



Published in final edited form as:

*Cancer Res.* 2015 March 15; 75(6): 1035–1045. doi:10.1158/0008-5472.CAN-13-1625.

## ERBB3 Independent Activation of the PI3K Pathway in EGFR Mutant Lung Adenocarcinomas

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### Abstract

ERBB3, a member of the Epidermal Growth Factor Receptor (EGFR) family of receptor tyrosine kinases, has been implicated in activation of the phosphatidylinositol 3-kinase (PI3K) pathway in human lung adenocarcinomas driven by *EGFR* mutations. We investigated the contribution of ERBB3 to the initiation, progression and therapeutic response of EGFR-induced lung adenocarcinomas using tetracycline- and tamoxifen- inducible transgenic mouse models. Deletion of *ErbB3* at the time of induction of mutant EGFR had no effect on tumorigenesis, demonstrating that ERBB3 is not required to initiate tumorigenesis. Tumors that developed in the absence of ERBB3 remained sensitive to EGFR TKIs and retained activation of the PI3K/AKT pathway. Interestingly, acute loss of *ErbB3* suppressed further growth of established EGFR<sup>L858R</sup>-mediated lung tumors. Four weeks after deletion of *ErbB3*, the tumors exhibited phosphorylation of EGFR, of the adaptor proteins GAB1 and GAB2 and, of the downstream signaling molecules AKT and ERK suggesting that alternative signaling pathways could compensate for loss of *ErbB3*. Similar to our observations with mouse tumors, we found that GAB adaptor proteins play a role in ERBB3 independent activation of the PI3K pathway by mutant EGFR in *EGFR* mutant human cell lines. Finally, in such cell lines, increased levels of phosphorylation of ERBB2 or MET were associated with reduced sensitivity to acute loss of ERBB3, suggesting remarkable plasticity in the signaling pathways regulated by mutant EGFR with important therapeutic implications.

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**Conflicts of Interest:** Rights to a patent application for *EGFR*<sup>T790M</sup> testing were licensed on behalf of KP and HV by Memorial Sloan-Kettering Cancer Center to MolecularMD. KP has served as a consultant for Takeda and her lab receives research support from AstraZeneca.

## Introduction

The epidermal growth factor receptor (EGFR) is the prototypical member of a family of four receptor tyrosine kinases: EGFR, ERBB2, ERBB3 and ERBB4. EGFR-induced signaling is initiated upon ligand binding, with the formation of EGFR homodimers or heterodimers with other members of the EGFR family (1). This leads to phosphorylation of residues on the cytoplasmic tail of the receptor that are then recognized and bound by intracellular signaling molecules. The four members of the EGFR family have distinctive properties. For example, ERBB2 is unable to bind any known ligands for this family, and ERBB3 lacks intrinsic tyrosine kinase activity (2). Further, the receptors contain different combinations of protein docking sites in their cytoplasmic domains. These features greatly increase the diversity of signals that can be transduced from specific homo- and hetero- dimers.

EGFR can form heterodimers with all three of the other EGFR family members (1). These heterodimers may have distinct and important roles in EGFR-mediated signaling in both normal cellular processes and during carcinogenesis. Evidence for these roles is especially provocative for the EGFR-ERBB3 heterodimer that is the focus of this report.

Mutations in exons encoding the tyrosine kinase domain of *EGFR* are found in approximately 10–15% of lung adenocarcinomas in the United States and over 40% in Asia (3–6). Two types of mutations account for 90% of all lung adenocarcinoma-associated *EGFR* mutations and are associated with sensitivity to treatment with the tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib: i) small in-frame deletions in exon 19 that lead to elimination of an LREA motif in the protein (DEL) and ii) a point mutation in exon 21 that substitutes an arginine for a leucine at position 858 in the protein (L858R). As a result of these changes, both the EGFR<sup>DEL</sup> and EGFR<sup>L858R</sup> mutants have the ability to transform cells and initiate tumorigenesis when overexpressed in transgenic mouse models (7–9). Human lung tumors bearing *EGFR* mutations show radiographic responses to TKIs that meet RECIST (“response and evaluation criteria in solid tumors”) criteria in about 70% of cases (10). Eventually, resistance to these TKIs emerges and is most frequently associated with the presence of a secondary mutation in *EGFR* (T790M) (11).

Studies with human lung cancer cell lines carrying *EGFR* mutations indicate that the phosphoinositide 3-kinase (PI3K) and signal transducer and activator of transcription (STAT) signaling pathways are important downstream mediators of cell survival (12). Although EGFR itself can activate the PI3K pathway through the adaptor protein GAB1 (GRB2-associated binding protein 1) (13), several lines of evidence indicate that ERBB3 might be the major activator of PI3K/AKT signaling induced by EGFR. First, ERBB3 has seven Tyr-X-X-Met motifs in ERBB3 that upon phosphorylation are recognized by the PI3K regulatory subunit p85, but these motifs are not found in EGFR and ERBB2 (14). Second, gefitinib-sensitive lung cancer cell lines have been shown to use ERBB3 to activate the PI3K pathway (15). Third, in a subset of *EGFR* mutant TKI-resistant lung cancers with *MET* amplification, *MET* dimerizes with ERBB3 to activate the PI3K pathway and thus confers resistance to gefitinib (16). Reduced expression of *ERBB3* with siRNAs in these cells reduces the activity of Akt (15,16). Finally, combined treatment of erlotinib-resistant EGFR<sup>L858R+T790M</sup>-induced tumors with the EGFR antibody cetuximab and an anti-ERBB3

antibody MM-121 causes tumor regression (17). Together these studies have led to the hypothesis that therapeutically targeting of ERBB3 and EGFR together, a strategy currently in clinical trials, may be superior to inhibition of EGFR alone.

To formally test whether the EGFR-ERBB3 heterodimer is the functional oncogenic element in mutant EGFR-driven lung cancer, we investigated the requirement for ERBB3 for lung adenocarcinoma formation in a previously generated transgenic mouse model of EGFR mutant lung adenocarcinoma (9). In this model, human *EGFR* mutants are regulated by a tetracycline-responsive element (*TetO*), and the reverse tetracycline transactivator (*rtTA*) is controlled by the *CCSP* (clara cell specific protein) promoter. (*rtTA* is expressed in type II pneumocytes in this transgenic strain.) Delivery of doxycycline activates expression of mutant *EGFR* transgenes. These mice subsequently develop lung adenocarcinomas that are responsive to EGFR TKI treatment (9).

We used these mouse models to delete mouse *ErbB3* in the same cells that express mutant EGFR prior to tumor formation and in established tumors to test its role in tumor initiation and maintenance, respectively. Our findings show that *ErbB3* is not required to initiate mutant EGFR-driven lung tumorigenesis when over-expressed. Tumors that formed in the presence or absence of *ErbB3* had similar histology and did not differ in their response to the TKI erlotinib. Interestingly, ERBB3 loss in established tumors restrained tumor growth. We also explored the significance of these findings with respect to intracellular signaling as presented below.

## Materials and Methods

### Mouse husbandry

All animals were kept in pathogen-free housing under guidelines approved by the Yale and Memorial Sloan-Kettering Cancer Center Animal Care and Use Committees. Doxycycline was administered by feeding mice with doxycycline-impregnated food pellets after mice were weaned (625 ppm; Harlan-Teklad). The tetracycline-inducible *EGFR* transgenic models (9), *ErbB3* floxed mice (18), *Nkx2.1-Cre<sup>ERT2</sup>* mice (19) and *TetO-Cre* (20) mice were all previously described.

