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PIK3CA^{H1047R} and Her2 initiated mammary tumors escape PI3K dependency by compensatory activation of MEK-ERK signaling

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Abstract

Human breast cancers that have HER2 amplification/overexpression frequently carry *PIK3CA* mutations, and are often associated with a worse prognosis. However, the role of *PIK3CA* mutations in the initiation and maintenance of these breast cancers remains elusive. In the present study, we generated a compound mouse model that genetically mimics HER2 positive breast cancer with coexisting *PIK3CA*^{H1047R}. Induction of *PIK3CA*^{H1047R} expression in mouse mammary glands with constitutive expression of activated Her2/Neu resulted in accelerated mammary tumorigenesis with enhanced metastatic potential. Interestingly, inducible expression of mutant PIK3CA resulted in a robust activation of PI3K/AKT signaling but attenuation of Her2/Her3 signaling, and this can be reversed by deinduction of *PIK3CA*^{H1047R} expression. Strikingly, while these Her2^{+} PIK3CA^{H1047R} initiated primary mammary tumors are refractory to HER2-targeted therapy, all tumors responded to inactivation of the oncogenic *PIK3CA*^{H1047R}, a situation closely mimicking the use of a highly effective inhibitor specifically targeting the mutant PIK3CA/p110a. Notably, these tumors eventually resumed growth, and a fraction of them escaped PI3K dependence by compensatory ERK activation, which can be blocked by combined inhibition of Her2 and MEK. Together, these results suggest that PIK3CA-specific inhibition as a monotherapy followed by combination therapy targeting MAPK and HER2 in a timely manner may be an

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Conflict of interest

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effective treatment approach against HER2 positive cancers with coexisting *PIK3CA*-activating mutations.

Keywords

PI3K; Her2 positive breast cancer; genetic mouse model; drug resistance; targeted therapy

Introduction

HER2 amplification/overexpression occurs in 15–20% of breast cancers.[1] The introduction of HER2-targeted therapies, such as trastuzumab, lapatinib, and pertuzumab, has changed the natural history of HER2 positive breast cancers and provided considerable clinical benefits for patients.[1, 2] However, some patients with early-stage disease still relapse despite standard adjuvant therapy, and in the metastatic setting, resistance to HER2-targeted therapies nearly always develops over time.[1–3] Multiple lines of evidence have suggested that the activation of the phosphatidylinositol-3-kinase (PI3K) signaling pathway correlates with resistance to HER2-directed therapies, although clinical data in the neoadjuvant and adjuvant settings have been somewhat conflicting.[4–11]

The PI3K pathway is genetically altered in more than 70% of breast cancers with *PIK3CA*, the gene that encodes the p110α catalytic subunit of PI3K, being one of the most commonly mutated genes.[12, 13] It has been found that cancer-associated *PIK3CA* mutations are present in hot spots, mainly E545K/E542K and H1047R.[14] We recently established a genetic mouse model of breast cancer that allows conditional expression of human $PIK3CA^{H1047R}$ in a doxycycline-inducible manner.[15] This genetic mouse model provides a unique tool to explore the molecular mechanisms underlying breast cancer tumorigenesis and progression. Interestingly, tumor responses to the inactivation of $PIK3CA^{H1047R}$ were heterogeneous,[15] suggesting that additional molecular drivers may regulate the degree to which tumor cells are addicted to the initiating oncogene. Of note, the finding that a fraction of $PIK3CA^{H1047R}$-initiated mammary tumors showed a complete response to transgene inactivation lends further credence to ongoing efforts towards the development of p110a mutant-specific inhibitors.[16, 17] Such inhibitors are particularly attractive therapeutic targets as they would presumably avoid the toxicity caused by inhibition of the wild-type form of PI3K-p110a.[13]

HER2 is a receptor tyrosine kinase that enhances the PI3K/AKT signaling pathway and the MAPK signaling pathway mainly through HER2/HER3 hetero-dimerization[2], or activation of the MAPK signaling pathway preferentially through HER2 homodimerization.[18] Recent comprehensive genomic characterization revealed that up to 40% of *HER2* positive breast cancers carry mutations in *PIK3CA*,[19] and that this circumstance correlates with lymph node metastases and poor patient outcome.[20–22] Such findings suggested that *PIK3CA* mutations may represent an important determinant of resistance to anti-HER2 therapies, thereby justifying further investigation of PI3K as a therapeutic target in HER2 positive breast cancer.

