Uncoupling of PI3K from ErbB3 Impairs Mammary Gland Development but Does Not Impact on ErbB2-Induced Mammary Tumorigenesis

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Abstract

The formation of ErbB2/ErbB3 heterodimers plays a critical role in ErbB2-mediated signaling in both normal mammary development and mammary tumor progression. Through 7 phosphoinositide 3-kinase (PI3K) phosphotyrosine-binding sites, ErbB3 is able to recruit PI3K and initiate the PI3K/AKT signaling pathway. To directly explore the importance of the ErbB3/PI3K pathway in mammary development and tumorigenesis, we generated a mouse model that carries a mutant ErbB3 allele lacking the seven known PI3K-binding sites (ErbB3Δ85). Mice homozygous for the ErbB3Δ85 allele exhibited an initial early growth defect and a dramatic impairment of mammary epithelial outgrowth. Although homozygous adult mice eventually recovered from the growth defect, their mammary glands continued to manifest the mammary outgrowth and lactation defects throughout their adult life. Interestingly, despite the presence of a profound mammary gland defect, all of the female ErbB3Δ85 mice developed metastatic ErbB2-induced mammary tumors secondary to mammary epithelial expression of an activated ErbB2 oncoprotein capable of compensatory PI3K signaling from both EGF receptor and ErbB2. Our findings therefore indicate that, although ErbB3-associated PI3K activity is critical for mammary development, it is dispensable for ErbB2-induced mammary tumor progression. Cancer Res; 72(12); 3080–90. © 2012 AACR.

Introduction

ErbB2 is a member of the EGF receptor (EGFR) family of receptor tyrosine kinases (RTK), composed of 4 closely related type 1 RTKs including the EGFR (ErbB1/HER1), ErbB2 (Neu/HER2), ErbB3 (HER3), and ErbB4 (HER4; ref. 1). Lacking a direct ligand, ErbB2 is activated through heterodimerization with other EGFR family members (2). Similarly, transphosphorylation and activation of ErbB3 depends on its capacity to heterodimerize with other EGFR family members because it lacks intrinsic tyrosine kinase activity (3). An abundance of evidence has highlighted an important role for the ErbB2/ErbB3 heterodimer in human breast cancer (4–8). It is well-established that 20% to 30% of tumors have genomic amplification of ERBB2 (9), which inversely correlates with patient survival (10). Increased ErbB3 expression relative to normal tissue is also observed in 20% to 30% of breast cancer cases (11–13) where it is frequently co-overexpressed with ErbB2 (12, 14, 15). Conversely, inactivation of ErbB2 by blocking antibodies decreases ErbB3 tyrosine phosphorylation and inhibits cell proliferation (16), whereas transcriptional repression of ErbB3 expression blocks ErbB2-induced proliferation in cell culture models (17). Evidence supporting the importance of ErbB2/ErbB3 signaling in breast cancer is also derived from numerous transgenic mouse models (18). Analysis of transgenic mouse models of ErbB2-driven breast cancer has revealed constitutive tyrosine phosphorylation of ErbB3 and a 10- to 20-fold elevation in ErbB3 expression compared with adjacent normal tissue (15). More recently, mammary-specific ablation of ErbB3 has been shown to impair mammary tumor development in the polyomavirus middle T model of mammary tumor progression (19).

Mechanistically, the apparent requirement for coexpression of ErbB2 and ErbB3 in both human and transgenic breast cancers may reflect their activation of distinct but complimentary signaling pathways that cooperate during mammary tumor progression. Consistent with this view, ErbB2 recruits adapter proteins that primarily function through the Ras signaling pathway (20), whereas ErbB3 recruits the regulatory p85 subunit of phosphoinositide 3-kinase (PI3K; refs. 21, 22). Thus, in the context of cancer, the role of ErbB3 may be to couple activated ErbB2 to PI3K/AKT signaling and thereby promote ErbB2-mediated tumor progression.

