Targeting ERBB Receptors Shifts Their Partners and Triggers Persistent ERK Signaling through a Novel ERBB/EFNB1 Complex

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Abstract

Most squamous cell carcinomas of the head and neck (HNSCC) overexpress ERBB1/EGFR, but EGF receptor (EGFR)-targeted therapies have yielded disappointing clinical results in treatment of this cancer. Here, we describe a novel interaction between EGFR and the ligand EphrinB1 (EFNB1), and we show that EFNB1 phosphorylation and downstream signaling persists in the presence of cetuximab. Mechanistically, cetuximab drives a shift in EGFR dimerization partners within the signaling complex, suggesting that targeted drugs may trigger partner rearrangements that allow persistent pathway activation. EFNB1 attenuation slowed tumor growth and increased survival in a murine model of HNSCC, suggesting a substantial contribution of EFNB1 signaling to HNSCC development. Together, our findings suggest that EFNB1 is part of the EGFR signaling complex and may mediate drug resistance in HNSCC as well as other solid tumors. Cancer Res; 73(18); 1–11. ©2013 AACR.

Introduction

A greater understanding of the molecular pathways driving tumor initiation and proliferation has heralded an age of targeted therapies for cancer. The therapeutic successes of these drugs are satisfying from both the scientific and medical perspectives. Their failures indicate an incomplete understanding of macromolecular complexes, the oncogenic pathways they drive, their cross-talk, and drug-driven compensatory mechanisms. We have recently identified a novel component of one such macromolecular complex in breast cancer (1). Here, we further characterize its role in the context of squamous cell carcinoma of the head and neck (HNSCC).

In human papillomavirus (HPV)-associated HNSCC, the high-risk HPV type 16 (HPV16) E6 oncoprotein targets the cellular phosphatase, PTPN13, for degradation (2, 3). PTPN13 functions as a tumor suppressor (4–9), yet the molecular mechanisms it modulates and how they become altered in cancer remain unclear. One important PTPN13 phosphatase substrate that may influence cancer initiation and/or progression is the signaling ligand EphrinB1 (EFNB1). EFNB1 belongs to a ligand family that binds and activates Eph receptor tyrosine kinases. Unlike most ligands, Ephrin ligands initiate their own downstream signaling following receptor engagement, a process called "reverse signaling" (10). PTPN13 transiently interacts with phosphorylated EFNB1, shutting "reverse signaling" off (11). Thus, in tumors with impaired PTPN13 expression or function, EFNB1 signaling may persist. In addition, Ephrin ligands are promiscuous in their associations (12, 13); for example, EFNB1 interacts with ERBB2 (14). Moreover, EFNB1 activation correlates with phosphorylation of extracellular signal-regulated kinase (ERK)1/2 (14). Together, these studies suggest that the complex consisting of ERBB2/EFNB1 together with PTPN13 regulates intracellular signals critical for epithelial tumorigenesis.

ERBB2 belongs to the ERBB family of receptor tyrosine kinases, which includes ERBB1/EGFR/HER1. More than 90% of HNSCC cases show upregulation of ERBB1 (15, 16), which may impact tumor growth and patient outcomes. ERBB1-targeted therapies such as cetuximab (Erbitux), a chimeric anti-ERBB1 monoclonal antibody, and erlotinib (Tarceva), an ERBB1 kinase inhibitor, are used as therapies in patients with HNSCC although with modest clinical outcomes (17). These clinical findings emphasize the need to better define components of macromolecular complexes active in disease, understand the molecular pathways they drive, and characterize how they influence tumor initiation and progression. In the case of HNSCC, these types of studies may help define what role, if any, ERBB1 upregulation plays in disease initiation and/or progression.