Recent studies have shown that PI3K/AKT inhibition induces the expression and phosphorylation of multiple receptor tyrosine kinases, including HER3, and this may attenuate their antitumor effects.[23, 24] While these studies elegantly delineated the oncogenic rewiring of signaling pathways in cancer cell models as a result of targeted inhibition of PI3K/AKT signaling, direct in vivo evidence that supports this notion is still lacking. Thus, an appropriate animal model that recapitulates genetic and molecular aspects of human cancers is needed to test this hypothesis in a physiological context.

Clinical trials testing PI3K inhibitors alone or in combination with HER2-directed therapies are ongoing (*clinicaltrials.gov*). Hence, knowledge gained from understanding the impact of PI3K activation on HER2 positive breast cancer can be used to optimize HER2-targeted cancer therapies, leading to rational design of effective treatment strategies for this subtype of breast cancer also harboring oncogenic *PIK3CA* mutations. We generated a compound mouse model of mammary tumors in which an oncogenic mutation of human *PIK3CA*, H1047R, is expressed in a doxycycline-inducible manner while the activated *Her2/Neu* is constitutively expressed. Using this model, we investigated the impact of PI3K activation on tumor initiation and maintenance of HER2 positive breast cancer. In addition, we explored molecular mechanisms of tumor escape from PI3K targeted treatment in HER2 positive breast cancer with co-existing *PIK3CA*-activating mutations.

Results

Mutant *PIK3CA* cooperates with Her2 to accelerate mammary tumor formation

Recent comprehensive characterization of the breast cancer genome revealed that a significant fraction of HER2 positive breast tumors harbor activating PIK3CA mutations,[19] suggesting that oncogenic *PIK3CA* and *Her2/Neu*, two of the most common genetic alterations in breast cancer, may cooperate in breast tumorigenesis. To test this hypothesis, we set out to generate a compound genetic mouse model that mimics Her2 positive breast cancers coexpressing an oncogenic *PIK3CA* mutation. *MMTV-NIC* (hereafter designated *NIC*) is a transgenic mouse model of breast cancer with sustained expression of activated *Her2/Neu* in mammary epithelial cells.[25] We crossed this mouse line with our recently established bitransgenic female mouse line, *iPIK3CA^{H1047R} (MTB/TetO-PIK3CA^{H1047R})*, in which the expression of the transgene *PIK3CA^{H1047R}*, an activating *PIK3CA* mutant, is under the control of a doxycycline-inducible promoter in mouse mammary epithelium.[15] *PIK3CA^{H1047R}* expression is coupled with that of a downstream luciferase reporter allowing transgene expression to be followed by *in vivo* bioluminescence imaging. The resulting triple transgenic mouse strain *MMTV-NIC; MTB/TetO-PIK3CA^{H1047R}* was designated *NIC/iPIK3CA^{H1047R}* (Supplementary Figure 1).

To determine the impact of *PIK3CA^{H1047R}* expression on *Her2*-dependent mammary tumorigenesis, we treated female *NIC/iPIK3CA^{H1047R}* mice with doxycycline to turn on *PIK3CA^{H1047R}* expression starting at the age of 6 weeks. Induction of the *PIK3CA^{H1047R}* transgene expression was confirmed by real time RT-PCR analysis, bioluminescence imaging as well as immunohistochemical staining (Supplementary Figure 2). All examined mice, *NIC/iPIK3CA^{H1047R}* and *NIC*, with or without doxycycline administration, developed mammary tumors with 100% penetrance (Figure 1a). Compared with our previously