Importantly, besides their role in mammary gland pathology, ErbB2 and ErbB3 are also required for normal mammary gland development (23, 24). Although germline ablation of erbB2 or erbB3 causes embryonic lethality (25), Cre-mediated recombination has been used to induce mammary-specific...
deletion of erbB2, causing a striking ductal elongation defect and reduced branching (4). Furthermore, transgenic mice expressing a dominant-negative ErbB2 in the mammary gland have defective lobuloalveolar development and reduced milk protein secretion (26) and transplanted mammary buds from genetically rescued ErbB2-deficient embryos are unable to support ductal outgrowth during puberty (27). It has more recently been shown that ErbB3-null mammary buds present a very similar phenotype that is maintained through adulthood (28).

To directly explore the importance of the ErbB3/PI3K pathway in mammary development and tumorogenesis, we have studied mammary ductal outgrowth and ErbB2-induced mammary tumor progression in mice bearing an erbB3 knock-in allele lacking the 7 PI3K-binding sites (herein referred to as ErbB3D85). In contrast to the normal ductal outgrowth that is observed in both wild-type (WT) and heterozygous ErbB3D85/wt females, mice homozygous for ErbB3D85 alleles exhibit a marked and persistent inhibition of mammary ductal outgrowth. This was also associated with a non-Mendelian pattern of inheritance indicating a degree of embryonic lethality. Interestingly, our data also showed that all female ErbB3D85 mice developed metastatic ErbB2-induced mammary tumors, albeit with a delayed onset. Our results indicate that, although ErbB3-associated PI3K activity is critical for mammary development, it is dispensable for ErbB2-induced mammary tumor progression.

Materials and Methods

Generation of the ErbB3D85 allele

A cDNA containing the full open reading frame of human ErbB3 and the 3'-untranslated region was obtained from Y. Yarden. Tyrosine-encoding codons at positions 941, 1054, 1197, 1222, 1260, 1276, and 1289 [within YXXM PI3K consensus binding sites (29)], were mutated at the second position (A to T) into phenylalanine-encoding codons. The targeting strategy is described further in Supplementary Material.

Transgenic mice

ErbB3D85 mice were interbred with MMTV-NDL2-5 mice (15) and genotyped by PCR. Experimental and control virgin mice were monitored for mammary tumor formation by twice-weekly palpation. All experiments involving animals were conducted in accordance with McGill University (Montreal, QC, Canada) animal care guidelines.

Tissue preparation and histology

Mammary gland wholemounts were prepared as previously described (30). For developmental studies, mammary glands were harvested from 4-, 6-, 8-, and 12-week-old virgin mice, lactating mice at day 3 and 9, and involuting glands were harvested at day 1, 3, 5, 7, 10, and 14 postweaning. For histology, tissues were sectioned at 5 μm and hematoxylin and eosin (H&E)-stained. Pulmonary metastases were identified by microscopic analysis of five 50 μm step-sections from lungs of mice bearing tumors for 8 weeks.

Immunohistochemical analysis of tissue sections and in situ apoptosis assays

For immunohistochemistry, tissue sections were prepared as described previously (30). For primary antibody details, see Supplementary Material. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays were conducted using the Apoptag Peroxidase In Situ Apoptosis Detection Kit (S7100, Millipore) as per the manufacturer’s protocol. Sections were scanned using a ScanScope XT Digital Slide Scanner (Aperio) and a nuclear algorithm was conducted on at least 10 independent fields for Ki67 and TUNEL quantifications. For immunohisto-fluorescence, sections were incubated with Alexa Fluor secondary antibodies (Molecular Probes) for 1 hour at room temperature followed by incubation with DAPI (Sigma) for 10 minutes and visualized using a Zeiss LSM 510 META confocal microscope.

Transplantation experiments

Mammary epithelial cells were prepared as previously described (30) from two 10- to 12-week-old control and 3 age-matched ErbB3D85 mice per experiment. Then, 100,000 cells were injected into surgically cleared left inguinal glands of prepubescent (3 weeks) control or ErbB3D85 female recipients. Reconstituted mammary glands were harvested 8 weeks postimplantation. Contra-lateral glands from the same recipient were used as controls.