Our previous finding that EFNB1 associates with ERBB2 prompted us to ask whether it associates with other ERBB family members. Here, we report that, like ERBB2, ERBB1 associates with EFNB1. Moreover, in the absence of PTPN13 function, EFNB1 phosphorylation is enhanced and ERK1/2 signaling is potentiated. These data suggest that EFNB1 exists in a complex together with ERBB1, ERBB2, or both. A combination of these associations likely exists that together regulate...
complex intracellular signals potentiated in the absence of PTPN13 function. Importantly, we show that antibody therapies such as cetuximab (Erbitux, anti-ERBB1) and trastuzumab (Herceptin, anti-ERBB2) potently block receptor activation, they do not attenuate EFN1 activation or ERK1/2 phosphorylation. Moreover, we show that these ERBB-targeted drugs promote the shifting of partners within ERBB/EFNB1 complexes and suggest that this mechanism supports persistent EFN1 signaling despite potent ERBB receptor blockade. Thus, we propose that EFN1 activation mediates signal transduction and drug resistance. In addition, we show that knockdown of EFN1 significantly slows tumor growth and improves survival in a murine model of HNSCC. Together, our findings support a tumor-suppressive role for PTPN13, suggest that EFN1 may be a useful therapeutic target in HNSCC and other solid tumors, and explain at least one mechanism by which HNSCCs fail cetuximab therapy.

Materials and Methods

Cell culture
HEK293, SCC1, and SCC47 cells were maintained with Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal calf serum and 1% penicillin/streptomycin. Primary murine tonsil epithelia (1° MTE) and human tonsil epithelia (1° HTE) were maintained with Keratinocyte Serum-Free Medium. 1MTE stably expressing HPV16 E6 and E7 together with RAS and luciferase (MEERL cells) and 1 HTE stably expressing HPV16 E6 and E7 alone (HEE cells) were maintained with E-medium (DMEM/Hams F12, 10% fetal calf serum, 1% penicillin/streptomycin, 0.5 μg/mL hydrocortisone, 8.4 ng/mL cholera toxin, 5 μg/mL transferrin, 5 μg/mL insulin, 1.36 ng/mL tri-iodo-thyonine, and 5 ng/mL EGF). HEE cells were generated from 1° MTE cells harvested from tonsillectomies conducted for noncancerous reasons and collected under Institutional Review Board approval with written consent at the University of Iowa (Iowa City, IA; ref. 3). The stable HEE cells were generated as described earlier. Twenty-four hours posttransfection, cells were slides and treated as described followed by processing for PLA analysis by immunoprecipitation and Western blot analysis. For analysis by PLA, cells were seeded on 16-well chamber slides and treated as described followed by processing for PLA (as described earlier).

Plasmids

EFNB1-mutant constructs and PTPN135–28 have been previously described (6, 14). Wild-type (wt) human ERBB1 cDNA was obtained from Addgene (#11011) and cloned by PCR into pCMV-HA (Clontech). ERBB1 transmembrane mutants were generated from the hemagglutinin (HA)-tagged wt ERBB1 construct using the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies).

Transfection
HEK293 cells were transfected using PolyFect Transfection Reagent as per the manufacturer’s instructions (Qiagen).

Immunoprecipitation and Western blot analysis
Cells were lysed in lysis buffer (50 mmol/L NaF, 10 mmol/L NaPPi, 10% glycerol, and 1% Triton X-100) and soluble proteins assayed by BCA protein assay (Pierce). Equal total protein was used for immunoprecipitation and immunoblot analysis by standard methodology. Antibodies for immunoprecipitation were as follows: anti-ERBB1 (Becton Dickinson), anti-ERBB2 (Dako), and anti-EFN1 (Santa Cruz Biotechnology); antibodies used for immunoblot were as follows: anti-ERBB1 (Upstate), anti-ERBB2 (Invitrogen), anti-phospho-tyrosine (Upstate), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Ambion), anti-EFN1 (Santa Cruz Biotechnology), anti-EFN1 (Sigma), anti-phospho-ERK1/2 (Cell Signaling Technology), anti-ERK1/2 (Cellbiochem), anti-phospho-Tyrosine137 EFN1 (Santa Cruz Biotechnology), anti-HA (Sigma), and anti-FLAG (Sigma).

