghpeykar, S., Jaladurgam, P., Hung, M.C., Coleman, R.L., Liu, J., Li, C., Urbauer, D., Lopez-Berestein, G., Jackson, D.B. & Sood, A.K. Erythropoietin Stimulates Tumor Growth via EphB4. *Cancer cell* **28**, 610-622 (2015).

19. Ding, J., Wang, X., Zhang, Y., Sang, X., Yi, J., Liu, C., Liu, Z., Wang, M., Zhang, N., Xue, Y., Shen, L., Zhao, W., Luo, F., Liu, P. & Cheng, H. Inhibition of BTF3 sensitizes luminal breast cancer cells to PI3Kalpha inhibition through the transcriptional regulation of ERalpha. *Cancer letters* (2018).
20. Li, Y., Wang, Y., Dong, M., Zou, H. & Ye, M. Sensitive Approaches for the Assay of the Global Protein Tyrosine Phosphorylation in Complex Samples Using a Mutated SH2 Domain. **89**, 2304-2311 (2017).
21. Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S. & Mesirov, J.P. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 15545-15550 (2005).
22. Hatzis, C., Pusztai, L., Valero, V., Booser, D.J., Esserman, L., Lluch, A., Vidaurre, T., Holmes, F., Souchon, E., Wang, H., Martin, M., Cotrina, J., Gomez, H., Hubbard, R., Chacon, J.I., Ferrer-Lozano, J., Dyer, R., Buxton, M., Gong, Y., Wu, Y., Ibrahim, N., Andreopoulou, E., Ueno, N.T., Hunt, K., Yang, W., Nazario, A., DeMichele, A., O'Shaughnessy, J., Hortobagyi, G.N. & Symmans, W.F. A genomic predictor of response and survival following taxane-anthracycline chemotherapy for invasive breast cancer. *Jama* **305**, 1873-1881 (2011).
23. Gyorffy, B., Lanczky, A., Eklund, A.C., Denkert, C., Budczies, J., Li, Q. & Szallasi, Z. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. *Breast cancer research and treatment* **123**, 725-731 (2010).
24. Desmedt, C., Piette, F., Loi, S., Wang, Y., Lallemand, F., Haibe-Kains, B., Viale, G., Delorenzi, M., Zhang, Y., d'Assignies, M.S., Bergh, J., Lidereau, R., Ellis, P., Harris, A.L., Klijn, J.G., Foekens, J.A., Cardoso, F., Piccart, M.J., Buyse, M. & Sotiriou, C. Strong time dependence of the 76-gene prognostic signature for node-negative breast cancer patients in the TRANSBIG multicenter independent validation series. *Clinical cancer research : an official journal of the American Association for Cancer Research* **13**, 3207-3214 (2007).
25. Kao, K.J., Chang, K.M., Hsu, H.C. & Huang, A.T. Correlation of microarray-based breast cancer molecular subtypes and clinical outcomes: implications for treatment optimization. *BMC cancer* **11**, 143 (2011).
26. Chakrabarty, A., Rexer, B.N., Wang, S.E., Cook, R.S., Engelman, J.A. & Arteaga,

C.L. H1047R phosphatidylinositol 3-kinase mutant enhances HER2-mediated transformation by heregulin production and activation of HER3. *Oncogene* **29**, 5193-5203 (2010).

27. Weigelt, B. & Bissell, M.J. Unraveling the microenvironmental influences on the normal mammary gland and breast cancer. *Seminars in cancer biology* **18**, 311-321 (2008).
28. Yamada, K.M. & Cukierman, E. Modeling tissue morphogenesis and cancer in 3D. *Cell* **130**, 601-610 (2007).
29. Debnath, J. & Brugge, J.S. Modelling glandular epithelial cancers in three-dimensional cultures. *Nature reviews. Cancer* **5**, 675-688 (2005).
30. Bardelle, C., Coleman, T., Cross, D., Davenport, S., Kettle, J.G., Ko, E.J., Leach, A.G., Mortlock, A., Read, J., Roberts, N.J., Robins, P. & Williams, E.J. Inhibitors of the tyrosine kinase EphB4. Part 2: structure-based discovery and optimisation of 3,5-bis substituted anilinopyrimidines. *Bioorganic & medicinal chemistry letters* **18**, 5717-5721 (2008).
31. Bardelle, C., Cross, D., Davenport, S., Kettle, J.G., Ko, E.J., Leach, A.G., Mortlock, A., Read, J., Roberts, N.J., Robins, P. & Williams, E.J. Inhibitors of the tyrosine kinase EphB4. Part 1: Structure-based design and optimization of a series of 2,4-bis-anilinopyrimidines. *Bioorganic & medicinal chemistry letters* **18**, 2776-2780 (2008).
32. Pylayeva-Gupta, Y., Grabocka, E. & Bar-Sagi, D. RAS oncogenes: weaving a tumorigenic web. *Nature reviews. Cancer* **11**, 761-774 (2011).
33. Downward, J. Targeting RAS signalling pathways in cancer therapy. *Nature reviews. Cancer* **3**, 11-22 (2003).
34. Bian, Y., Li, L., Dong, M., Liu, X., Kaneko, T. & Cheng, K. Ultra-deep tyrosine phosphoproteomics enabled by a phosphotyrosine superbinder. **12**, 959-966 (2016).
35. Dong, M., Bian, Y., Wang, Y., Dong, J., Yao, Y., Deng, Z., Qin, H., Zou, H. & Ye, M. Sensitive, Robust, and Cost-Effective Approach for Tyrosine Phosphoproteome Analysis. **89**, 9307-9314 (2017).
36. Yao, Y., Dong, J., Dong, M., Liu, F., Wang, Y., Mao, J., Ye, M. & Zou, H. An immobilized titanium (IV) ion affinity chromatography adsorbent for solid phase extraction of phosphopeptides for phosphoproteome analysis. *Journal of chromatography. A* **1498**, 22-28 (2017).
37. Araki, T., Nawa, H. & Neel, B.G. Tyrosyl phosphorylation of Shp2 is required for normal ERK activation in response to some, but not all, growth factors. *The Journal of biological chemistry* **278**, 41677-41684 (2003).
38. Lu, W., Gong, D., Bar-Sagi, D. & Cole, P.A. Site-specific incorporation of a phosphotyrosine mimetic reveals a role for tyrosine phosphorylation of SHP-2 in cell signaling. *Molecular cell* **8**, 759-769 (2001).
39. Lu, W., Shen, K. & Cole, P.A. Chemical dissection of the effects of tyrosine phosphorylation of SHP-2. *Biochemistry* **42**, 5461-5468 (2003).
40. Neel, B.G., Gu, H. & Pao, L. The 'Shp'ing news: SH2 domain-containing

tyrosine phosphatases in cell signaling. *Trends in biochemical sciences* **28**, 284-293 (2003).