Preparation of mammary epithelial cells, tumor tissue harvesting, Western blotting, and immunoprecipitations

Mammary epithelial cells and tumor tissues were isolated and lysed as described previously (15, 30). Antibodies for Western blots are described in Supplementary Material. Immunoprecipitations were carried out as described previously (15). Immunoblots were visualized using horseradish peroxidase–conjugated secondary antibodies (Jackson ImmunoResearch) and enhanced chemiluminescence (Amersham).

PI3K activity assay

Immunoprecipitates (from 500 μg mammary gland protein) were extensively washed and resuspended in 50 μL of kinase assay buffer (20 mmol/L Tris-HCl, pH 8.0, 100 mmol/L NaCl, 1 mmol/L EGTA) containing 0.5 mg/mL 1-α-phosphatidylinositol sodium salt (Avanti Polar Lipids), 20 mmol/L MgCl2 and 10 μCi ATP [γ32P] (Perkin Elmer) and assayed for PI3K activity as described elsewhere (31).

Results

Mice homozygous for the ErbB3D85 exhibit an early growth defect

To directly assess the in vivo contribution of coupling the PI3K to ErbB3, we have generated a knock-in allele of erbB3 in which the tyrosine residues in the 7 PI3K-binding sites are mutated to alanine (Fig. 1A and Supplementary Fig. S1). Because genetic background can influence the phenotypes obtained after EGFR targeted disruption (32), we introgressed the ErbB3D85...
strain to a uniform FVB genetic background and evaluated whether we could recover viable homozygous ErbB3\textsuperscript{Δ85} animals. Mice heterozygous for the ErbB3\textsuperscript{Δ85} allele were healthy and fertile. However, we observed an abnormal segregation of genotypes in the progeny of crosses between 2 heterozygous parents (Supplementary Table S1). Mating between heterozygotes produced homozygous pups at a reduced frequency (10% instead of the expected 25% Mendelian ratio).

Although viable mice homozygous for the ErbB3\textsuperscript{Δ85} allele could be generated, both males and females had lower weights at birth and exhibited a significant growth defect compared with WT littermates (Fig. 1B and C). However, after 6 weeks the homozygous ErbB3\textsuperscript{Δ85} animals had regained normal growth rates (Fig. 1D). These observations indicate that although loss of ErbB3-associated PI3K is compensated at puberty, it is associated with both reduced viability and early growth defects that suggest a critical role for ErbB3–PI3K signaling in the early stages of development and prepubertal growth.

Loss of ErbB3-associated PI3K activity results in a severe mammary ductal outgrowth defect

Given the growth phenotype observed in ErbB3\textsuperscript{Δ85} animals, we next evaluated whether mammary gland development was...
perturbed in these mice by conducting wholemount analyses. In contrast to WT or ErbB3Δ85 heterozygous female mice, ErbB3Δ85 homozygous mammary epitheloid cells of control or heterozygous ErbB3Δ85/wt and homzygous ErbB3Δ85/Δ85 6-week-old mice. The immunoprecipitates were then subjected to PI3K activity assays (PI3P). Representative autoradiogram of PI3K activity assay (n = 3) on anti-ErbB3 is shown. As a control, ErbB3 abundance in the IP material was revealed by Western blotting (WB) and part of the total lysate was probed for cytokeratin-8 (CK8).

Figure 2. ErbB3-associated PI3K activity is required for mammary gland development. A, representative mammary gland wholemounts prepared from virgin control (ErbB3wt/wt) and heterozygous ErbB3Δ85/wt or homozygous ErbB3Δ85/Δ85 mice at 4, 6, 8, and 12 weeks of age. White arrows represent the distance from the lymph node to the end of the terminal end bud. B, quantification of ductal growth is shown—midpoint of the lymph node was used as the reference. Values are presented as mean ± SEM. Both no. 4 inguinal glands of 4 mice/genotype at each time point were analyzed. C, ErbB3 was immunoprecipitated (IP) from isolated mammary epithelial cells of control or heterozygous ErbB3Δ85/wt and homzygous ErbB3Δ85/Δ85 6-week-old mice. The immunoprecipitates were then subjected to PI3K activity assays (PI3P). Representative autoradiogram of PI3K activity assay (n = 3) on anti-ErbB3 is shown. As a control, ErbB3 abundance in the IP material was revealed by Western blotting (WB) and part of the total lysate was probed for cytokeratin-8 (CK8).
from females homozygous for the ErbB3Δ85 allele exhibited the same ductal outgrowth defect in the cleared fat pads from either WT or ErbB3Δ85 recipient mice (Fig. 3A). These data indicate that the ductal outgrowth defect observed in ErbB3Δ85 mammary glands is mammary epithelial cell-autonomous.