Immunofluorescence and proximity ligation assay
Antibodies for immunofluorescence were as follows: anti-ERBB1 (Becton Dickinson), anti-ERBB2 (Dako), anti-HA (Sigma), anti-EFN1 (Santa Cruz Biotechnology), and anti-EFN1 (Sigma). Surface EphrinB ligands were bound by EphB1-Fc (R&D System) on unfixed, unpermeabilized cells and detected with Millipore anti-human immunoglobulin G–fluorescein isothiocyanate (IgG-FITC). Antibodies used for proximity ligation assay (PLA) were as follows: anti-ERBB1 (Becton Dickinson), anti-ERBB2 (Dako), anti-ERBB2 (Invitrogen), and anti-EFN1 (Santa Cruz Biotechnology). Standard immunofluorescence and PLA protocols were followed.

Quantification of PLA

PLA-positive signals (visualized as fluorescent red dots) were analyzed by confocal microscopy (Olympus Fluoview1000, ×60 oil objective, ×2.5 magnified; Alexa Fluor 568 detector). At least three stacked Z series for each condition were analyzed, confocal IOB files converted to tiff format, and then analyzed by DuoLink Imagentool software (Olink Biosciences). The number of PLA-positive signals from at least three different Z-stacked images per condition was averaged. Experiments were repeated at least three times with similar results. Student t test was used to analyze the data and test for significance.

Targeted therapy treatment

Cells were grown on 60-mm tissue culture dishes to 80% confluence and treated with recombinant EGF (SAFC Biosciences) in the presence or absence of cetuximab (20 μg/mL; Imclone), trastuzumab (10 μg/mL; Dako), or their combination for 1 hour at 37°C. Control cells received either no treatment or EGF alone. Cells were harvested as described earlier and analyzed by immunoprecipitation and Western blot analysis. For analysis by PLA, cells were seeded on 16-well chamber slides and treated as described followed by processing for PLA (as described earlier).

Generating stable cell lines

Cells were seeded onto 6-well tissue culture dishes and transfected using PolyFect Transfection Reagent as described earlier. Twenty-four hours posttransfection, cells were placed under antibiotic selection with Zeocin (250, 500, or

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Vermeer et al.

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1,000 µg/mL; Invitrogen), clones were picked and transferred to 48-well dishes, maintained under selection, and passaged up to 100-mm dishes, at which time one dish was lysed for biochemical analysis and another frozen for later use.

In vivo studies

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee. Cells were trypsinized and harvested from tissue culture plates and resuspended. An 18-gauge needle with 100,000 cells was injected subcutaneously into the right hind limb of each C57Bl/6 mouse (N = 5/group). C57Bl/6 male mice were 4 to 6 weeks of age, weighed at least 20 to 25 g, and were purchased from The Jackson Laboratory. Injected cells included: parental cells, nonsilencing, wt mEfraB1, or short hairpin (sh) EfraB1. Weekly caliper measurements were taken and tumor volume was calculated as follows: (width)² × (depth). Mice were humanely euthanized when tumors reached 2 cm in the greatest dimension or the animal became emaciated or had functional impairment of the leg.

Results

**EFNB1 associates with ERBB1 and ERBB2**

To test whether ERBB1 and EFNBI interact, two HNSCC cell lines were processed for immunofluorescence: the SCC1 cell line is HPV negative (HPV⁻), whereas the SCC47 cell line is HPV-positive (HPV⁺). Surface, rather than total, EphrinB was localized because cells robustly express intracellular EphrinB ligands making the distinction of surface expression difficult. EphB1-Fc binding followed by IgG-FITC localizes surface EphrinB ligands. Both cell lines express ERBB1 and EphrinB, which colocalize at cell–cell borders (Fig. 1A and B).

To verify that the colocalization by immunofluorescence involved ERBB1 and EFNBI specifically, PLA and coimmunoprecipitation studies were conducted. Figure 1C shows positive PLA signals for EFNBI and ERBB1 in SCC1 (HPV⁻) and SCC47 (HPV⁺) cells. The previously discovered ERBB2/EFNB1 association (14) was confirmed and the presence of ERBB1/ERBB2 heterodimers was determined (Fig. 1C). In addition, stained Z-stacked images in the AZ plane indicate that these interactions are not restricted to the cell surface but also occur within the cytoplasm (Fig. 1D). These intracellular PLA signals likely represent internalized complexes. Importantly, PLA quantification shows that infection with HPV16 correlates with enhanced ERBB1/EFNB1 interactions (Fig. 1E). EFNBI, ERBB1, and ERBB2 protein levels in SCC1 (HPV⁻) and SCC47 (HPV⁺) cells are very similar (Fig. 3), thus increased protein expression cannot account for the enhanced interactions evident in SCC47 (HPV⁺) cells.