41. Cunnick, J.M., Dorsey, J.F., Munoz-Antonia, T., Mei, L. & Wu, J. Requirement of SHP2 binding to Grb2-associated binder-1 for mitogen-activated protein kinase activation in response to lysophosphatidic acid and epidermal growth factor. *The Journal of biological chemistry* **275**, 13842-13848 (2000).
42. Cunnick, J.M., Mei, L., Doupnik, C.A. & Wu, J. Phosphotyrosines 627 and 659 of Gab1 constitute a bisphosphoryl tyrosine-based activation motif (BTAM) conferring binding and activation of SHP2. *The Journal of biological chemistry* **276**, 24380-24387 (2001).
43. Furcht, C.M., Buonato, J.M. & Lazzara, M.J. EGFR-activated Src family kinases maintain GAB1-SHP2 complexes distal from EGFR. *Science signaling* **8**, ra46 (2015).
44. Gu, H. & Neel, B.G. The "Gab" in signal transduction. *Trends in cell biology* **13**, 122-130 (2003).
45. Aceto, N., Sausgruber, N., Brinkhaus, H., Gaidatzis, D., Martiny-Baron, G., Mazzarol, G., Confalonieri, S., Quarto, M., Hu, G., Balwierz, P.J., Pachkov, M., Elledge, S.J., van Nimwegen, E., Stadler, M.B. & Bentires-Alj, M. Tyrosine phosphatase SHP2 promotes breast cancer progression and maintains tumor-initiating cells via activation of key transcription factors and a positive feedback signaling loop. *Nature medicine* **18**, 529-537 (2012).
46. Hogarty, M.D., Norris, M.D., Davis, K., Liu, X., Evageliou, N.F., Hayes, C.S., Pawel, B., Guo, R., Zhao, H., Sekyere, E., Keating, J., Thomas, W., Cheng, N.C., Murray, J., Smith, J., Sutton, R., Venn, N., London, W.B., Buxton, A., Gilmour, S.K., Marshall, G.M. & Haber, M. ODC1 is a critical determinant of MYCN oncogenesis and a therapeutic target in neuroblastoma. *Cancer research* **68**, 9735-9745 (2008).
47. Shim, H., Dolde, C., Lewis, B.C., Wu, C.S., Dang, G., Jungmann, R.A., Dalla-Favera, R. & Dang, C.V. c-Myc transactivation of LDH-A: implications for tumor metabolism and growth. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 6658-6663 (1997).
48. Filippakopoulos, P., Qi, J., Picaud, S., Shen, Y., Smith, W.B., Fedorov, O., Morse, E.M., Keates, T., Hickman, T.T., Felletar, I., Philpott, M., Munro, S., McKeown, M.R., Wang, Y., Christie, A.L., West, N., Cameron, M.J., Schwartz, B., Heightman, T.D., La Thangue, N., French, C.A., Wiest, O., Kung, A.L., Knapp, S. & Bradner, J.E. Selective inhibition of BET bromodomains. *Nature* **468**, 1067-1073 (2010).
49. Yeh, E., Cunningham, M., Arnold, H., Chasse, D., Monteith, T., Ivaldi, G., Hahn, W.C., Stukenberg, P.T., Shenolikar, S., Uchida, T., Counter, C.M., Nevins, J.R., Means, A.R. & Sears, R. A signalling pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells. *Nature cell biology* **6**, 308-318 (2004).
50. Cheng, H., Liu, P., Ohlson, C., Xu, E., Symonds, L., Isabella, A., Muller, W.J., Lin,

N.U., Krop, I.E., Roberts, T.M., Winer, E.P., Arteaga, C.L. & Zhao, J.J. PIK3CA(H1047R)- and Her2-initiated mammary tumors escape PI3K dependency by compensatory activation of MEK-ERK signaling. *Oncogene* **35**, 2961-2970 (2016).

51. Jensen, J.D., Knoop, A., Laenkholm, A.V., Grauslund, M., Jensen, M.B., Santoni-Rugiu, E., Andersson, M. & Ewertz, M. PIK3CA mutations, PTEN, and pHER2 expression and impact on outcome in HER2-positive early-stage breast cancer patients treated with adjuvant chemotherapy and trastuzumab. *Annals of oncology : official journal of the European Society for Medical Oncology* **23**, 2034-2042 (2012).
52. Cizkova, M., Dujaric, M.E., Lehmann-Che, J., Scott, V., Tembo, O., Asselain, B., Pierga, J.Y., Marty, M., de Cremoux, P., Spyratos, F. & Bieche, I. Outcome impact of PIK3CA mutations in HER2-positive breast cancer patients treated with trastuzumab. *British journal of cancer* **108**, 1807-1809 (2013).
53. Majewski, I.J., Nuciforo, P., Mittempergher, L., Bosma, A.J., Eidtmann, H., Holmes, E., Sotiriou, C., Fumagalli, D., Jimenez, J., Aura, C., Prudkin, L., Diaz-Delgado, M.C., de la Pena, L., Loi, S., Ellis, C., Schultz, N., de Azambuja, E., Harbeck, N., Piccart-Gebhart, M., Bernards, R. & Baselga, J. PIK3CA mutations are associated with decreased benefit to neoadjuvant human epidermal growth factor receptor 2-targeted therapies in breast cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **33**, 1334-1339 (2015).
54. Baselga, J., Lewis Phillips, G.D., Verma, S., Ro, J., Huober, J., Guardino, A.E., Samant, M.K., Olsen, S., de Haas, S.L. & Pegram, M.D. Relationship between Tumor Biomarkers and Efficacy in EMILIA, a Phase III Study of Trastuzumab Emtansine in HER2-Positive Metastatic Breast Cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **22**, 3755-3763 (2016).
55. Brantley-Sieders, D.M., Zhuang, G., Hicks, D., Fang, W.B., Hwang, Y., Cates, J.M., Coffman, K., Jackson, D., Bruckheimer, E., Muraoka-Cook, R.S. & Chen, J. The receptor tyrosine kinase EphA2 promotes mammary adenocarcinoma tumorigenesis and metastatic progression in mice by amplifying ErbB2 signaling. *The Journal of clinical investigation* **118**, 64-78 (2008).
56. Miao, H., Li, D.Q., Mukherjee, A., Guo, H., Petty, A., Cutter, J., Basilion, J.P., Sedor, J., Wu, J., Danielpour, D., Sloan, A.E., Cohen, M.L. & Wang, B. EphA2 mediates ligand-dependent inhibition and ligand-independent promotion of cell migration and invasion via a reciprocal regulatory loop with Akt. *Cancer cell* **16**, 9-20 (2009).
57. Zhuang, G., Brantley-Sieders, D.M., Vaught, D., Yu, J., Xie, L., Wells, S., Jackson, D., Muraoka-Cook, R., Arteaga, C. & Chen, J. Elevation of receptor tyrosine kinase EphA2 mediates resistance to trastuzumab therapy. *Cancer research* **70**, 299-308 (2010).
58. Miao, B., Ji, Z., Tan, L., Taylor, M., Zhang, J., Choi, H.G., Frederick, D.T., Kumar,

R., Wargo, J.A., Flaherty, K.T., Gray, N.S. & Tsao, H. EPHA2 is a mediator of vemurafenib resistance and a novel therapeutic target in melanoma. *Cancer discovery* **5**, 274-287 (2015).