### Cell culture and transient transfections

PC9, H3255, H1975, and HCC827 cells have been maintained in the Politi laboratory since 2010 and were obtained from the Varmus Laboratory. PC9BRC1 cells were obtained from William Pao (21). All cells were grown in RPMI 1640 medium containing 10% FBS and penicillin/streptomycin in a 37°C incubator with 5% CO<sub>2</sub>. Cells were examined regularly for mycoplasma contamination and authenticated by STR profiling (GenePrint® 10 System) at the Yale University DNA Analysis Facility in December 2014.

### Drug treatment

Mice were treated with 25mg/kg/day of erlotinib for either 5 days or for 4–6 weeks (9). Tamoxifen was administered 5mg/day in 250 µl corn oil for two days to induce deletion of

*ErbB3*. Tumor regression was monitored using MRI as described in the Supplementary Materials and Methods.

### Immunoblotting

Whole lung lysates were made as described for the RTK arrays in the Supplementary Materials and Methods. Protein concentrations were quantified using the Bio-Rad Dc protein assay reagent. For each sample, 50  $\mu$ g of protein lysate were loaded onto 4–20% gradient gels (Bio-Rad precast) and electrophoresis was performed followed by transfer to nitrocellulose. Membranes were blocked in 0.1 % Tween-20 with 5% w/v nonfat dry milk then incubated with specific antibody to each target protein at 4°C overnight followed by 2 hours of incubation with a secondary antibody and visualization using ECL.

## Results

### EGFR mutant tumors form at normal rate in the absence of *ErbB3*

As a first step in establishing whether ERBB3 plays a role in lung tumorigenesis driven by mutant *EGFR* in mice, we examined whether ERBB3 is produced in tumors induced by mutant *EGFR*. Immunohistochemical staining of lung tumors obtained from *CCSP-rtTA +; TetO-EGFR<sup>L858R</sup> +* mice (9) revealed strong cytoplasmic staining for ERBB3 protein in tumor tissue and minimal staining in normal lung (Fig. 1A).

Since the transgenic mice express a human *EGFR* cDNA, we also examined whether transgenic human mutant EGFR interacts with mouse ERBB3 in both cultured cells and mutant *EGFR*-driven mouse lung tumors that contain both proteins. We first confirmed that human EGFR<sup>L858R</sup> can bind to murine ERBB3 by overexpressing human *EGFR<sup>L858R</sup>* and murine *ERBB3*, individually or together, in 293T cells via transient transfection. Antibodies that specifically recognize ERBB3, EGFR or only EGFR<sup>L858R</sup> (L858R) were used for co-immunoprecipitation and immunoblotting. EGFR was detected after immunoprecipitation with the ERBB3 antibody only when both proteins were present (Fig. 1B). In reciprocal experiments, ERBB3 was detected in precipitates formed with an antibody against EGFR<sup>L858R</sup> (Supplementary Fig. 1). Furthermore, we found that ERBB3 co-precipitated with mutant EGFR from lysates of mouse lung tumors (Fig. 1C). These data indicate that mutant human EGFR is able to interact with mouse ERBB3, that these heterodimers form in mouse tumors induced by mutant human EGFR, and that mutant human EGFR could, in principle, signal through ERBB3 during lung tumorigenesis.

Given these results, we set out to determine whether ERBB3 is functionally important during tumorigenesis initiated by mutant EGFR in mice by deleting *ErbB3* in the same cells in which mutant *EGFR* is expressed. To do this, we crossed *CCSP-rtTA +; TetO-EGFR<sup>L858R</sup> +* mice with mice that carry floxed alleles of *ErbB3* (18) and a *TetO-Cre* transgene (20), creating mice that carry one copy of each of the three transgenes and are homozygous for the floxed allele of *ErbB3*. In the absence of the tetracycline analog, doxycycline, mutant *EGFR* is not expressed and the *ErbB3* gene remains intact (Fig. 1D). Feeding doxycycline to experimental mice leads to expression of mutant *EGFR* and *Cre* from the tetracycline-inducible promoters in type II pneumocytes, with subsequent excision

of the floxed portion of the *ErbB3* gene (Fig. 1D). Western blotting of whole lung extracts demonstrated that Cre-mediated deletion of floxed *ErbB3* alleles causes the loss of ERBB3 protein but production of mutant *EGFR* is unaffected (Fig. 1E).

To assess whether loss of *ErbB3* had an effect on EGFR-induced lung carcinogenesis, we monitored a cohort of *CCSP-rtTA +; TetO-EGFR<sup>L858R</sup> +; TetO-Cre +; ErbB3 fl/fl* mice and littermates of control genotypes for signs of advanced cancer. All mice that carried the *CCSP-rtTA* and *TetO-EGFR<sup>L858R</sup>* transgenes became cachectic, showed decreased activity and had to be sacrificed, regardless of whether they also carried intact *ErbB3* alleles. Survival curves derived from mice of the different genotypes did not differ significantly in two different *TetO-EGFR<sup>L858R</sup>* transgenic lines (Fig. 2A and Supplementary Fig. 2), implying that deletion of *ErbB3* had little or no effect on survival. At necropsy we observed enlarged lungs with macroscopically visible tumor nodules. Histological sections showed adenocarcinomas irrespective of genotype (Fig. 2B). Similar results were observed with mice carrying a transgene with the *EGFR* exon19 deletion (*CCSP-rtTA +; TetO-EGFR<sup>747-752</sup> +; TetO-Cre +; ErbB3 fl/fl*) (Fig. 2C).

### EGFR mutant tumors lacking *ErbB3* retain sensitivity to erlotinib

EGFR TKIs block activation of the ERBB3-mediated PI3K/AKT pathway in TKI-sensitive, human lung adenocarcinoma cell lines carrying either wild type or mutant *EGFR* (15). This implies that a potentially important aspect of therapeutic response is dependent on EGFR-mediated signaling via ERBB3. Therefore, we asked whether mouse lung tumors that arose without ERBB3 responded differently to TKI treatment than those arising with ERBB3. Mice with *EGFR<sup>L858R</sup>*- or *EGFR<sup>747-752</sup>*-induced lung tumors, both with and without intact *ErbB3* genes, were treated with erlotinib for 5 days or 4 weeks, and monitored with magnetic resonance imaging (MRI) before and during treatment. Tumor volumes for each mouse were quantified to evaluate the drug response. After 5 days of erlotinib treatment, tumors regressed in two of three *CCSP-rtTA +; TetO-EGFR<sup>L858R</sup> +; TetO-Cre +; ErbB3 fl/fl* mice (Fig. 3A, Supplementary Table 1) and in twelve out of thirteen *CCSP-rtTA +; TetO-EGFR<sup>L858R</sup> +; TetO-Cre +; ErbB3 fl/fl* mice after four weeks (Figs. 3A, 3C, Supplementary Table 1). These findings were not significantly different from results with control mice that retained intact *ErbB3* alleles (*CCSP-rtTA +; TetO-EGFR<sup>L858R</sup> +; TetO-Cre -; ErbB3 fl/fl*): tumors regressed in two of two mice after 5 days treatment (P-value= 0.83) and six of seven mice after four weeks treatment (P-value=0.92, Fig. 3D and Supplementary Table 1). *EGFR<sup>747-752</sup>*-induced lung tumors also showed a radiographic response to erlotinib, regardless of *ErbB3* status (Figs. 3B, 3C, Supplementary Table 1). In addition, all animals showed histological evidence of tumor regression, such as scarring, after 4 weeks of drug treatment, irrespective of genotype (Fig. 3A, 3B). Thus deletion of *ErbB3* does not impair the response of *EGFR* mutant tumors to TKIs.