**EFNB1 and ERBB1 association is likely mediated via their transmembrane domains**

Because of the overwhelming expression of ERBB1 in HNSCCs, we focused further analysis on ERBB1 and EFNBI. To define the domains necessary and sufficient for ERBB1/EFNB1 association, we generated deletion mutants (Fig. 2A). All ERBB1 mutants, including the full-length wt, were HA-tagged; constructs express well, run at the predicted molecular weight, and possess the HA tag (Fig. 2B). To test whether any of the ERBB1 deletions compromise its interaction with EFNBI, HEK293 cells were transfected, lysates immunoprecipitated for EFNBI and immunoblotted for HA. Surprisingly, no loss of coimmunoprecipitation with any ERBB1 mutants occurred, suggesting that the extracellular ligand-binding domains of ERBB1 are not required for its association with EFNBI (Fig. 2B). These data are consistent with our previous finding for the ERBB2/EFNB1 interaction (14). EFNBI deletion mutants and wt constructs have been previously described and were all FLAG-tagged (14). Briefly, either the entire extracellular domain (EFNB1-ED) or just the intracellular PDZ-binding motif (EFNB1-PDZ) of EFNBI was deleted. HEK293 cells were transfected with the EFNBI constructs. Because HEK293 cells express nearly undetectable endogenous ERBB1, cells were also transfected with untagged wt ERBB1. Immunoprecipitation for ERBB1 followed by Western blot analysis for FLAG shows no loss of coimmunoprecipitation with any of the EFNBI mutants, suggesting that neither the extracellular domain nor the PDZ motif is required for its association with ERBB1 (Fig. 2C).

ERBB receptor transmembrane domains mediate homo- and hetero-dimerization (19, 20). In fact, ERBB transmembrane domains contain two motifs: one mediating heterodimerization, the other homodimerization (21). To test whether ERBB1’s transmembrane domain mediates its interaction with EFNBI, two additional ERBB1 mutants were generated: one within the first transmembrane motif and another within the second transmembrane motif. These constructs were HA-tagged and transfected into HEK293 cells. Control cells were transfected with wt HA-tagged ERBB1 or enhanced GFP (eGFP). Lysates were immunoprecipitated for EFNBI and analyzed by Western blot analysis for HA. Although the first ERBB1 transmembrane motif mutant, T648I G652I, retains coimmunoprecipitation with EFNBI, mutation within the second transmembrane motif (A661I G665I) loses it (Fig. 2D). These data were verified by immunofluorescence. HEK293 cells were transfected as described and cells processed for immunofluorescence using an anti-HA antibody for ERBB1 detection and an anti-EFNBI antibody for EFNBI detection. Expression of the ERBB1 T648I G652I mutant (green) colocalizes with endogenous EFNBI (red), evident in the merged panel (yellow). Expression of the ERBB1 A661I G665I mutant (green) does not colocalize with EFNBI (red; Fig. 2E). Together, these data suggest that ERBB1’s second transmembrane motif mediates the EFNBI association.

Expression of HPV oncogenes correlates with EFNBI and ERK1/2 phosphorylation in HNSCC. We and others have shown that PTPN13 regulates EFNBI phosphorylation. Moreover, the loss of PTPN13 expression, as seen in basal-like breast cancer, correlates with enhanced EFNBI and ERK1/2 phosphorylation (14, 22). Importantly, breast tumors compromised for PTPN13 expression are associated with decreased overall survival (23). A similar dysregulation in signaling occurs in HNSCC, both in a human cell line (SCC84) and a murine model of head and neck cancer (14). Thus, we wondered whether EFNBI’s phosphorylation state modulates its interactions with ERBB receptors. In addition, given that tonsil epithelial cells
are the major site for HPV⁺ HNSCC, we tested whether ERBB/EFNB1 interactions exist in these cells. Thus, 1°C14HTE and HTEs stably expressing HEE were tested. Consistent with our previous studies, ERBB1 and ERBB2 associate with EFNB1 in both cell types (Fig. 3A). In addition, expression of E6 correlates with enhanced phosphorylation of EFNB1 and ERK1/2 consistent with previous reports (2, 3). These data show that ERBB1 and ERBB2 associate with EFNB1 in primary human tonsil cells as well as those expressing HPV oncogenes. Moreover EFNB1 associates with its ERBB partners regardless of its phosphorylation status. Importantly, expression of HPV16 oncogenes (HEE cells) enhances EFNB1 activation and subsequent ERK1/2 phosphorylation. These data are consistent with our previous findings that EFNB1 signals down the mitogen-activated protein kinase (MAPK) pathway (14). Similar results were evident in 1°CTE cells and a previously characterized mouse model of HPV-related HNSCC (MTEs expressing MEERL cells; Supplementary Fig. S1; refs. 18, 24, 25).