Given that the ErbB3Δ85 allele is unable to couple to PI3K cell survival signaling, we determined whether the ErbB3Δ85 mammary glands exhibited elevated levels of apoptosis or defective proliferation by measuring cleaved caspase-3 and Ki67 staining in mammary epithelium. ErbB3Δ85 mammary glands exhibited comparable Ki67 staining to WT controls but significantly elevated caspase-3 staining (Fig. 3B and C). Taken together, these results argue that the dramatic ductal outgrowth defect in ErbB3Δ85 mammary glands is because of impairment of the ErbB3/PI3K cell survival pathway.

Loss of ErbB3-associated PI3K activity impairs lactation and results in accelerated involution

To determine whether the observed ductal outgrowth defect in mutant ErbB3Δ85 mice could affect lactation, we conducted whole mount analyses on mammary glands from day-3 lactating WT and ErbB3Δ85 mice. Although ErbB3Δ85 mice formed secretory terminal alveoli, they appeared less expanded and failed to fill the fat pad to the same extent as in control mice (Supplementary Fig. S2A). Histologic analyses of day-3 lactating control glands showed fully distended alveoli and flattened epithelium with negligible presence of adipose tissue. However, corresponding mammary tissue from ErbB3Δ85 dams contained thicker epithelium with sparser and less expanded alveoli, smaller alveolar cavities, and large areas of adipose tissue (Supplementary Fig. S2B and S2C). Moreover, large cytoplasmic lipid droplets within...
the epithelial cells suggested a lower milk accumulation in the alveolar lumens (Supplementary Fig. S2C). Immunohistochemical analyses of sections of lactating mammary glands using antibodies against 2 different milk proteins (β-casein and WAP) revealed decreased expression of these proteins in ErbB3Δ85 epithelial cells compared with WT epithelium (Supplementary Fig. S2D), confirming a block in differentiation. Furthermore, β-casein and WAP, which were abundant and present in all alveolar lumens of lactating WT glands, were absent in a large proportion of the ErbB3Δ85 alveolar luminal spaces (Supplementary Fig. S2C and S2D). Overall, these results indicate coupling of PI3K to ErbB3 is required for the mammary epithelial differentiation process during lactation.

**Figure 4.** Involution is accelerated in mammary glands from ErbB3Δ85 mice. A, representative H&E-stained sections of mammary glands from control WT and ErbB3Δ85 mice at days 1, 3, 5, 7, 10, and 14 of involution (I). Three mice/genotype at each time point were analyzed. Scale bar, 200 μm. B, paraffin-embedded sections of mammary glands from control and ErbB3Δ85 mice at days 1, 3, 7, and 10 of involution (I) were analyzed by TUNEL assay. Three mice/genotype at each time point were analyzed. Scale bar, 100 μm. C, quantification of the reappearance of adipose tissue is represented as percentage area (±SEM) occupied by adipocytes and was calculated after counting multiple fields from 3 animals/genotype at each time point. *, P < 0.05; **, P < 0.01 (t test). D, quantifications of the TUNEL-positive cells are represented as percentage of epithelial luminal mammary cells (±SEM) and were calculated following counting multiple fields from 3 animals/genotype at each time point. ***, P < 0.01 (t test).
To determine the effect of the ErbB3<sup>Δ85</sup> mutation on epithelial cell apoptosis and mammary tissue remodeling during involution, pups were withdrawn from day-12 lactating control and ErbB3<sup>Δ85</sup> dams. Histologic examination of control and ErbB3<sup>Δ85</sup> mammary gland sections at day-1 of involution revealed that the majority of the secretory epithelium was composed of alveoli with ErbB3<sup>Δ85</sup> glands containing fewer and smaller alveoli and larger areas of adipose tissue. As involution progressed in control mice, most of the alveoli had collapsed between day-7 and day-10 (Fig. 4A). In contrast, ErbB3<sup>Δ85</sup> mice showed an accelerated involution with largely complete remodeling of lobuloalveolar structures by day-3, resulting in glands that resembled the morphology seen on day-5 in control glands containing fewer and smaller alveoli and larger areas of adipose tissue. As involution progressed in control mice, most of the alveoli had collapsed by day-3 and day-7 involuting glands possessed a higher proportion of apoptotic cells than control glands (Fig. 4B and D). At day-10 of involution, the apoptotic rates were similar between control and ErbB3<sup>Δ85</sup> mice, consistent with the fact that the majority of the gland had been remodeled (Fig. 4B and D). These results indicate that ErbB3<sup>Δ85</sup> glands exhibit enhanced involution due to an increase in apoptotic death.