### The PI3K signaling pathway is activated despite deletion of *ErbB3*

ERBB3 is a strong activator of the PI3K signaling pathway. Therefore we examined whether activation of this pathway is altered in tumors arising in the absence of ERBB3. We used phosphorylation of Akt as measured by Western blotting of lung tumor lysates from two groups of mice (*CCSP-rtTA +; TetO-EGFR<sup>L858R</sup> +; TetO-Cre +; ErbB3 fl/fl* and *CCSP-rtTA*



and absence of ERBB3 (Fig. 4D). Both EGFR and ERBB3 were detected in the p85 immunoprecipitates from lysates of ERBB3-containing tumors. In reciprocal experiments, p85 was detected in EGFR<sup>L858R</sup> immunoprecipitates in ERBB3-containing and ERBB3-depleted mouse tumors (Supplementary Fig. 4A). Similar results showing that EGFR<sup>L858R</sup> co-precipitates with p85 from extracts of cultured 293T cells that lack endogenous ERBB3 (Supplementary Fig. 1). In summary, in the absence of ERBB3, the PI3K signaling pathway is active; this activation could be mediated by a direct or indirect association of mutant EGFR with p85. ERBB2 may also participate in PI3K activation in this setting, however we did not find conclusive evidence in this regard.

### Gab Family Proteins Couple the PI3K Pathway to Mutant EGFR

Since EGFR (and ERBB2) lacks a canonical p85-binding site, adaptor proteins are likely to bridge the association between EGFR and p85. Docking proteins---including the GRB2-associated binder protein (GAB) and insulin receptor substrate (IRS) protein families---have been implicated as activators of the PI3K signaling pathway after EGF stimulation (13,25,26)(27)(28).

To examine whether mutant EGFR activates the PI3K pathway via these mediators, we first determined the level of these proteins in mutant *EGFR*-driven lung tumors by Western blotting. Lysates of EGFR-mutant mouse lung tumors contain GAB1, GAB2, IRS1 and IRS2, and the levels of these proteins are unaffected by the absence of ERBB3 (Fig. 5A). To identify which of these proteins might activate PI3K in this context, we determined which adapter proteins and receptors are associated with p85 (Fig. 5B). Interestingly, p85 co-precipitated with IRS1 and GAB2 in the lysates from tumors independent of ERBB3 status (Fig. 5B, 5C, Supplementary Fig. 4B). In the reciprocal experiment, p85 precipitated with GAB2 in mouse tumors (Fig. 5D, Supplementary Fig. 4C). Moreover, EGFR co-precipitated, albeit weakly, with GAB2 in EGFR mutant lung tumors regardless of the presence of ERBB3 (Fig. 5C) while co-precipitation of IRS1 and EGFR was barely detectable (Supplementary Fig. 4B).

Taken together, these results indicate that both p85 and EGFR co-precipitate in a GAB1/GAB2-containing complex, which might promote activation of PI3K by EGFR. Consistent with this hypothesis, GAB1 and GAB2 are expressed and phosphorylated in several human lung cancer cell lines (Fig. 5E, 7A) and GAB2 co-precipitates with p85 in these lines regardless of the presence of ERBB3 (Supplementary Figure 4D). Interestingly, HCC827 cells (insensitive to ERBB3 loss) exhibited higher levels of GAB2 and IRS1 than PC-9 cells (partially sensitive to ERBB3 loss; Supplementary Figure 4D). Furthermore, siRNA knock-down of EGFR greatly diminished phosphorylation of GAB1 and GAB2 indicating that phosphorylation of GAB1 and GAB2 is regulated by EGFR in human cancer cells as well (Fig. 5E and Supplementary Fig. 5A). In summary, it seems likely that mutant EGFR can activate the PI3K pathway through at least two different mechanisms: 1) via ERBB3, which binds p85 directly and 2) by binding the adaptor proteins GAB1 or GAB2 to recruit p85.

### Acute inactivation of *ErbB3* restrains the growth of EGFR mutant lung tumors

To assess the therapeutic potential of targeting ERBB3 in lung cancer, we tested whether it is required for the survival of established mouse lung tumors. We crossed *CCSP-rtTA* +; *TetO-EGFR<sup>L858R</sup>* + mice to mouse lines with *Nkx2.1-Cre<sup>ERT2</sup>* (19) and *ErbB3 flox/flox* alleles (Fig. 6A) to generate *CCSP-rtTA* +; *TetO-EGFR<sup>L858R</sup>* +; *Nkx2.1-Cre<sup>ERT2</sup>*; *ErbB3 flox/flox* mice and appropriate controls. Tumors were induced using doxycycline to activate transcription of the mutant *EGFR* transgenes. To restore enzymatic activity to *Cre<sup>ERT2</sup>* and delete *ErbB3* from both normal and transformed mouse lung epithelial cells, tamoxifen was administered to tumor-bearing mice by oral gavage. As controls, mice of the same genotype were treated with corn oil and mice without the *Nkx2.1-Cre<sup>ERT2</sup>* allele were treated with tamoxifen. The former control is especially important because *Nkx2.1* heterozygosity has been shown affect the formation of mutant EGFR-induced tumors (29). Lung tumor volume was assessed weekly for 4 weeks following tamoxifen administration. Overall during the 4-week period of observation following acute *ErbB3* loss, tumor size was stable (Fig. 6B). By contrast, in mice with wild type ERBB3, the tumors continued to grow. These data indicate that *ErbB3* signaling contributes to the overall growth of established EGFR mutant tumors.

To explore the molecular changes in mouse lung tumors after loss of *ErbB3*, we compared signaling pathway activation in mouse lung tissue 4 weeks after corn oil or tamoxifen treatment. As expected, *ErbB3* was effectively deleted in mouse lung tissue (Fig. 6C). Upon *ErbB3* deletion, the levels of EGFR, GAB1, GAB2, AKT and ERK phosphorylation were maintained and in some cases increased (Fig. 6C), consistent with our observations in tumors initiated in the absence of *ErbB3* (Fig. 4C). In summary, acute loss of *ErbB3* was associated with sustained EGFR pathway signaling, consistent with the notion that mutant EGFR can use GAB adaptor proteins to activate the PI3 kinase pathway and transduce an oncogenic signal.

### ERBB2 and MET phosphorylation levels correlate with the sensitivity of EGFR-mutant human lung adenocarcinomas to loss of ERBB3

The studies described above demonstrate that acute loss of ERBB3 can, at least partially, restrict tumor growth. We used a panel of established EGFR mutant human lung cancer cell lines to further investigate factors associated with the sensitivity of EGFR mutant tumors to the loss of ERBB3. Western blots of lysates from five such lines revealed that all contain ERBB3 and that it is phosphorylated in each cell line, albeit at varying levels (Fig. 7A). We studied the effect of acute loss of ERBB3 in the cell lines by transiently knocking down expression of *ERBB3* with siRNAs. Western blotting showed that ERBB3 protein levels were efficiently reduced by siRNA in all cell lines analyzed (Fig. 7B and Supplementary Fig. 5). Similar reduction in EGFR resulted from introduction of EGFR-specific siRNAs (Fig. 7B). However, while loss of EGFR protein reduced cell viability by 50–75%, loss of ERBB3 had a more limited effect, with 0–40% loss of viability, indicating that tumor cell survival depends more heavily on mutant EGFR than on ERBB3, findings consistent with our data in mice. HCC827, H3255 and H1975 cells were the least affected by reduction of ERBB3 levels, and they demonstrated the highest levels of phosphorylation of MET or ERBB2 and of total EGFR (Fig. 7A). Furthermore, knockdown of ERBB3 had the strongest effect on phosphorylation of AKT and ERK in human lung cancer cell lines with relatively



low levels of MET and ERBB2 phosphorylation (Fig. 7B, Supplementary Fig. 5). These data suggest that there is intrinsic variability in EGFR mutant tumors with regard to their dependence on ERBB3 for survival and that a normal level of signaling through ERBB3 is not a prerequisite for survival of cells transformed by mutant EGFR. This may be especially so in cells that can sustain signaling through other trans-membrane receptors, such as MET and ERBB2. Of note, our data in HCC827 cells contrast published results in which ERBB3 knock-down was shown to have an effect on the viability of these cells (16). Technical differences in our approaches (siRNA vs. shRNA knock-down and timing of the assays) as well as divergence in our cell lines could account for the discrepancy in these results.