PTPN13 regulates EFNB1 phosphorylation and reverse signaling
HPV16 E6 mediates the degradation of PTPN13 (2, 3). The data suggest that enhanced EFNB1 activation and downstream signaling are a consequence of this degradation (Fig. 3A). To directly test this, we conducted a series of transfections in HEK293 cells, which express nearly undetectable ERBB receptors allowing us to control expression of the proteins involved. Briefly, HEK293 cells were transfected with wt ERBB1,
wt EFNB1, and PTPN13 (either wt or a phosphatase null, PTPN13(C/S)) and lysates analyzed by Western blot analysis. In eGFP-transfected controls, endogenous EFNB1 is phosphorylated (Fig. 3B). The multiple bands likely represent different phosphorylated forms of EFNB1 as suggested by Xu and colleagues (26). In addition, a low level of EFNB1 coimmunoprecipitates with ERBB1 yet ERK1/2 is not phosphorylated. Transfection with wt ERBB1, wt EFNB1, and wt PTPN13 decreases EFNB1 phosphorylation without altering its association with ERBB1; ERK1/2 remains unphosphorylated. Interestingly, transfection with wt ERBB1, wt EFNB1, and PTPN13(C/S), a phosphatase null PTPN13-mutant, increases EFNB1 phosphorylation as well as its association with ERBB1 and initiates ERK1/2 phosphorylation. Together, these data are consistent with those of others showing that PTPN13 regulates EFNB1 phosphorylation. In addition, the data suggest that phosphorylated EFNB1 associates more readily with ERBB1 and that the loss of PTPN13 function potentiates not only EFNB1 phosphorylation but also ERK1/2 signaling.

ERBB1 and ERBB2 antibody blockade do not attenuate downstream signaling

ERBB1- and ERBB2-targeted therapies are in clinical use for treatment of a variety of solid cancers including HNSCC. Our data suggest that PTPN13 modulates EFNB1-mediated signaling and that EFNB1 associates with ERBB1 and ERBB2. To test whether ERBB1- and/or ERBB2-targeted therapies alter EFNB1 activation and downstream signaling, we analyzed SCC1 (HPV⁻/C0⁻) and SCC47 (HPV⁺) cells. Cells were treated with EGF to stimulate ERBB receptor activation and then segregated into the following groups: (i) untreated (EGF alone, control), (ii) treated with cetuximab, (iii) treated with trastuzumab, or (iv) treated with both cetuximab and trastuzumab. Unstimulated (no EGF) and untreated cells (EGF alone) served as control. Figure 4 shows Western blot analysis of these groups. Both SCC1 (HPV⁻) and SCC47 (HPV⁺) cells show nearly undetectable endogenous ERBB1 or ERBB2 phosphorylation (unstimulated). In addition, and consistent with clinical data, cells abundantly express ERBB1 and modestly express ERBB2.
(15, 16, 27–31). EGF stimulation activates ERBB1 and ERBB2, suggesting that at least a fraction of ERBB1 heterodimerizes with ERBB2. Treatment with cetuximab alone potently attenuates EGF-mediated ERBB1 and ERBB2 phosphorylation, suggesting that ERBB2 phosphorylation following EGF stimulation occurs via heterodimerization with ERBB1. As expected, trastuzumab has no effect on ERBB1 phosphorylation but attenuates ERBB2 activation. Treatment with a combination of cetuximab and trastuzumab is not additive. In other words, treatment with drugs in combination does not enhance the effect of each drug when given in isolation. Importantly, despite the ability of cetuximab and trastuzumab to attenuate receptor activation, neither showed an effect on ERK phosphorylation; that is, ERK1/2 remains phosphorylated despite the presence of drug. These data suggest that persistent signaling via EFNB1 drives ERK1/2 phosphorylation despite blockade of ERBB1 and ERBB2.