reported *MTB/TetO-PIK3CA^{H1047R}* mice (mean time to tumor onset 208 days following doxycycline administration)[15] and *NIC* mice (mean time to tumor onset =102 days), the time course of tumor development in the *NIC/iPIK3CA^{H1047R}* mice maintained on doxycycline is significantly shortened (44 days following doxycycline administration) (Figure 1a, *** *P <0.001*), indicating the much more aggressive nature of these tumors. For each genotype, seven tumor-bearing mice were sacrificed 6 weeks after initial tumor detection, and tumors and lungs were harvested for further analysis. *NIC/iPIK3CA^{H1047R}* mice displayed significantly increased tumor burden and tumor volume when compared to *NIC* mice (Figure 1b). Of note, *NIC^{+}PIK3CA^{H1047R}* tumors at harvest are usually with large volume and often present with larger necrotic areas when compared to *NIC^{+}* tumors (Supplementary Figure 3). Histological examination of the *NIC^{+}PIK3CA^{H1047R}* mammary tumors revealed a morphology similar to that observed in the NIC mammary tumors with manifestations of multifocal, solid nodular adenocarcinoma (Figure 1c). There is a significant increase in the number of CD31+ blood vessels/field in *NIC^{+}PIK3CA^{H1047R}* tumors when compared to *NIC^{+}* tumors (Figure 1d), consistent with the role of PI3K activation in angiogenesis.[26] In addition, the percentage of mice with lung metastases was increased in the *NIC/iPIK3CA^{H1047R}* mice when compared to the *NIC* mice (Figure 1e). Taken together, these results constitute genetic evidence that mutational activation of p110α cooperates with activated *Her2/Neu* to accelerate the formation of metastatic mammary adenocarcinomas.

Inducible expression of mutant *PIK3CA* resulted in activation of PI3K/AKT signaling but attenuation of Her2/Her3 signaling

To understand the molecular mechanism underlying the rapid onset of mammary adenomcarcinomas in the *NIC/iPIK3CA^{H1047R}* mice, we first examined the status of PI3K signaling in these mammary tumors. Upon doxycycline administration, inducible expression of *PIK3CA^{H1047R}* resulted in activation of the PI3K/AKT signaling pathway in mouse mammary epithelium of the *iPIK3CA^{H1047R}* mice (Figure 2a and Supplementary Figure 4).[15] In line with this, the *NIC^{+}PIK3CA^{H1047R}* primary mammary tumors also exhibited a significant increase in phosphorylation levels of Akt and its downstream targets FoxO1/FoxO3a when compared to the *NIC^{+}* mammary tumors (Figure 2a and Supplementary Figure 5), indicating activation of the PI3K/AKT signaling pathway. However, in these *NIC^{+}PIK3CA^{H1047R}* tumors, PI3K activation did not have additional impact on phosphorylation of S6rp, an effector downstream of Akt/mTor signaling (Figure 2a). It is worth noting that phosphorylation of p42/p44 Mapk, downstream of ErbB2-mediated signaling, was also not affected by sustained PI3K activation (Figure 2a). Interestingly, while the *NIC^{+}PIK3CA^{H1047R}* mammary tumors showed comparable levels of the proliferative marker Ki67 to *NIC^{+}* mammary tumors, analysis of their surrounding mammary glands displayed a significant increase in Ki67-positive nuclei within early MINs (mammary intraepithelial neoplasia) and hyperplasia when compared to the *NIC^{+}* mammary counterparts (Figure 2b). Together, these results suggest that the activation of PI3K/Akt signaling in response to inducible expression of *PIK3CA^{H1047R}* contributed to the accelerated tumorigenesis in the *NIC/iPIK3CA^{H1047R}* mice.

We next assessed the effect of PI3K/AKT activation on Her2/Her3 signaling. Interestingly, when compared to the *NIC^{+}* tumors, the abundance of both Her2 and Her3 proteins, and

accordingly phospho-Her2 and phospho-Her3, was significantly reduced in the *NIC*$^{+}$*PIK3CA*^{H1047R} tumors with sustained induction of *PIK3CA*^{H1047R} expression (Figure 2c). Quantitative reverse transcription PCR analysis revealed a significant decrease in *Her3* mRNA in the *NIC*$^{+}$*PIK3CA*^{H1047R} tumors (Figure 2d), consistent with the notion that PI3K/AKT-mediated feedback suppression of Her3 transcription.[23, 24, 27] Of note, AKT inhibitor treatment on primary cells isolated from *NIC*$^{+}$*PIK3CA*^{H1047R} tumors maintained on doxycycline led to a significant increase in Her3 expression (Supplementary Figure 6), suggesting an AKT-dependent effect. However, the mRNA levels of both endogenous Her2/Neu and transgenic NIC remained largely unchanged (Supplementary Figure 7), suggesting that the reduced Her2 protein abundance is likely due to a post-transcriptional regulation. It has been shown that FoxO transcription factors could modulate *HER3* expression[23, 24], indeed, we found that induction of PI3K/AKT signaling resulted in substantially increased phospho-FoxO proteins, and attenuated nuclear localization of FoxO proteins in *NIC*$^{+}$*PIK3CA*^{H1047R} tumors when compared to *NIC*$^{+}$ tumors (Figures 2a and e). In line with this finding, analysis of TCGA database revealed that HER3 mRNA levels are significantly reduced in human HER2/PIK3CA mutation positive breast cancers (n=37) when compared to those in HER2/PIK3CA WT breast cancers (n=99) (Supplementary Figure 8, $*P < 0.05$). Thus, while previous findings demonstrated that PI3K/AKT inhibition led to upregulation of upstream RTKs, including HER2/HER3, using human breast cancer cell lines,[23, 24] our results revealed that PI3K/AKT activation suppresses upstream receptor tyrosine kinase expression and their activity, supporting the negative feedback loop of PI3K/AKT signaling and RTKs.