We also noticed that the adapter proteins GAB1 and GAB2 remain phosphorylated in cells after knockdown of ERBB3 with inhibitory RNAs (Fig. 7B and Supplementary Fig. 5). To explore the role of these proteins more directly as activators of the PI3K pathway in EGFR-mutant, human lung cancer cell lines, we knocked down GAB1 alone or both GAB1 and GAB2 in several cell lines. Phosphorylation of Akt and Erk declined in H3255, PC9, and PC9BRc1 cells after GAB1 knock down (Fig. 7B). In H3255 and H1975 cells, which were amongst the least affected by ERBB3 knockdown, combined knockdown of GAB1 and GAB2 reduced viability by 40–50% (Fig. 7B, Supplementary Fig. 5). In contrast, in PC9 and PC9BRc1 cells, which were most susceptible to ERBB3 inhibition, knockdown of GAB1 or GAB1+GAB2 reduced viability by only 20–30%. Combined knockdown of ERBB3, GAB1, and GAB2 did not reduce viability further in any of the cell lines tested except in H3255 cells, suggesting that multiple mechanisms may be available to sustain viability, perhaps through the PI3K pathway.

To test whether inhibition of EGFR and ERBB3 had a synergistic effect on the viability of EGFR mutant lung cancer cells, we knocked down both EGFR and ERBB3 in PC9 and H3255 cells. However, the double knock-down did not affect viability more than knockdown of EGFR alone (Fig. 7C and Supplementary Fig. 6A and 6B). Knockdown of ERBB2 only slightly reduced the viability of these EGFR mutant cell lines, regardless of whether ERBB3 was present (Fig. 7C and Supplementary Fig. 6A and 6B), suggesting that ERBB2/ERBB3 heterodimers do not have an important role in activating survival signaling, presumably through PI3K, in these cells.

To model combinatorial treatments designed to block both EGFR and ERBB3 signaling, we evaluated the effect of the EGFR TKI erlotinib and *ERBB3* knock-down in PC9 cells, which were the most sensitive to loss of ERBB3 (Fig. 7D). We found that reducing ERBB3 levels did not enhance the effects of erlotinib at concentrations well below physiological drug concentrations (30).

## Discussion

### **ERBB3 is not required for mutant EGFR-induced tumorigenesis but its loss can slow the growth of established tumors**

Most lung adenocarcinomas with sensitizing *EGFR* mutations are exquisitely sensitive to tyrosine kinase inhibitor (TKI) treatment (3–5). In such tumors, mutant EGFR activates critical pathways, such as the PI3K/AKT, MEK/ERK and JAK/STAT pathways to promote

transformation and tumor cell growth and survival. EGFR activates its downstream signaling pathways by homo- and/or heterodimerizing with other EGFR family receptors including ERBB3. However, prior to this study, very little was known about which dimers of mutant EGFR are the essential oncogenic units during cell transformation. Our data demonstrate that in mice, ERBB3 is not required to initiate tumorigenesis driven by mutant *EGFR* in a transgenic mouse model. Established tumors, in contrast, partially depend on ERBB3: loss of *ErbB3* stabilizes tumor growth. We also show that in human tumor cell lines and in adenocarcinomas in mouse models, response of EGFR-driven tumor cells to the tyrosine kinase inhibitor, erlotinib, is independent of the presence or absence of ERBB3. In contrast to our results with lung cancer, deletion of ERBB3 in the *Apc<sup>Min</sup>* mouse model of colon cancer decreased PI3K signaling, promoted apoptosis, and reduced tumor size (18). These results suggest that the requirement for ERBB3 may depend upon both the driver mutations and the tumor cell lineage.

In our mouse models of mutant *EGFR*-driven lung cancer, few receptors other than EGFR, ERBB2 and ERBB3 (at low levels) are tyrosine phosphorylated. This indicates that mutant *EGFR* does not require activation of other RTKs for lung tumorigenesis. Both EGFR and ERBB2 were highly phosphorylated in mutant *EGFR*-driven lung tumors, and phosphorylation increased in tumors when *ErbB3* was deleted. It is likely that loss of ERBB3 leads to the increased formation of EGFR homodimers or EGFR/ERBB2 heterodimers, which cause increased phosphorylation of EGFR and ERBB2. The functional consequences of this increase in EGFR and ERBB2 phosphorylation, however, remain to be determined.

These observations raise the possibility that mutant EGFR might cooperate with ERBB2 in certain settings to transform cells. This is supported by the presence of high levels of phosphorylation of ERBB2 in EGFR mutant lung tumors. However, knockdown of *ERBB2* or *ERBB2/ERBB3* in PC9 and H3255 cells did not decrease cell viability (Fig. 7C and Supplementary Fig. 6). Currently we are performing studies to establish the role of ERBB2 in tumorigenesis driven by mutant EGFR *in vivo*.

### ERBB3-independent activation of the PI3K pathway

It has been shown that activation of the PI3K pathway is coupled to ERBB3 in EGFR mutant lung adenocarcinoma cell lines and that this interaction is sensitive to TKI treatment (15). Our results with mouse models, however, demonstrate that mutant EGFR does not depend upon ERBB3 to transduce its oncogenic signal and suggest that early studies in cell lines may have overestimated the contribution of ERBB3 on PI3K pathway activation in EGFR mutant tumors. Most strikingly, activation of the PI3K signaling pathway was unperturbed in lung tumors arising in the absence of ERBB3, and active in established tumors upon loss of ERBB3. This latter result is especially surprising since these tumors showed stable growth: it is possible that at the 4 week time-point analyzed the tumors were beginning to regrow (as evidenced by a slight increase in the tumor volume between 2–4 weeks following tamoxifen treatment, Fig. 6B) and thus show phosphorylation of Akt. Together, these data strongly suggest that compensatory mechanisms capable of PI3K activation occur in the absence of functional ERBB3.

There are two ways that EGFR can activate the PI3K/AKT pathway: 1) by heterodimerizing with ERBB3 (14) or 2) by directly recruiting adapters (e.g. GAB1, GAB2) that couple to p85 (26). We observed a physical interaction between EGFR and ERBB3 in EGFR mutant mouse lung tumors and also detected the association of EGFR with GAB1/GAB2 in the presence and absence of *ErbB3*. These results suggest that mutant EGFR uses both ERBB3 and GAB1/GAB2 to activate the PI3K pathway. Based on these results, we propose that mutant EGFR activates the PI3K/AKT pathway through both ERBB3-dependent and ERBB3-independent mechanisms. When ERBB3 is not present, mutant EGFR uses GAB1/GAB2 mediated signaling to activate the PI3K pathway. We cannot exclude that other signaling mechanisms that also engage adapter proteins (including GAB1/GAB2 and IRS1) may also contribute to activation of the PI3K pathway in these tumors. Moreover, increased Ras activation could also contribute to PI3K signaling. In a similar study, *ErbB3*-mediated PI3K signaling was shown to be dispensable for *ErbB2*-induced mammary tumorigenesis (31).