59. Paraiso, K.H., Das Thakur, M., Fang, B., Koomen, J.M., Fedorenko, I.V., John, J.K., Tsao, H., Flaherty, K.T., Sondak, V.K., Messina, J.L., Pasquale, E.B., Villagra, A., Rao, U.N., Kirkwood, J.M., Meier, F., Sloot, S., Gibney, G.T., Stuart, D., Tawbi, H. & Smalley, K.S. Ligand-independent EPHA2 signaling drives the adoption of a targeted therapy-mediated metastatic melanoma phenotype. *Cancer discovery* **5**, 264-273 (2015).
60. Zhou, X. & Agazie, Y.M. Molecular mechanism for SHP2 in promoting HER2-induced signaling and transformation. *The Journal of biological chemistry* **284**, 12226-12234 (2009).
61. Zhou, X.D. & Agazie, Y.M. Inhibition of SHP2 leads to mesenchymal to epithelial transition in breast cancer cells. *Cell death and differentiation* **15**, 988-996 (2008).

Figure legends

Fig. 1 *EphB4* expression is significantly elevated in HER2-positive breast cancer and high expression levels of EphB4 strongly correlates with poor prognosis. **A** *EphB4* expression levels were significantly higher in HER2-positive breast carcinoma (n = 58) than in normal breast tissues (n = 22) in the TCGA (The Cancer Genome Atlas) breast cancer cohort. The median with interquartile range is indicated by black lines in each group. ** $P < 0.01$, (Student's *t* test). **B** *EphB4* expression levels in five distinct molecular subtypes of breast cancer classified by PAM50 (20-22). The median with interquartile range is indicated by a black line in each group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ by a one-way ANOVA followed by Tukey's multiple comparison tests. **C-D** Kaplan–Meier analysis of overall survival **(C)** and metastasis-free survival **(D)** of breast cancer patients in three independent cohorts. **E** Kaplan–Meier analysis of overall survival and metastasis-free survival in HER2-positive breast cancer patients from the KM-plotter_Breast cohort . All samples were classified into two groups with high and low expression levels of EphB4. Log-rank *p* value and hazard ratio (HR) are shown. 202894_s_at probe set was used for the analysis.

Fig. 2 EphB4 confers resistance to Lapatinib in HER2-positive breast cancer. A Western blot analysis of the proteins as indicated in the shControl or shEphB4-expressing HCC1954 cells treated with or without Lapatinib (1 μM) for 48 hours. Vinculin served as a loading control. **B** Cell viability was measured by the CCK8 assay for cells as in **(A)** treated with or without Lapatinib for 72 hours. **C** Representative images of the 3D culture of cells as in **(A)** grown on Matrigel and treated with or without Lapatinib (0.5 μM). **D** Western blot analysis of proteins as indicated in HCC1954 cells treated with Lapatinib (1 μM), BHG712 (2 μM), or the combination for 48 hours. Note, the level of EphB4 phosphorylation was examined by phosphotyrosine (pTyr) immunoprecipitation followed by western blot analysis for EphB4. **E** Cell viability was measured by the crystal violet assay for cells as in **(D)** for 14 days. Fresh medium was replaced every three days. The bar graphs indicate means ±S.D. of three independent experiments, ** $P < 0.01$ and *** $P < 0.001$ (Student's *t* test). **F** Representative images of the 3D culture of HCC1954 cells grown on Matrigel treated with vehicle, Lapatinib (0.5 μM), BHG712 (1 μM) or the combination. **G** Western blot analysis of the proteins as indicated in the vector or EphB4-overexpressing BT474 cells treated with or without Lapatinib (0.15 μM). Levels of EphB4 phosphorylation was examined as in **(D)**. **H** Cell viability was measured by the CCK8 assay for cells as in **(G)** treated with Lapatinib for 72 hours. **I** Representative images of the 3D culture of cells treated as in **(G)**. For the 3D cell culture experiments, representative images of scored structures (intact, semi-disintegrated, and disintegrated) and quantification for structural integrity are shown. Scale bars, 50 μm.

Fig. 3 Overexpression of EphB4 activates KRAS signaling in HER2-positive breast cancer. A Gene set enrichment analysis of KRAS regulated gene signatures in the shControl-expressing HCC1954 cells versus the shEphB4-expressing cells. **B** Gene set enrichment analysis of KRAS regulated

gene signatures in EphB4-overexpressing BT474 cells versus the control cells (vector-expressing BT474). Normalized enrichment score (NES) and False discovery rate (FDR) *q* value of correlation are shown. **C** Western blot analysis of the proteins as indicated in the shControl or shEphB4-expressing HCC1954 cells. **D** Western blot analysis of the proteins as indicated in the vector or EphB4-overexpressing BT474 and MCF10A/HER2 cells. Vinculin served as a loading control. **E** Representative images of the 3D culture of EphB4-overexpressing BT474 cells grown on Matrigel treated with vehicle, Lapatinib (0.15 μM), MK2206 (2 μM) and MEK162 (2 μM), either alone or in combination as indicated. Representative images of scored structures (intact, semi-disintegrated, and disintegrated) and quantification for structural integrity are shown. Scale bars, 50 μm. **F** Western blot analysis of the proteins as indicated in EphB4-overexpressing BT474 cells treated with vehicle, Lapatinib (0.15 μM), MK2206 (1 μM) and MEK162 (1 μM), either alone or in combination as indicated. Vinculin served as a loading control.

Fig. 4 Overexpression of EphB4 engages the SHP2/GAB1-MEK signaling axis in HER2-positive breast cancer. A Quantitative tyrosine phosphoproteomics analysis identified enhanced phosphorylation in KRAS signaling-related proteins in EphB4 overexpressed BT474 cells. Fold changes (FC) in the abundance of phosphorylation sites in EphB4 versus Vector overexpressed BT474 cells are shown as a function of significance. EphB4 overexpression resulted in 105 up-regulated phosphorylation sites. Significance cutoffs were set by *p*-value = 0.05 (student's *t* test) and FC = 2 or 0.5 (n = 3). **B** Western blot analysis of the proteins as indicated in the vector or EphB4-overexpressing BT474 cells treated with or without Lapatinib (0.15 μM). Vinculin served as a loading control. **C** Representative images of the 3D culture of cells as indicated in the vector or EphB4-overexpressing BT474 cells with co-expression of SHP2 shRNA or GAB1 shRNA grown on Matrigel treated

with vehicle or Lapatinib (0.15 μM). Representative images of scored structures (intact, semi-disintegrated, and disintegrated) and quantification for structural integrity are shown. Scale bars, 50 μm.