Loss of ErbB3 PI3K-coupled signaling is dispensable for ErbB2 mammary tumor induction

We next investigated whether expression of the ErbB3<sup>Δ85</sup> alleles would impact ErbB2-induced mammary tumor progression. To accomplish this, MMTV-activated erbB2 (NDL2-5 strain) males (15) were interbred with females heterozygous or homozygous for ErbB3<sup>Δ85</sup> alleles. Cohorts of female mice bearing the activated erbB2 transgene were subsequently monitored for mammary tumor development by physical palpation. Compared to NDL2-5 mice bearing WT erbB3 alleles, development of mammary tumors was significantly delayed in homozygous ErbB3<sup>Δ85</sup> females (Fig. 5A). Mice heterozygous for the ErbB3<sup>Δ85</sup> allele also developed tumors with delayed onset albeit at an intermediate point between control and homozygous ErbB3<sup>Δ85</sup> carriers (Fig. 5A). Consistent with the delay in tumor onset, whole mount analyses of mammary glands derived from non-tumor-bearing females at 6 months of age revealed that uncoupling ErbB3 from PI3K signaling resulted in a significant reduction in the number of early mammary hyperplastic lesions (Fig. 5B and C). Nevertheless, the results revealed that all ErbB3<sup>Δ85</sup> female mice eventually developed mammary tumors (Fig. 5A).

Histologic examination of tumors derived from ErbB3<sup>Δ85</sup>/NDL2-5 mice revealed a morphology similar to that observed in control tumors (Supplementary Fig. 5A). ErbB3<sup>Δ85</sup> tumors showed the typical luminal phenotype composed of large nodular nests with central necrosis exhibited by the NDL2-5 tumors (Supplementary Fig. 5A) and both control and ErbB3<sup>Δ85</sup> tumors were uniformly cytokeratin-8 positive (Supplementary Fig. 5B). Moreover, tumors from both genotypes exhibited comparable proliferative activity (Supplementary Fig. S5A) and low levels of apoptotic cell death (Supplementary Fig. S5B). These data indicate that although loss of ErbB3 PI3K signaling does impact on ErbB2 tumor onset, it is ultimately dispensable for ErbB2 tumor induction.

Given that loss of ErbB3–PI3K association had only a modest effect on the initiation of ErbB2-induced tumors, we next examined whether ErbB3 mutation altered the metastatic capacity of the resulting tumors. Lungs from control and ErbB3<sup>Δ85</sup>/NDL2-5 tumor-bearing mice at end-stage were scored for the presence of metastatic lesions (Supplementary Fig. S3A and S3B). Although ErbB3<sup>Δ85</sup>/NDL2-5 mice exhibited a slightly reduced level of metastasis, the percentage of 8-week tumor-bearing mice that developed lung metastases as well as the number of metastases present in the lungs were not significantly different than controls.
ErbB3 activity assays (PI3P). Representative autoradiogram of PI3K activity of NeuNDL2-5 tumor lysates. Immunoprecipitates were subjected to PI3K assay done from 3 samples/genotype is shown. B, ErbB3 IP from ErbB3<sup>wt/wt</sup>/NDL2-5 tumors (Supplementary Fig. S6). Moreover, the levels of expression and phosphorylation of p60Src kinase, which can be involved in activation of PI3K signaling by several RTKs, and ShcA, which can function as an adaptor in the initiation of PI3K-dependant signaling pathways (34) remained similar between control and ErbB3<sup>Δ85</sup> tumor lysates (Supplementary Fig. S6).