**Treatment with cetuximab or trastuzumab (together or in combination) drives the shifting of partners within ERBB/EFNB1 complexes**

Given that EFNB1 associates with ERBB1 and ERBB2, we wondered whether treatment with targeted drugs mediates changes in partner associations within these complexes that might account for sustained signaling in the presence of drug. To test this, the experiments carried out in Fig. 4 were duplicated on 16-well chamber slides, cells were processed for PLA, and quantified. In SCC1 (HPV−) cells, stimulation with EGF alone (control; light gray) results in modest association of ERBB1 with EFNB1 (Fig. 5A). ERBB2 interactions with EFNB1 are more abundant in comparison, suggesting that the preferred partner for EFNB1 is ERBB2. In addition, ERBB1 and ERBB2 heterodimers are in the minority. Treatment of cells with EGF + cetuximab (black bars) leads to a significant increase in ERBB1/EFNB1 interactions \((P = 0.03)\), a significant decrease in ERBB2/EFNB1 interactions \((P = 0.0001)\), whereas...
Brie EFNB1 phosphorylation and downstream ERK1/2 signaling with PTPBL (the murine homolog of PTPN13) results in enhanced signaling with ERBB2 decreases (P < 0.01). Finally, treatment with a combination of EGF, cetuximab, and trastuzumab (blue bars) has no effect on the number of interactions formed between ERBB1 and EFNB1; however, EFNB1’s association with ERBB2 decreases (P = 0.03), whereas ERBB1/ERBB2 heterodimers significantly increase (P = 0.01). Interactions between ERBB2 and EFNB1 remain unchanged. In SCC47 (HPV+) cells (Fig. 5B), treatment with targeted drugs showed similar trends as those evident with SCC1 (HPV-) cells though significance was not reached in any condition. Nonetheless, these PLA data together with the biochemical analysis of these experiments (Fig. 4) suggest that partner shifting occurs in the presence of targeted therapies and may explain the sustained activation of EFNB1 and ERK1/2 evident in the presence of drug.

**Loss of EFNB1 decreases tumor growth in an in vivo model of HNSCC**

The data suggest that EFNB1 associates with ERBB1 and ERBB2 (alone or in combination) and that PTPN13 modulates these interactions by regulating EFNB1 phosphorylation and downstream signaling. Although ERBB1 and ERBB2 roles in tumor growth have been appreciated for some times, we wanted to directly test EFNB1’s function in tumor growth using a previously described murine model of HPV+ HNSCC (18, 24, 25). In these cells, HPV16 E6–mediated degradation of PTPN13 (the murine homolog of PTPN13) results in enhanced EFNB1 phosphorylation and downstream ERK1/2 signaling (14). Briefly, MTE cells stably expressing MEERL cells were transfected with the pcDNA3.1 Zeocin expression vector containing either wt murine Efnb1 (wt mEfnb1) or an short hairpin RNA (shRNA)-targeting Efnb1 (shEfnb1). Transfection with a nonsilencing shRNA (nonsilencing) as well as the parental cells served as controls. Cells were ring-cloned and clones tested biochemically. More than 50 clones were analyzed by Western blot analysis with only modest overexpression or knockdown of EFNB1 evident. However, immunofluorescence localization of surface expression shows abundant increase in wt mEfnb1 clones and decrease in shEfnb1 clones (Fig. 6A). These findings are consistent with our previous observations that abundant intracellular EFNB1 expression precludes Western blot analysis detection of surface expression changes. Thus, selection of clones was based predominantly on immunofluorescence surface EFNB1 localization. To test the role of EFNB1 in tumor growth in vivo, 100,000 cells of each clone were injected into the hind limb of C57Bl/6 mice, N = 5 mice per group. Tumor growth was measured weekly, wt mEfnb1 tumors grew significantly faster than parental and shEfnb1 tumors (P < 0.001). However, tumor growth of the wt mEfnb1 group was not significantly different than that of the nonsilencing control group (P = 0.42). Given that the wt mEfnb1 group was significantly different than the parental group, we speculate that the nonsilencing construct mediates off-targets effects that account for this difference in tumor growth with the wt mEfnb1 group. Importantly, mice bearing shEfnb1 tumors had significantly slower tumor growth compared with all other groups (P < 0.001) at all time points up to 20 days, at which time mice from the other groups reached sacrifice criteria (Fig. 6B).