Fig. 5 EphB4 engages c-MYC activation in HER2-positive breast cancer. **A** GSEA of MYC-regulated gene signatures in shControl-expressing HCC1954 cells versus shEphB4-expressing cells. NES and FDR q value of correlation are shown. **B** Quantitative RT-PCR analysis of c-MYC mRNA levels in the shControl or shEphB4-expressing HCC1954 cells. *β-actin* served as an internal control. The bar graphs indicate the means ± S.D. of three independent experiments. **C** Western blot analysis of proteins as indicated in cells as in **(B)**. Vinculin served as a loading control. **D** Quantitative RT-PCR analysis of *ODC1* and *LDHA* mRNAs in cells as in **(B)**. The bar graphs indicate the means±S.D. of three independent experiments. **E** Western blot analysis of proteins as indicated in HCC1954 cells treated with vehicle, Lapatinib (1 μM) and JQ1 (2 μM), either alone or in combination as indicated for 48 hours. Vinculin served as a loading control. **F** Representative images of the 3D culture of HCC1954 cells grown on Matrigel treated as in **(E)**. Representative images of scored structures (intact, semi-disintegrated, and disintegrated) and quantification for structural integrity are shown. Scale bars, 50 μm. **G** Quantitative RT-PCR analysis of *c-MYC* mRNA levels in the control or EphB4-overexpressing BT474 cells. The bar graphs indicate the means±S.D. of three independent experiments. **H** Western blot analysis of the proteins as indicated in cells as in **(G)**. **I** Western blot analysis of proteins as indicated in EphB4-overexpressing BT474 cells treated with vehicle, Lapatinib (0.15 μM), JQ1 (2 μM) or the combination. **J** Representative images of the 3D culture of HCC1954 cells grown on Matrigel treated as in **(I)**. Scale bars, 50 μm. **K** Western blot analysis of the proteins as indicated in the inducible shEphB4-expressing HCC1954 cells with or without overexpression of c-MYC

T58A in the presence or absence of Doxycycline (DOX, 100 ng/ml). **L** The cells as in **(K)** were cultured for 14 days. Fresh medium was replaced every three days. Cell viability was measured by the crystal violet assay. The bar graphs indicate means±S.D. of three independent experiments, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ (Student's *t* test).

Fig. 6 EphB4 knockdown improved the anti-tumor effect of Lapatinib. A Mice bearing HCC1954 TetO-shEphB4 orthotopic breast tumors were randomly divided into four groups: vehicle, Doxycycline (DOX), Lapatinib, or the combination. Lapatinib, 100mg/kg/day, QD; DOX, 2 mg/ml in drinking water and was replaced every 3 days. The graph shows the fold change in tumor volume, with respect to the initial treatment at day 0. Data are means ± S.E.M, n = 11/group. ** $P < 0.01$ and *** $P < 0.001$ by a one-way ANOVA followed by Tukey's multiple comparison tests. **B** The xenograft tumor-bearing mice were treated as in **(A)** for 3 days and sacrificed 3.5 hours after the last dose. Tumor lysates were collected and subjected to western blot analysis of the proteins as indicated. Vinculin served as a loading control. **C** Schematics illustrating the identified signaling cascade triggered by EphB4 overexpression and that inhibition of EphB4 or its downstream effectors (e.g. MEK inhibition by MEK162 or MYC inhibition by JQ-1) sensitizes EphB4-overexpressing HER2-positive breast cancer cells to Lapatinib.