The observation that EGFR co-precipitates with p85, GAB1 and GAB2, even in the presence of ERBB3, suggests that activation of PI3K signaling can also occur via the mutant receptor. It is unclear how the proportion of ERBB3-dependent and ERBB3-independent mechanisms of activation of PI3K is determined for receptors like EGFR that have multiple ways to activate the pathway. Published evidence suggests that the mechanism used by EGFR to activate the PI3K/AKT pathway is directly affected by the cellular levels of adapter proteins like GAB1 and ERBB3 (32). In the mutant *EGFR*-mediated lung tumors and cancer cell lines we tested, ERBB3, GAB1 and GAB2 were all highly expressed and phosphorylated (Fig. 6). Our data point to a prominent role for GAB proteins in AKT activation, since knock-down of GAB proteins in EGFR mutant cell lines reduced phosphorylation of AKT.

The results of our studies have important implications for the treatment of *EGFR* mutant lung cancer. Our results predict that blocking ERBB3 function alone might stabilize tumors (at least transiently), but not lead to regressions in lung adenocarcinomas with *EGFR* mutations. Further, it appears that such tumors can adapt to the loss of ERBB3. We also note that the effect of blocking ERBB3 function in human EGFR mutant lung tumors may be predicted from the activation status of MET and ERBB2, since highly phosphorylated MET and ERBB2 proteins are associated with independence from ERBB3 (Fig. 7).

Moreover, we found that EGFR mutant lung tumors exhibit similar sensitivity to erlotinib regardless of the presence or absence of ERBB3 and there is a lack of synergy of ERBB3 knock-down with erlotinib (Fig. 7D) suggesting that combined blockade of ERBB3 with an EGFR TKI like erlotinib may not be beneficial in the TKI-naïve setting. Experiments to test this conclusively are planned. Studies with mouse models have shown that combined treatment with the EGFR antibody cetuximab and the ERBB3 antibody MM-121, leads to tumor regression in erlotinib-resistant L858R+T790M-induced tumors (17). This could indicate that ERBB3 plays a more prominent role in transformation when the T790M mutation is present. Future studies aimed at understanding the role of ERBB3 in tumors that are resistant to TKIs are underway. Indeed, ERBB3 has been suggested to play both direct and indirect roles in resistance to TKIs. In a subset of *EGFR* mutant TKI-resistant lung

cancers with *MET* amplification, *MET* dimerizes with *ERBB3* to activate the *PI3K* pathway and in this way confers resistance to gefitinib (16). *ERBB3*, however, does not always mediate *PI3K* activation: resistance to *EGFR* TKIs in *EGFR* mutant lung cancer cells resulting from Hepatocyte Growth Factor (*HGF*)-mediated *MET* activation led to *PI3K* activation via *GAB1* (33).

In summary our data point to remarkable resiliency and redundancy in activation of the *PI3K* signaling pathway in lung cancer and highlight the need for treatment strategies that are effective at blocking this pathway, regardless of how it is activated, to produce sustained tumor regression in patients. Moreover, more precise knowledge of the effects of the *EGFR* heterodimerization partner *ERBB3* on mutant *EGFR* signaling in lung cancer may guide the therapeutic application of *EGFR* family antagonists.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

**Financial Support:** This work was funded by R00CA131488 (KP), R01CA120247 (HV and KP), R01CA092479 (DWT), Yale University and by grants R24 CA83084 (MSKCC) and P30 CA08748 (MSKCC), which provided partial support for the core facilities used in conducting this investigation. XS received financial support from the Yale Cancer Center and Uniting Against Lung Cancer.

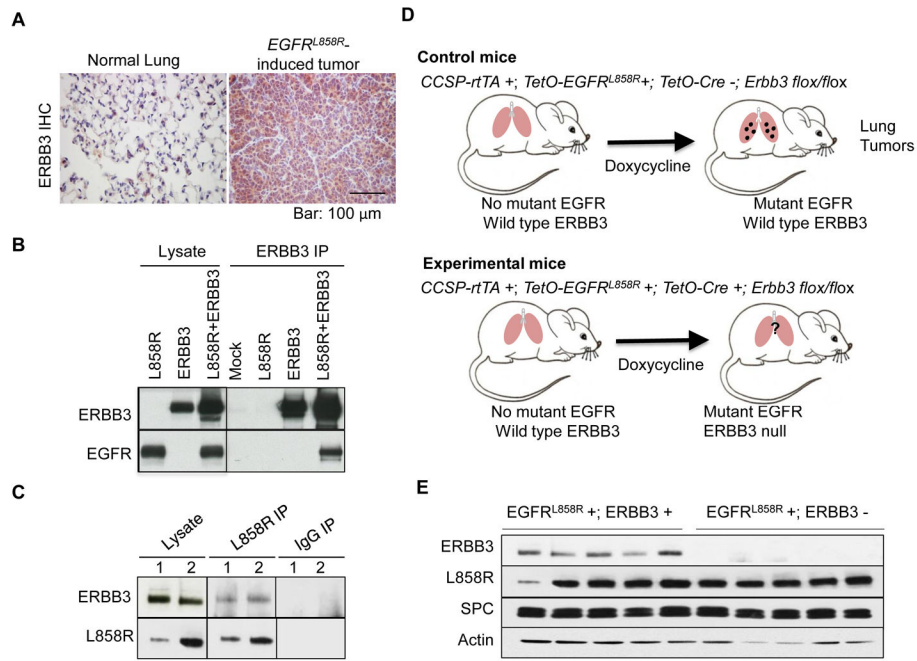
The authors thank Don Nguyen for critical reading of the manuscript and Mary Ann Melnick for expert technical assistance.