Another possible mechanism for ErbB2 mammary tumors to circumvent the requirement for ErbB3-associated PI3K signaling is that other EGFR family dimerization partners are capable of activating the PI3K pathway independently of ErbB3. For example, both EGFR and ErbB2 can recruit docking proteins such as Gab1 that in turn can bind the regulatory p85 subunit of PI3K, which can be involved in activation of PI3K signaling by several RTKs, and ShcA, which can function as an adaptor in the initiation of PI3K-dependant signaling pathways (34). To test this possibility, we measured the levels of PI3K activity associated with either ErbB2 or EGFR immunoprecipitates derived from control and ErbB3<sup>Δ85</sup> mammary tumors. The results showed that ErbB2 and EGFR immunoprecipitates from both genetic backgrounds possessed comparable levels of PI3K-associated activity (Fig. 7A and C). Furthermore, both ErbB2 and EGFR immunoprecipitates were found in a complex with p85 subunit (Fig. 6B) and exhibited very low levels of ErbB3 tyrosine phosphorylation (Fig. 6B and C), although tumors from all genetic backgrounds expressed comparable protein levels of ErbB3 and ErbB2 (Fig. 6C). Consistent with a lack of effect on tumor cell proliferation and death, comparable levels of ERK activity and expression of the proliferation markers cyclin D1 and PCNA and the apoptosis marker cleaved-caspase-3 were detected (Fig. 6C and Supplementary Fig. S5C). ErbB3<sup>Δ85</sup> tumors expressed comparable levels of EGFR, ErbB2, and ErbB4 to controls, with no apparent differences in tyrosine phosphorylation of ErbB2 and EGFR (Supplementary Fig. S4C). Together, these data strongly suggest that oncogenic signaling by other EGFR family members is preserved in the absence of ErbB3–PI3K coupling.

Although upregulation of the expression or activity of other EGFR family members was not observed in ErbB3<sup>Δ85</sup> tumors, it is possible that the requirement for ErbB3-associated PI3K signaling can be bypassed by upregulating components involved in the PI3K–AKT signaling pathway (33). Examination of the levels and state of activation of immediate molecular partners directly involved in regulating the activity of PI3K or AKT, such as PTEN and PDK1, or of effectors involved in metabolism (GSK-3), cell survival (Bad), or protein synthesis (p70 S6 kinase, mTOR, and 4E-BP1) revealed no substantial differences between WT and ErbB3<sup>Δ85</sup>/NDL2-5 tumors (Supplementary Fig. S6). Moreover, the levels of expression and phosphorylation of p60Src kinase, which can be involved in activation of PI3K signaling by several RTKs, and ShcA, which can function as an adaptor in the initiation of PI3K-dependant signaling pathways (34) remained similar between control and ErbB3<sup>Δ85</sup> tumor lysates (Supplementary Fig. S6).

To confirm that the mutant ErbB3 receptor expressed in the ErbB2-induced tumors was impaired in its capacity to activate PI3K, we measured the levels of PI3K activity associated with ErbB3 immunoprecipitates from the mammary tumors of control and ErbB3<sup>Δ85</sup> mutant animals. In contrast to the robust phosphorylation of PIP2 to PIP3 observed in the ErbB3 immunoprecipitates from control tumors, very low levels of phosphorylated PIP3 were detected in ErbB3 immunoprecipitates from ErbB3<sup>Δ85</sup>/NDL2-5 tumors (Fig. 6A). Consistent with the reduced level of PI3K activity, ErbB3 immunoprecipitates also showed loss of association with the p85 subunit (Fig. 6B) and exhibited very low levels of ErbB3 tyrosine phosphorylation (Fig. 6B and C), although tumors from all genetic backgrounds expressed comparable protein levels of ErbB3 and ErbB2 (Fig. 6C). Consistent with a lack of effect on tumor cell proliferation and death, comparable levels of ERK activity and expression of the proliferation markers cyclin D1 and PCNA and the apoptosis marker cleaved-caspase-3 were detected (Fig. 6C and Supplementary Fig. S5C). ErbB3<sup>Δ85</sup> tumors expressed comparable levels of EGFR, ErbB2, and ErbB4 to controls, with no apparent differences in tyrosine phosphorylation of ErbB2 and EGFR (Supplementary Fig. S4C). Together, these data strongly suggest that oncogenic signaling by other EGFR family members is preserved in the absence of ErbB3–PI3K coupling.