Figure 1

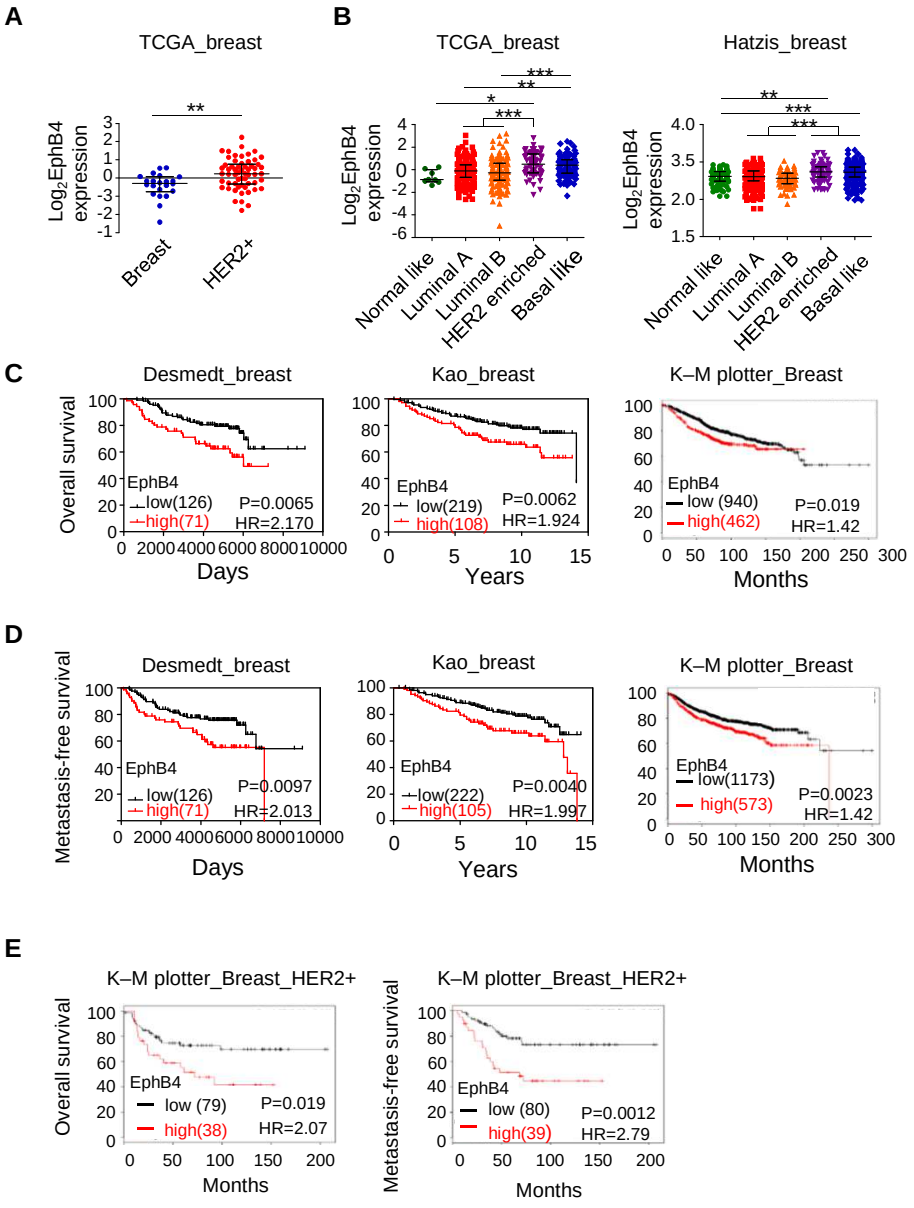

A
TCGA_breast
Log2EphB4 expression
Breast
HER2+
B
TCGA_breast
Hatzis_breast
Normal like
Luminal A
Luminal B
HER2 enriched
Basal like
C
Desmedt_breast
Kao_breast
K–M plotter_Breast
Overall survival
EphB4
low(126)
high(71)
P=0.0065
HR=2.170
Days
low(219)
high(108)
P=0.0062
HR=1.924
Years
low (940)
high(462)
P=0.019
HR=1.42
Months
D
Metastasis-free survival
low(126)
high(71)
P=0.0097
HR=2.013
low(222)
high(105)
P=0.0040
HR=1.997
low(1173)
high(573)
P=0.0023
HR=1.42
E
K–M plotter_Breast_HER2+
low (79)
high(38)
P=0.019
HR=2.07
low (80)
high(39)
P=0.0012
HR=2.79

Figure 2

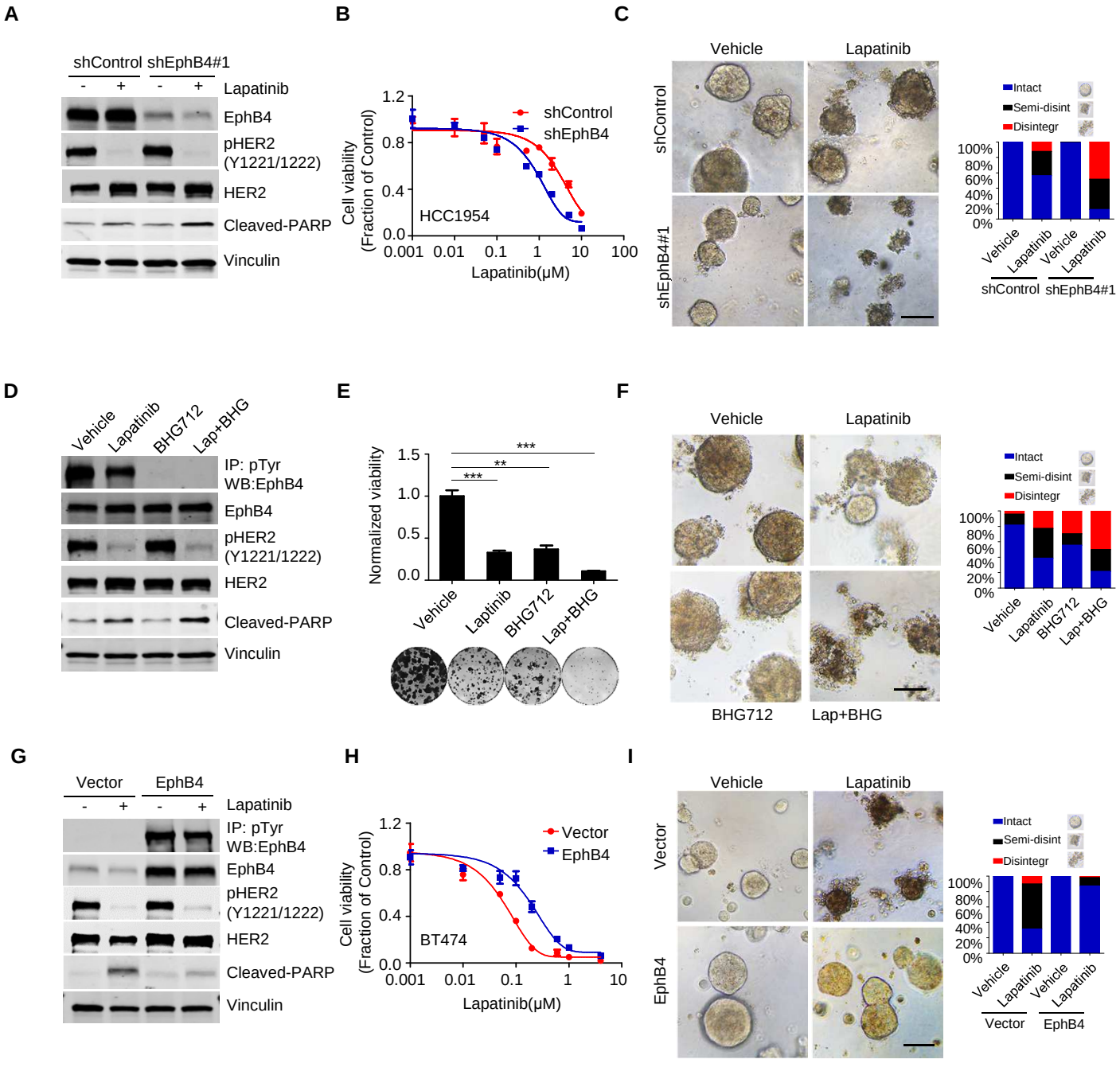

A
shControl
shEphB4#1
-
+
-
+
Lapatinib
EphB4
pHER2
(Y1221/1222)
HER2
Cleaved-PARP
Vinculin
B
Cell viability
(Fraction of Control)
1.2
0.8
0.4
0.0
0.001
0.01
0.1
1
10
100
Lapatinib(μM)
shControl
shEphB4
HCC1954
C
Vehicle
Lapatinib
shControl
shEphB4#1
Intact
Semi-disint
Disintegr
100%
80%
60%
40%
20%
0%
Vehicle
Lapatinib
Vehicle
Lapatinib
shControl
shEphB4#1
D
Vehicle
Lapatinib
BHG712
Lap+BHG
IP: pTyr
WB:EphB4
EphB4
pHER2
(Y1221/1222)
HER2
Cleaved-PARP
Vinculin
E
Normalized viability
1.5
1.0
0.5
0.0
***
**
***
Vehicle
Laptinib
BHG712
Lap+BHG
F
Vehicle
Lapatinib
BHG712
Lap+BHG
Intact
Semi-disint
Disintegr
100%
80%
60%
40%
20%
0%
Vehicle
Lapatinib
BHG712
Lap+BHG
G
Vector
EphB4
-
+
-
+
Lapatinib
IP: pTyr
WB:EphB4
EphB4
pHER2
(Y1221/1222)
HER2
Cleaved-PARP
Vinculin
H
Cell viability
(Fraction of Control)
1.2
0.8
0.4
0.0
0.001
0.01
0.1
1
10
Lapatinib(μM)
Vector
EphB4
BT474
I
Vehicle
Lapatinib
Vector
EphB4
Intact
Semi-disint
Disintegr
100%
80%
60%
40%
20%
0%
Vehicle
Lapatinib
Vehicle
Lapatinib
Vector
EphB4