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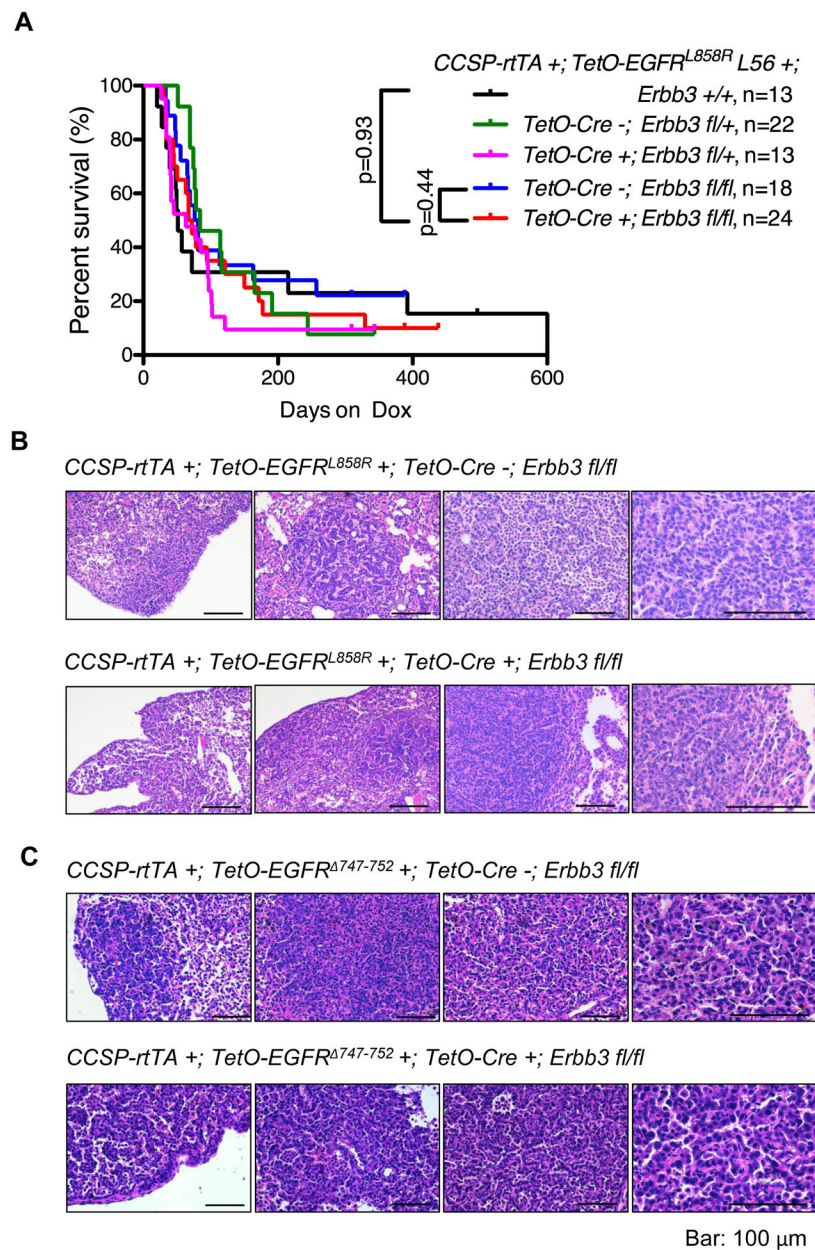
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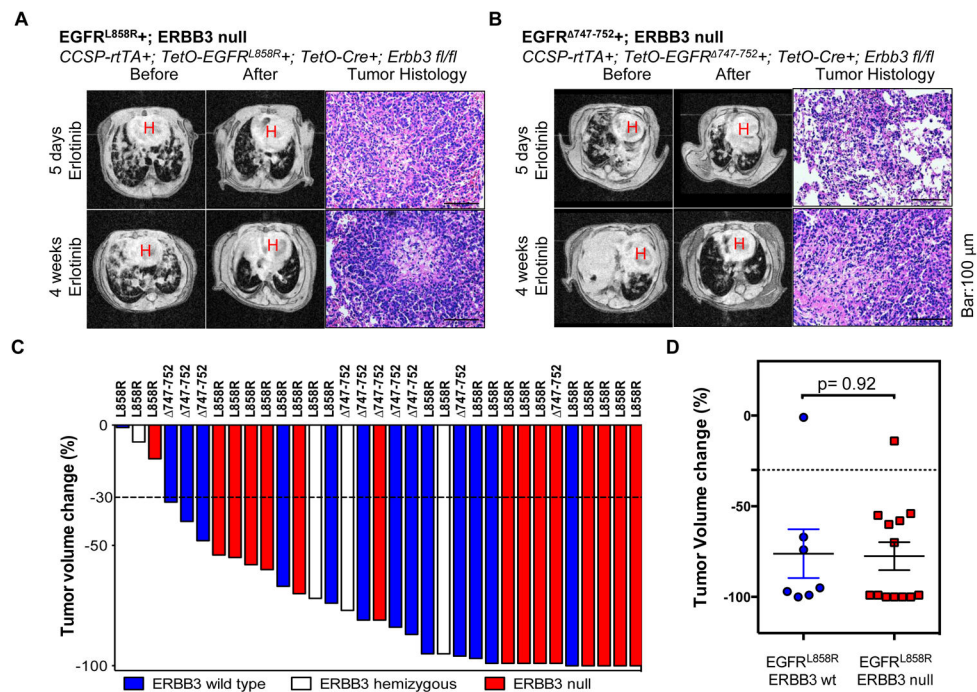
**Figure 1. ERBB3 is present in murine EGFR mutant lung adenocarcinomas and interacts with mutant EGFR**

(A) Immunohistochemical staining for ERBB3 is strong in *EGFR<sup>L858R</sup>*-induced lung tumors (right) but weak or non-existent in normal lung from control mice (left). (B) *EGFR<sup>L858R</sup>* interacts with ERBB3 in transfected cells. Plasmids encoding human *EGFR<sup>L858R</sup>*, mouse ERBB3 or both were transfected into 293T cells. Two days after transfection, an antibody to ERBB3 was used to co-precipitate ERBB3 and proteins associated with it. Immunoprecipitates were blotted with antibodies to EGFR and ERBB3. Results from mock-transfected cells are shown as indicated. (C) Mutant *EGFR<sup>L858R</sup>* interacts with ERBB3 in mutant EGFR-induced lung tumors. *EGFR<sup>L858R</sup>* or IgG immunoprecipitates from two *CCSP-rtTA+; TetO-EGFR<sup>L858R</sup>+* mice (1 and 2) were immunoblotted with antibodies specific to mutant *EGFR<sup>L858R</sup>* and ERBB3. (D) Strategy used to conditionally delete *Erbb3* in type II pneumocytes in tetracycline-inducible mouse models of *EGFR* mutant lung cancer. Control mice (top) express mutant *EGFR* and wild-type *Erbb3* in the lung epithelium upon induction of doxycycline. Experimental mice (bottom) express mutant EGFR but not ERBB3 due to the presence of *Cre* recombinase, which deletes floxed sequences in the *Erbb3* locus. (E) ERBB3 protein is not detected in lungs of mice upon deletion of *Erbb3*. Immunoblots of lung protein extracts with antibodies to *EGFR<sup>L858R</sup>*, ERBB3, Surfactant Protein C (SPC) and Actin are shown. Protein levels were normalized for numbers of pneumocytes as judged by levels of surfactant protein C. *EGFR<sup>L858R</sup>+*; *Erbb3+* (control mice) and *EGFR<sup>L858R</sup>+*; *Erbb3-* (experimental mice). IP, immunoprecipitation.