**ErbB2 and EGFR can functionally compensate for the defective ErbB3<sup>Δ85</sup> allele**

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EGFR and ErbB2 can activate the PI3K signaling pathway at levels that functionally substitute for the defective erbB3 allele.

Discussion

Biochemical and genetic analyses of ErbB2-induced tumor progression have revealed that elevated levels of tyrosine phosphorylated ErbB3 are detected in ErbB2-driven tumors (15). Given that ErbB2/ErbB3 heterodimers have been considered the most potent oncogenic ErbB dimers (3), this suggests that ErbB3 plays a critical role in ErbB2-induced mammary tumorigenesis. Several studies have indicated that the potent transforming potential of the ErbB2/ErbB3 heterodimer is because of the ability of ErbB3 to recruit PI3K through multiple consensus binding sites for the p85 subunit located in its C-terminal tail (3, 5, 17). Although these studies provide evidence that ErbB3 is a key player in established breast cancer cell lines, whether the ErbB3/PI3K signaling axis is required for ErbB2-induced breast cancer progression remained to be established in vivo. Using a mouse model that carries an erbB3 knock-in allele where the known PI3K binding tyrosines were mutated to phenylalanines, we show herein that although ErbB3-associated PI3K activity is required for normal ductal morphogenesis as well as for proper lobuloalveolar differentiation during lactation and involution, expression of the erbB3<sup>Δ</sup> allele had only a modest impact on ErbB2 mammary tumor induction and progression. Our observations argue that, in the context of activated ErbB2 signaling, ErbB3–PI3K coupling is dispensable for ErbB2-induced mammary cancer progression.

Although viable mice carrying both ErbB<sub>3</sub><sup>ΔΔS</sup> alleles could be generated, these mice initially exhibited a generalized growth defect as well as a non-Mendelian pattern of inheritance indicative of a certain degree of embryonic lethality. This is consistent with observations of a late embryonic lethal phenotype in ErbB3-null mice because of severe neuropathies (36). The sub-Mendelian ratio of homozygous ErbB3<sup>ΔΔS</sup> pups may reflect a less severe manifestation of this phenotype. The observation that surviving ErbB3<sup>ΔΔS</sup> mice exhibited a growth defect raises the intriguing possibility that ErbB3/PI3K is required for ductal outgrowth in an epithelial cell autonomous manner is consistent with recent studies where ErbB3-null embryos were implanted into the cleared fat pads of immunodeficient mice (28). Like the ErbB3<sup>ΔΔS</sup> strain, the erbB3-null mammary epithelium exhibited a dramatic ductal outgrowth defect that persisted throughout adulthood and was cell autonomous in origin (28). In contrast to ErbB3, genetic ablation of ErbB2 resulted in a transient ductal outgrowth defect (4, 27). The inability of the ErbB3 defective mammary epithelium to recover indicates that, unlike ErbB2,
the hormonal stimuli that occur during sexual maturation cannot overtake the requirement for ErbB3-coupled cell survival pathways, thus leading to the observed increase in apoptotic cell death (Fig. 3).

In addition to the profound defect in ductal morphogenesis, the ErbB3<sup>–/–</sup> female mice exhibit accelerated mammary gland involution and impaired lactation (Supplementary Figs. S2 and S4). As with the ductal outgrowth defect, the accelerated involution defect observed in ErbB3<sup>–/–</sup> female mice is associated with elevated apoptotic death (Fig. 4B and D). Although the molecular basis for the lactation defect is unclear, reduced β-casein production in the ErbB3<sup>–/–</sup> female indicates that ErbB3/PI3K signaling may also impact on the differentiation status of mammary epithelial cells. Because ErbB3 is a coreceptor for the heresulin growth factor family that plays an important role in mammary gland development (24, 39), it is not unexpected that disruption of this ErbB3/PI3K axis would induce such a severe defect in mammary epithelial development.