Figure 3

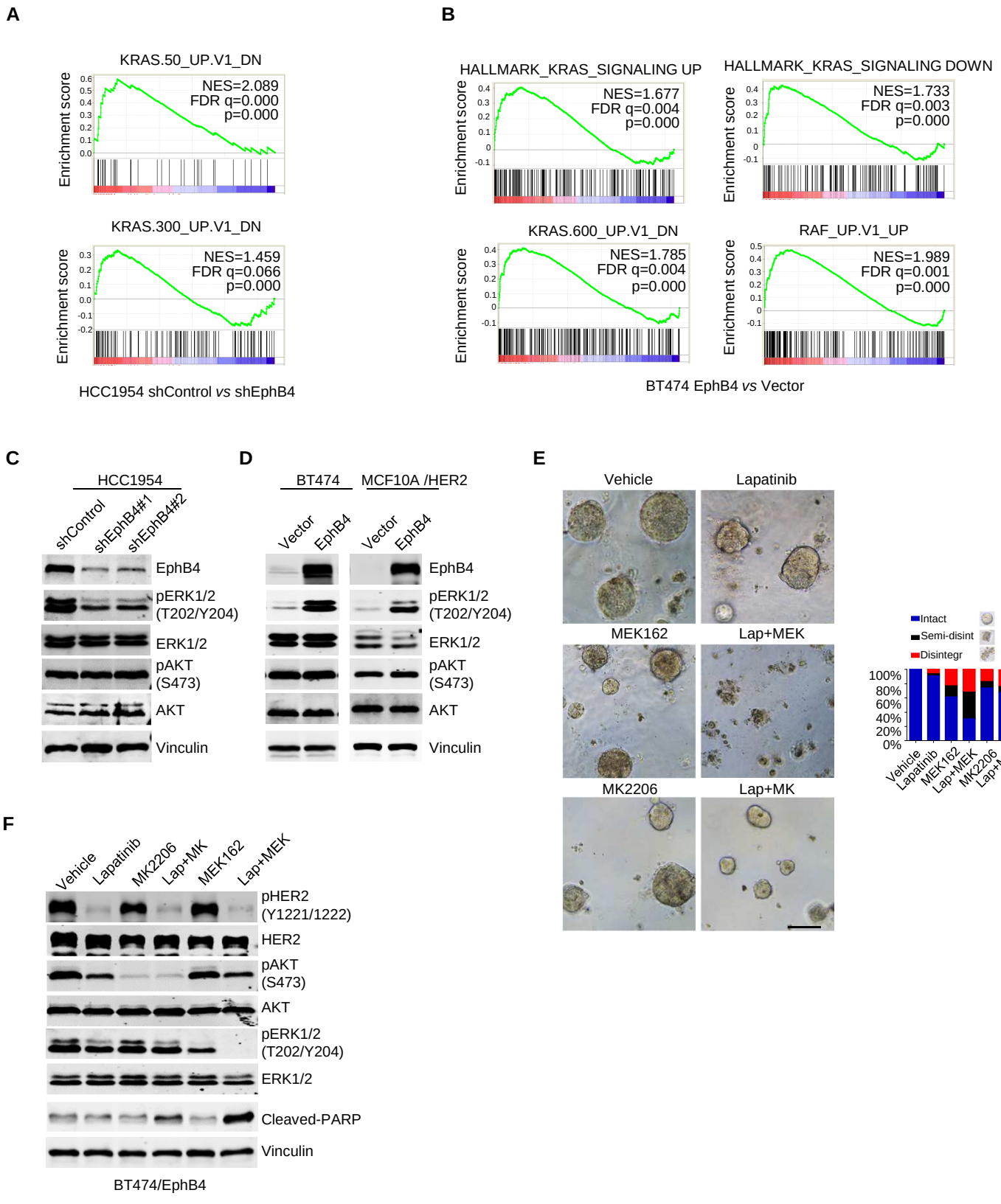

A
KRAS.50_UP.V1_DN
NES=2.089
FDR q=0.000
p=0.000
KRAS.300_UP.V1_DN
NES=1.459
FDR q=0.066
p=0.000
Enrichment score
HCC1954 shControl vs shEphB4
B
HALLMARK_KRAS_SIGNALING UP
NES=1.677
FDR q=0.004
p=0.000
HALLMARK_KRAS_SIGNALING DOWN
NES=1.733
FDR q=0.003
p=0.000
KRAS.600_UP.V1_DN
NES=1.785
FDR q=0.004
p=0.000
RAF_UP.V1_UP
NES=1.989
FDR q=0.001
p=0.000
BT474 EphB4 vs Vector
C
HCC1954
shControl
shEphB4#1
shEphB4#2
EphB4
pERK1/2 (T202/Y204)
ERK1/2
pAKT (S473)
AKT
Vinculin
D
BT474
MCF10A /HER2
Vector
EphB4
E
Vehicle
Lapatinib
MEK162
Lap+MEK
MK2206
Lap+MK
Intact
Semi-disint
Disintegr
100%
80%
60%
40%
20%
0%
F
Vehicle
Lapatinib
MK2206
Lap+MK
MEK162
Lap+MEK
pHER2 (Y1221/1222)
HER2
pAKT (S473)
AKT
pERK1/2 (T202/Y204)
ERK1/2
Cleaved-PARP
Vinculin
BT474/EphB4