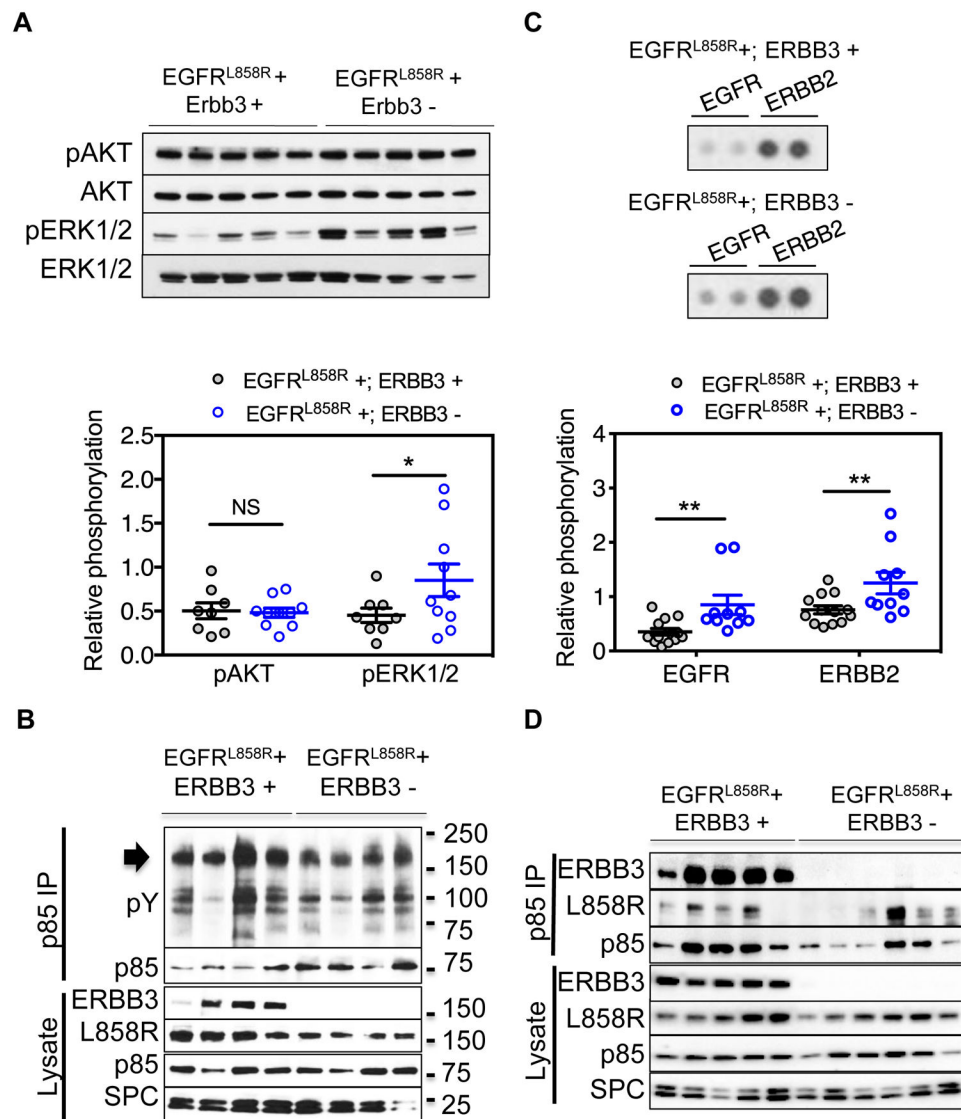


**Figure 2. Development of lung adenocarcinomas in mice lacking ERBB3**  
 (A) Kaplan-Meier survival curves comparing CCSP-rtTA+;TetO-EGFR<sup>L858R</sup>+ mice with (*ErbB3* +/+, *ErbB3*fl/fl; *Cre*-, *ErbB3* fl/+; *Cre*-), without (*ErbB3* fl/fl; *Cre*+) or heterozygous for (*ErbB3* fl/+; *Cre*+) *ErbB3*. L56, transgenic line 56. (B) Hematoxylin and Eosin staining of EGFR<sup>L858R</sup>-induced tumors that developed in the presence and absence of ERBB3. Representative images of mouse lungs from mice on doxycycline for approximately two months are shown. Bar, 100μm. (C) Hematoxylin and Eosin staining of EGFR<sup>747-752</sup>-induced tumors that developed in the presence and absence of ERBB3. Representative images of mouse lungs from mice on doxycycline for approximately four months are shown. Bar, 100μm.





**Figure 3. Lung tumors that develop in the absence of ERBB3 are sensitive to erlotinib**  
 (A) *CCSP-rtTA* +; *TetO-EGFR<sup>L858R</sup>* +; *TetO-Cre* +; *Erbb3fl/fl* mice respond to short- and long-term erlotinib treatment. Sick mice were treated with erlotinib for 5 days or 4 weeks before they were sacrificed. Mice were imaged prior to (before) and towards the end (after) of treatment. Representative MR images and H&E stained sections are shown. Images of residual tumors observed by histopathology are shown. Bar, 100μm. (B) *CCSP-rtTA* +; *TetO-EGFR<sup>Δ747-752</sup>* +; *TetO-Cre* +; *Erbb3fl/fl* mice respond to short- and long-term erlotinib treatment. Sick mice were treated with erlotinib for 5 days or 4 weeks before they were sacrificed. Mice were imaged prior to (before) and towards the end (after) of treatment. Representative MR images and H&E stained sections are shown. Images of residual tumors observed by histopathology are shown. Bar, 100μm. (C) Waterfall plot showing the change in tumor volume from baseline in mice with mutant *EGFR*-induced lung tumors (*EGFR<sup>L858R</sup>* or *EGFR<sup>Δ747-752</sup>*) upon treatment with erlotinib for 4 weeks. The *Erbb3* genotypes of individual mice are indicated. (D) Lung tumor volume change in mice with *EGFR<sup>L858R</sup>*-induced lung tumors upon treatment with erlotinib for 4 weeks.



#### Figure 4. Intact PI3K and MAPK Signaling in Lung Tumors Deficient for ERBB3

(A) Immunoblots of protein lysates from lungs of mice with EGFR<sup>L858R</sup>-induced lung tumors arising in the presence (EGFR<sup>L858R</sup>+; ERBB3+) or absence (EGFR<sup>L858R</sup>+; ERBB3-) of ERBB3 and probed with antibodies to pAKT, total AKT, pERK and total ERK (top). The data are presented as the mean  $\pm$  the standard error (bottom). (B) Immunoprecipitation of p85 from EGFR<sup>L858R</sup>-induced lung tumors. The immunoprecipitates were blotted with anti-phosphotyrosine and p85 antibodies. The arrow indicates bands the size of EGFR family receptors in the phosphotyrosine immunoblot. Immunoblots of lysates using antibodies to ERBB3, EGFR, p85 and SPC are shown. (C) Phospho-RTK arrays reveal increased phosphorylation of EGFR and ERBB2 upon deletion of ERBB3. Whole lung tumor lysates from eight ERBB3 wild type mice and ten ERBB3 null mice were hybridized to phospho-RTK arrays (top). Signals for EGFR family members on representative arrays are shown. Data are presented as the mean  $\pm$  standard error (bottom). (D) Immunoprecipitation of p85 from lung tumor lysates that develop in the

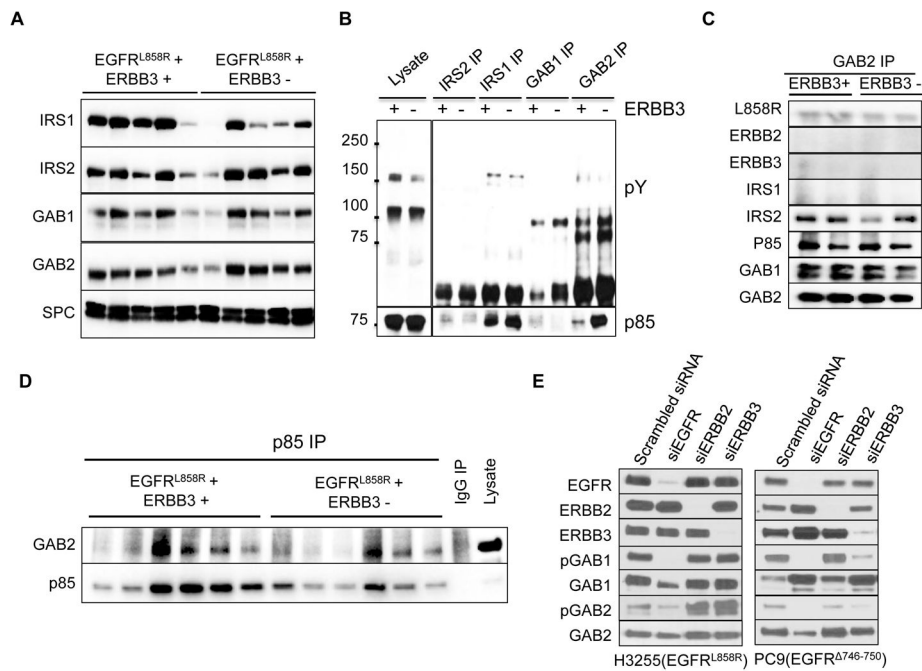
presence (EGFR<sup>L858R+</sup>; ERBB3+) and absence (EGFR<sup>L858R+</sup>; ERBB3-) of *ErbB3*. Immunoprecipitates were immunoblotted with antibodies to ERBB3, EGFR<sup>L858R</sup> and p85. IP, immunoprecipitation. \*indicates p-value  $\leq 0.05$ ; \*\* indicates p-value  $< 0.01$ ; NS, not significant.