Consistent with tumor growth, mice bearing parental tumors survived significantly longer than those bearing wt mEfnb1 or nonsilencing tumors (P < 0.001), whereas shEfnb1 bearing mice survived significantly longer (P < 0.001) than the other groups (Fig. 6C). These data show that EFNB1 significantly affects tumor growth and survival, suggesting it is a good candidate for therapeutic targeting in HNSCC and other solid tumors.

**Discussion**

We describe a novel association in HNSCC consisting of ERBB1 and EFNB1. ERBB2 is also a part of this complex, consistent with previous findings (14). Loss of PTPN13 function increases EFNB1 phosphorylation, enhances EFNB1’s interaction with ERBB1, and correlates with potentiated ERK1/2 activation. Our data do not test whether ERBB1 directly phosphorylates EFNB1 or whether other components exist within this complex that mediate EFNB1 phosphorylation. However, previous studies suggest that SRC kinase phosphorylates EFNB1 following cognate receptor engagement (10) and our previous studies support this role for SRC (14). Whether SRC is recruited to EFNB1 when complexed to ERBB1 remains to be tested. Regardless of the mechanism, increased EFNB1 phosphorylation correlates with ERK1/2 phosphorylation.

Importantly, we show that ERBB1’s transmembrane domain mediates the association with EFNB1. These data predict that ERBB1-targeted therapies (which bind extracellular, cetuximab, or intracellular, erlotinib, epitopes) alone will be inefficient in blocking intracellular signaling mediated by this complex, as EFNB1 is likely impervious to them. Cetuximab’s...
binding site on ERBB1 has been solved (33). The extracellular domain of ERBB1 is subdivided into four subdomains (I–IV; ref. 34). EGF binds epitopes on domains I and III, whereas cetuximab binds only to domain III, yet overlaps with the domain III epitope bound by EGF. Thus, cetuximab binding is sufficient to sterically inhibit EGF binding. ERBB1’s extracellular domains do not mediate its interaction with EFNB1, strongly suggesting that cetuximab binding will not inhibit it. Trastuzumab binds an epitope within the juxtamembrane region of extracellular domain IV of ERBB2 (35). Our previous findings show that ERBB2’s extracellular domains do not mediate its association with EFNB1 (14), and therefore, trastuzumab binding likely will not affect this interaction.

In cancers with decreased or lost PTPN13 function, ERBB/EFNB associations and signaling may be potentiated. We tested these possibilities and found that indeed, while treatment with cetuximab potently blocked ERBB1 activation, EFNB1 phosphorylation persisted as did that of ERK1/2. Trastuzumab treatment yielded similar results and the combination of these targeted therapies showed no ill effect on EFNB1 phosphorylation or downstream signaling. The findings were strikingly similar in both HPV+ and HPV− cells suggesting that HPV status does not alter drug efficacy. However, the PLA data show that HPV infection promotes ERBB/EFNB1 interactions (Fig. 1E), whereas analysis of these interactions in the context of drug treatment showed no significant differences in the HPV+ cells (Fig. 5B). We speculate this is a reflection of the increased number of associations due to HPV infection in these cells and suggest that a maximal threshold of total ERBB/EFNB1 interactions per cell may exist such that once this threshold is reached, conditions that would otherwise drive significant changes become difficult to discern. In addition, we noted a higher variability in the number of interactions for some conditions, which also affected attainment of statistical significance. Taken together, however, these data explain, at least in part, the modest clinical benefits evident with cetuximab in the HNSCC setting. In addition, the data suggest that dual targeting of ERBB1 and ERBB2 likely will
not yield better results than either drug alone. This has in fact been shown for HNSCC cell lines (36). Moreover, a randomized phase II study using lapatinib, a dual ERBB1 and ERBB2 inhibitor, showed no significant difference in objective response rate between lapatinib and placebo (37). These findings are not unique to HNSCC but seem to be the rule rather than the exception in a majority of solid tumors. For example, in a subset of molecularly classified breast tumors that overexpressed ERBB2, trastuzumab therapy proved beneficial for only a minority of patients, whereas most showed de novo or acquired trastuzumab resistance (38).