Figure 4

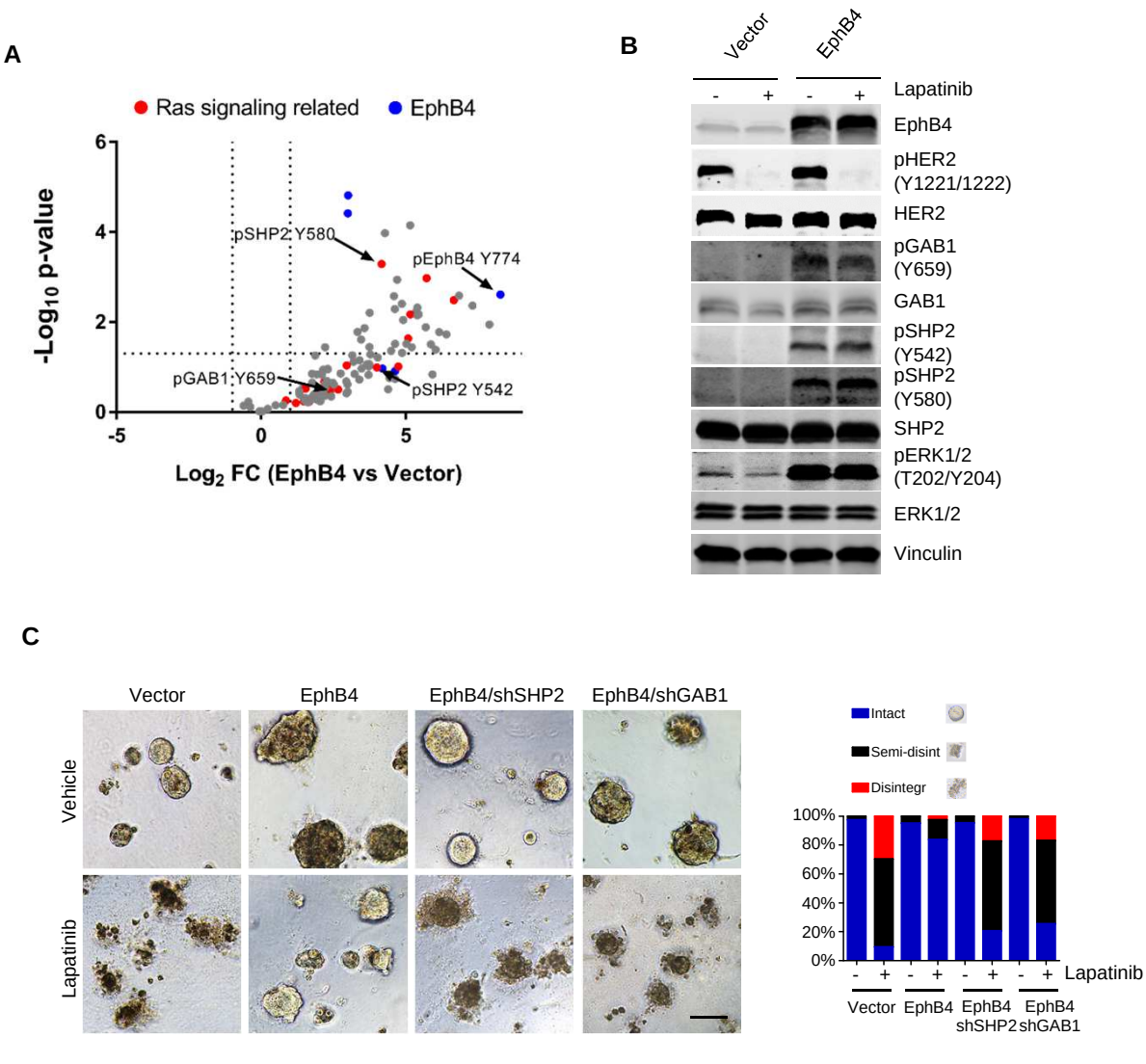

A
Ras signaling related
EphB4
-Log10 p-value
Log2 FC (EphB4 vs Vector)
pSHP2 Y580
pEphB4 Y774
pGAB1 Y659
pSHP2 Y542
B
Vector
EphB4
Lapatinib
EphB4
pHER2 (Y1221/1222)
HER2
pGAB1 (Y659)
GAB1
pSHP2 (Y542)
pSHP2 (Y580)
SHP2
pERK1/2 (T202/Y204)
ERK1/2
Vinculin
C
Vector
EphB4
EphB4/shSHP2
EphB4/shGAB1
Vehicle
Lapatinib
Intact
Semi-disint
Disintegr
Lapatinib
Vector
EphB4
EphB4 shSHP2
EphB4 shGAB1