In contrast to the profound effects of loss of ErbB3/PI3K signaling on mammary development, the effect on ErbB2-driven tumorigenesis was modest. After normalization for area of epithelial outgrowth, there was a small but statistically significant reduction in the burden of ErbB2 induced lesions observed in females homozygous for ErbB3<sup>–/–</sup> compared with the WT MMTV/ErbB2 mice. Consistent with this observation, expression of the ErbB3<sup>–/–</sup> allele resulted in a minor delay in the onset of ErbB2 expressing mammary tumors and no significant affect on metastatic potential. Despite the shown inability of ErbB3<sup>–/–</sup> to recruit the p85 subunit of PI3K, leading to a dramatic reduction of ErbB3-associated PI3K activity (Fig. 6), we show that the activity of the major PI3K effecter AKT is not affected in tumors from ErbB3<sup>–/–</sup> mice. Furthermore, ErbB2-induced mammary tumors derived from the ErbB3<sup>–/–</sup> genetic background did not exhibit any indication of enhanced apoptotic cell death, strongly suggesting preservation of PI3K/AKT-associated survival signaling.

Our data show that ErbB2 and EGFR are associated with significant PI3K activity in tumors expressing ErbB3<sup>–/–</sup>. This suggests that activated ErbB2 and EGFR can functionally compensate in this model by mediating levels of PI3K signaling sufficient to drive ErbB2 tumor progression. As we previously showed that ErbB2/EGFR heterodimers could be detected in tumors derived from an MMTV/activated ErbB2 strain, the PI3K activity shown in Fig. 7C could be due to formation of ErbB2/EGFR heterodimers and recruitment and tyrosine phosphorylation of the Gab1 scaffold protein (35). Because Gab adapter family are known to recruit the PI3K signaling pathway (35, 40), this could allow sustained recruitment of Gab-1 that indirectly activates the PI3K signaling. Consistent with the importance of adapter proteins as scaffolds involved in PI3K signaling, we recently showed that the SH2 domain of the ShcA adapter can activate the PI3K signaling pathway through a multiprotein complex comprising ShcA, 14-3-3<sub>ζ</sub>, and the p85 regulatory subunit of PI3K (41). Given that ErbB2 can recruit the ShcA through multiple tyrosine docking sites (42), the robust ErbB2/PI3K signaling activity observed in ErbB2/ ErbB3<sup>–/–</sup> tumors (Fig. 7A), reflects the indirect recruitment of PI3K to ErbB2 through the ShcA scaffold.

Although we clearly show that ErbB3/PI3K signaling is dispensable for ErbB2-mediated mammary tumorigenesis, it is intriguing that conditional ablation of the entire ErbB3 protein results in a profound defect in ErbB2-driven tumor progression that can be partially rescued by an ErbB3<sup>–/–</sup> mutant (R. Cook and C. Arteaga, personal communication). Taken together with our observations, these data argue that ErbB3 has important PI3K-independent functions in ErbB2 mammary tumor progression. Elevated levels of ErbB3 have also been linked to resistance to small molecule inhibitors that target ErbB2 or PI3K signaling (43, 44). The availability of humanized antibodies directed against ErbB3/ErbB2 (45) raises the intriguing possibility that a combination of antibody therapy and small molecule inhibitors will act synergistically to block ErbB2 tumor progression. Whether ErbB2 mammary tumor cells derived from ErbB3<sup>–/–</sup> mice are sensitized to these small molecule inhibitors awaits future experimental validation.

Disclosure of Potential Conflicts of Interest
W.J. Muller has ownership interest in Genentech. No potential conflicts of interests were disclosed by the other authors.

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Uncoupling of PI3K from ErbB3 Impairs Mammary Gland Development but Does Not Impact on ErbB2-Induced Mammary Tumorigenesis

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