The finding that targeted drugs promote shifting of partners within ERBB/EFNB1 complexes emphasizes the complexity of macromolecular complexes, their roles in disease and suggests that targeted drugs may drive the progression of tumors. As with other solid tumors, it is increasingly evident that targeting a single element lacks long-term clinical efficacy. These findings strongly show that silencing or eliminating a single oncogenic driver merely imposes evolutionary forces that promote alternative mechanisms to maintain, or even potentiate, the cancerous phenotype. This may be best exemplified by non–small cell lung cancer in which patients with classic ERBB1 mutations (L858R and exon 19 deletions) initially respond to ERBB1-targeted therapies, yet eventually acquire a secondary mutation, T790M, which confers resistance (39). Multipronged approaches for effective treatment have been proposed for several cancers (40–43). Our current findings that targeted therapies drive shifting of partners within a multikinase/ligand/phosphatase complex strongly support these proposals. Along these lines, the use of pertuzumab in combination with trastuzumab shows promise for patients with HER2 breast cancer (44, 45). Pertuzumab binds an extracellular epitope within domain II of ERBB2 and blocks dimerization with other ERBB family members (46). Our data show that in SCC1 cells, ERBB1/2 heterodimers are in the minority and only become a major dimer group following cetuximab treatment (Fig. 5A). The current study did not evaluate ERBB3 or ERBB4, therefore it is unclear what role they may play in the multikinase/ligand/phosphatase complex described. However, given our finding that the transmembrane domain of ERBB1 mediates the association with EFNB1, it seems unlikely that pertuzumab will abrogate ERBB/EFNB1 interactions. The role of ERBB transmembrane domains in mediating dimer formation is well established (47–49). Our finding that the transmembrane also is critical for EFNB1 associations, strongly suggests that small molecules able to abolish transmembrane interactions may effectively block dimer associations of multiple signaling molecules not limited to the ERBB family. Such drugs may have potent clinical efficacy. Our data also suggest that EFNB1-targeted therapy may be of clinical value. In all, the data suggest that blocking formation of the complex as opposed to blocking individual components within the complex may provide better tumor growth control and clinical outcomes.

In summary, the present findings are consistent with published data and support a tumor-suppressive function for PTPN13 (2, 14). These studies confirm our previous findings that EFNB1 associates with members of the ERBB receptor tyrosine kinases. For those cancers with upregulation of ERBB1 (such as HNSCC), its association with EFNB1 may significantly affect tumorigenesis. Moreover, acquisition of PTPN13 loss-of-function mutations or its decreased expression (due to HPV infection or epigenetic silencing) may further enhance ERBB1/EFNB1-mediated signals. Our study suggests that EFNB1 is an important target for therapeutic intervention and that combinatorial targeted therapy approaches must include ERBB1, ERBB2, as well as EFNB1 and possibly SRC for quenching
complex mediated oncogenic signals. Although these studies focus on HNSCC, EFNB1 complexes with ERBB2 in breast cancer where loss of PTPN13 correlates with decreased overall survival (14) and thus, we speculate that a similar complex exists in breast tumors (and possibly other solid tumors), suggesting that a multitargeted drug approach may be beneficial in this setting as well.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: P.D. Vermeer, P.L. Colbert, J.H. Lee
Development of methodology: P.D. Vermeer, P.L. Colbert, D.W. Vermeer
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P.D. Vermeer, P.L. Colbert, B.G. Wieking, D.W. Vermeer
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P.D. Vermeer, P.L. Colbert, D.W. Vermeer, J.H. Lee

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