Figure 5

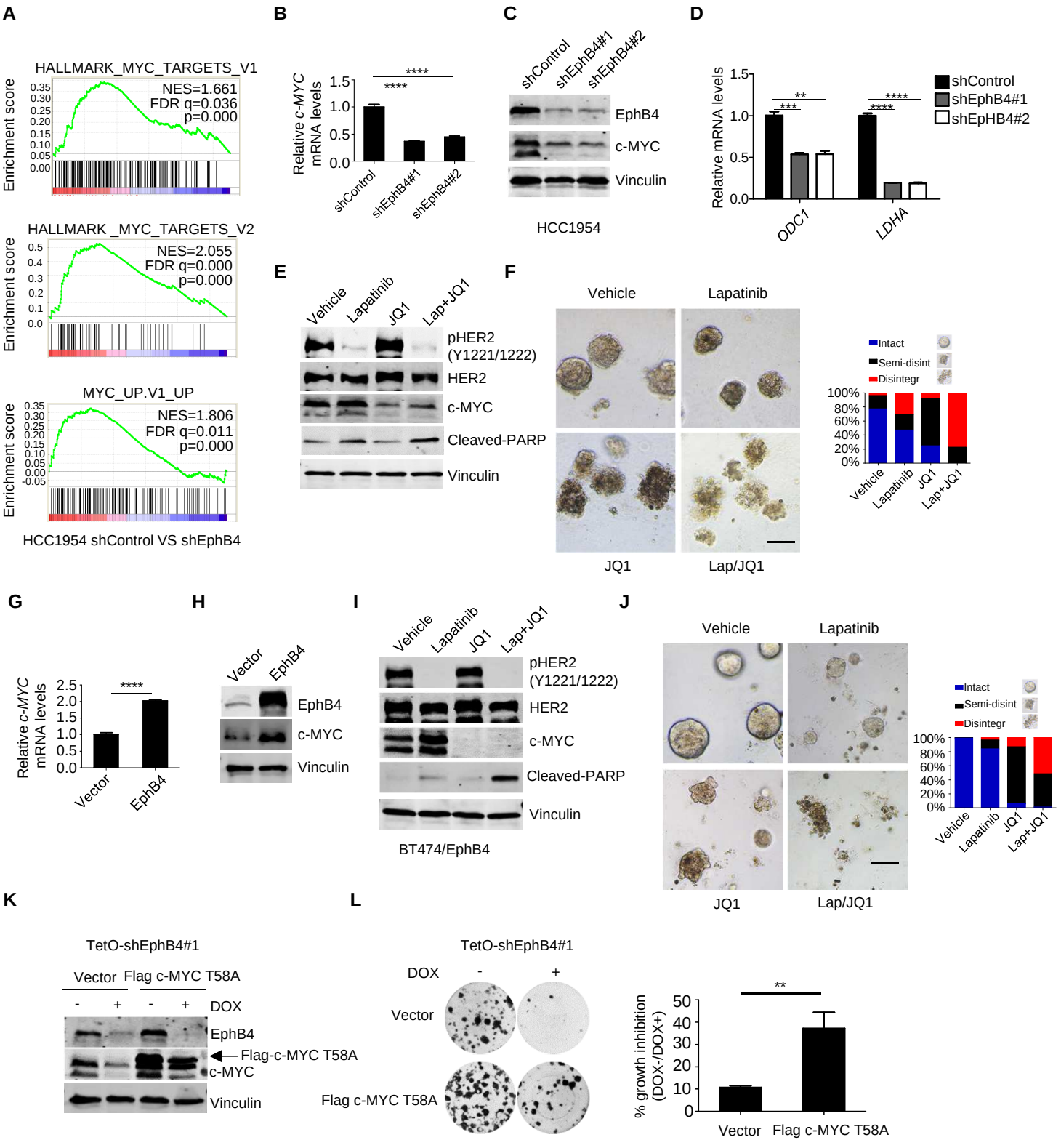
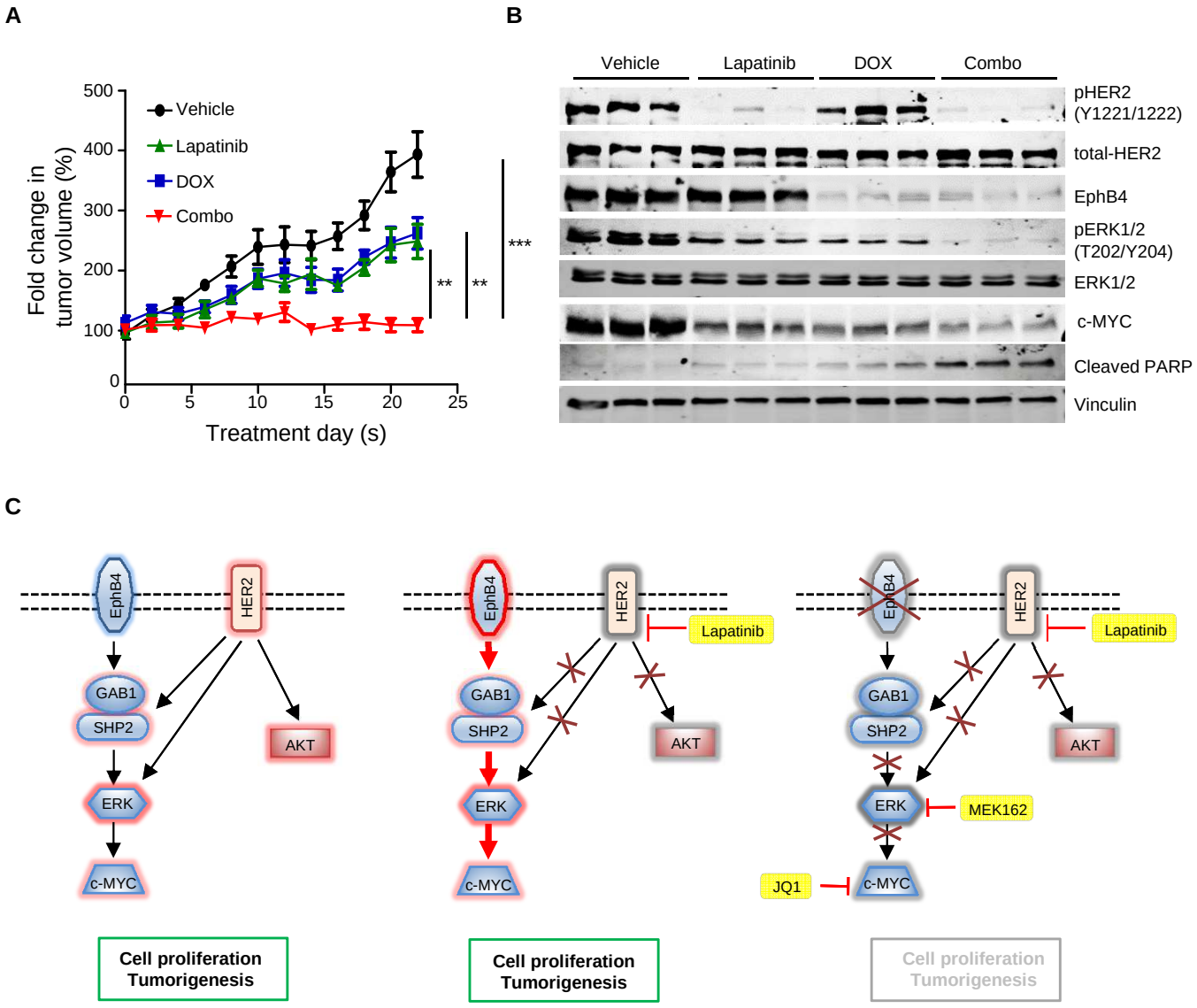

A
HALLMARK_MYC_TARGETS_V1
Enrichment score
NES=1.661
FDR q=0.036
p=0.000
HALLMARK _MYC_TARGETS_V2
NES=2.055
FDR q=0.000
p=0.000
MYC_UP.V1_UP
NES=1.806
FDR q=0.011
p=0.000
HCC1954 shControl VS shEphB4
B
Relative c-MYC mRNA levels
shControl
shEphB4#1
shEphB4#2
C
EphB4
c-MYC
Vinculin
HCC1954
D
Relative mRNA levels
ODC1
LDHA
shControl
shEphB4#1
shEphHB4#2
E
Vehicle
Lapatinib
JQ1
Lap+JQ1
pHER2 (Y1221/1222)
HER2
c-MYC
Cleaved-PARP
Vinculin
F
Vehicle
Lapatinib
JQ1
Lap/JQ1
Intact
Semi-disint
Disintegr
G
Relative c-MYC mRNA levels
Vector
EphB4
H
I
BT474/EphB4
J
K
TetO-shEphB4#1
Vector
Flag c-MYC T58A
DOX
EphB4
Flag-c-MYC T58A
c-MYC
Vinculin
L
TetO-shEphB4#1
DOX
Vector
Flag c-MYC T58A
% growth inhibition (DOX-/DOX+)

Figure 6


A
B
C
Vehicle
Lapatinib
DOX
Combo
Fold change in tumor volume (%)
Treatment day (s)
**
**
***
pHER2 (Y1221/1222)
total-HER2
EphB4
pERK1/2 (T202/Y204)
ERK1/2
c-MYC
Cleaved PARP
Vinculin
EphB4
HER2
GAB1
SHP2
AKT
ERK
c-MYC
Lapatinib
MEK162
JQ1
Cell proliferation Tumorigenesis
Cell proliferation Tumorigenesis
Cell proliferation Tumorigenesis

Highlights

- High levels of EphB4 correlate with poor prognosis in HER2-positive breast cancer
- EphB4 activation confers resistance to Lapatinib
- EphB4 activation engages the SHP2/GAB1/MEK/MYC signaling axis
- Inhibition of EphB4 sensitizes HER2-positive breast tumors to Lapatinib

Conflict of interest

The authors declare that they have no conflict of interest.