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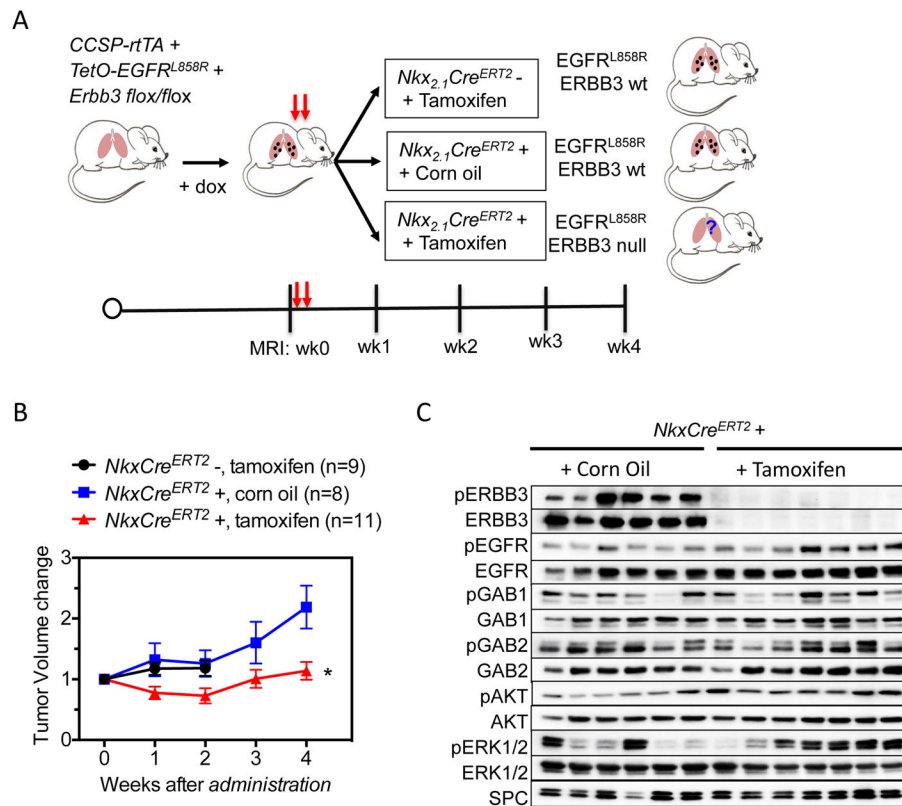
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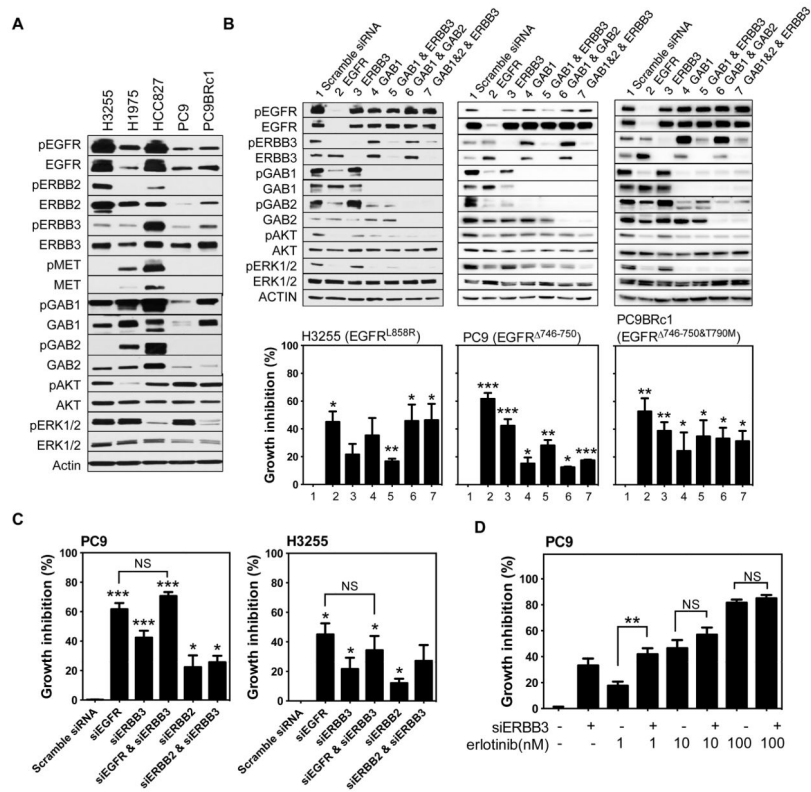


**Figure 5. Adapter proteins bind p85 *in vivo* in EGFR mutant lung tumors**

(A) Immunoblots of lung protein lysates from mice with EGFR<sup>L858R</sup>-induced tumors arising in the presence (EGFR<sup>L858R</sup>+; ERBB3+) and absence (EGFR<sup>L858R</sup>+; ERBB3-) of ERBB3 using antibodies to proteins that can directly bind p85 (IRS1, IRS2, GAB1 and GAB2). The same lysates were blotted with an antibody to SPC as a control for the fraction of pneumocytes present. (B) Co-immunoprecipitation of the adapter proteins IRS1, IRS2, GAB1 and GAB2 from lung protein lysates from mice with EGFR<sup>L858R</sup>-induced tumors arising in the presence (+) and absence (-) of ERBB3. The immunoprecipitates were blotted with antibodies to phosphotyrosine (pY, top) and p85 (bottom). (C) Co-immunoprecipitation of EGFR, GAB1, GAB2, IRS1, IRS2 and p85, with GAB2 from lung protein lysates from mice with EGFR<sup>L858R</sup>-induced tumors arising in the presence (+) and absence (-) of Erbb3. (D) Co-immunoprecipitation of GAB2 with p85 from lung protein lysates from mice with EGFR<sup>L858R</sup>-induced tumors arising in the presence (+) and absence (-) of Erbb3. IP, immunoprecipitation. (E) GAB1 and GAB2 are expressed in EGFR mutant lung cancer cell lines and phosphorylated by EGFR. H3255 and PC9 cells were transfected with either scrambled siRNA or siRNA of EGFR, ERBB2 or ERBB3. Total and phospho- protein levels were analyzed by Western blotting.



**Figure 6. Partial dependence of EGFR<sup>L858R</sup>-induced lung adenocarcinomas on ErbB3**  
 (A) Model for tamoxifen induced deletion of *ErbB3*. Mutant EGFR<sup>L858R</sup> tetra transgenic mice were fed doxycycline to induce tumors. When the tumor size reached a minimum volume of 100 mm<sup>3</sup>, experimental mice (*Nkx2.1-Cre<sup>ERT2</sup>+*) were administered tamoxifen by oral gavage at the doses of 250 mg/day for two days. Control animals were *Nkx2.1-Cre<sup>ERT2</sup>* negative and treated with tamoxifen or *Nkx2.1-Cre<sup>ERT2</sup>* positive treated with corn oil. After tamoxifen treatment, mice were imaged using MRI weekly for 4 weeks. The tumor burden for each group is shown in Fig. 6B. Mouse lungs were collected at the end of 4 weeks or when moribund and tumor extracts were analyzed by Western blot for the indicated proteins (Fig. 6C).



**Figure 7. ERBB3 knockdown partially affects the viability of EGFR mutant, human lung cancer cell lines**

(A) Western blots of protein lysates derived from EGFR mutant lung cancer cell lines probed with antibodies as indicated. (B) H3255, PC9 and PC9BRc1 cells were transiently transfected with pools of siRNAs against EGFR, ERBB3, GAB1, GAB1+ERBB3, GAB1+GAB2, GAB1+GAB2+ERBB3 or a scrambled siRNA control as indicated. Three days after transfection, cells were lysed in RIPA buffer and proteins were analyzed by Western blotting and cell viability was examined using the CellTiter blue assay. Experiments were performed at least three times for each cell line. (C) Viability of PC9 and H3255 cells after transfection with pools of siRNAs against EGFR, ERBB3, EGFR +ERBB3, ERBB2 and ERBB2+ERBB3. Cell viability was measured using the Cell Titer Blue Assay. (D) Growth inhibition in PC9 cells upon ERBB3 knockdown treated with varying concentrations of erlotinib as indicated. \*indicates p-value  $\leq 0.05$ , \*\*\* indicates p-value  $\leq 0.01$ . NS: not significant.