Removal of *PIK3CA*^{H1047R} induces tumor regression

To determine whether continued PI3K activation is still required for the progression of these fast growing mammary tumors, we withdrew doxycycline to turn off the expression of transgene *PIK3CA*^{H1047R}, mimicking the use of a highly effective PI3K inhibitor specifically targeting *PIK3CA*^{H1047R}. Strikingly, all *NIC*$^{+}$*PIK3CA*^{H1047R} primary mammary tumors displayed rapid regression within a week following deinduction of *PIK3CA*^{H1047R} expression (Figures 3a and b). Further analysis revealed that the expression of *PIK3CA*^{H1047R} is abrogated substantially after 3 days of doxycycline withdrawal, and levels of phosphorylated Akt and its downstream target FoxO1/FoxO3a are also significantly reduced (Figure 3c). Concordantly, in response to inactivation of *PIK3CA*^{H1047R}, the number of proliferating cells was significantly reduced (Figure 3d); conversely, apoptotic cell death as determined by TUNEL staining and cleaved Parp as determined by western blot analysis was dramatically increased (Figure 3e and Supplementary Figure 9). Therefore, reduced cellular proliferation and increased apoptosis contribute to the initial phase of tumor regression after *PIK3CA*^{H1047R} downregulation. Together, these results suggested that the vast majority of cells in the primary tumors are highly dependent on the expression of *PIK3CA*^{H1047R}, one of the initiating oncogenes, supporting *PIK3CA*^{H1047R} as a promising therapeutic target in HER2 positive breast cancers with co-existing oncogenic *PIK3CA*.

PI3K activation confers intrinsic resistance to Her2 inhibition by lapatinib

We next set out to determine the impact of inducible *PIK3CA* activation on the responses of the *NIC*$^{+}$*PIK3CA*^{H1047R} mammary tumors to Her2 directed therapy by lapatinib, a HER2/

EGFR kinase inhibitor by transplantation of primary tumors. Since the transplantation take-rate for the Her2/PIK3CA primary mammary tumors in syngeneic FVB female mice is low (~20%) with an unpredictable tumor latency, we opted to use an immunocompromised athymic mouse model, in which the tumors were engrafted with a full take rate and a latency for approximately 3 weeks. We found that while lapatinib treatment alone had little effect on tumor progression, inactivation of *PIK3CA*^{H1047R} by doxycycline withdrawal combined with lapatinib treatment significantly slowed down tumor growth (Figure 4a and Supplementary Figure 10). Interestingly, while the phospho-ERK signals in the *NIC*$^{+}$ *PIK3CA*^{H1047R} tumors maintained on doxycycline was markedly reduced in response to lapatinib (Figure 4b and Supplementary Figure 11A), the tumor growth remained unchanged (Figure 4a). Consistent with these findings, the transplanted tumors also did not respond to MEK inhibitor MEK162 (Supplementary Figure 11B). Thus, MAPK/ERK signaling does not appear to play a major role in supporting the growth of these Her2/PIK3CA primary mammary tumors.

In contrast, phospho-AKT signals were only moderately reduced in response to lapatinib (Figure 4b and Supplementary Figure 11A), likely an effect of sustained induction of *PIK3CA*^{H1047R} expression. Additional use of GDC-0941, a pan Class I PI3K inhibitor, completely abolished phospho-AKT signaling and showed a strong anti-tumor activity with partial tumor regression (Figures 4a and b, and Supplementary Figure 11A). Together, these results demonstrate that targeting PI3K activity overcomes the intrinsic mechanism of resistance to HER2-directed cancer therapy conferred by mutational activation of *PIK3CA*.

Prolonged inhibition of PI3K induces hyperactivation of MEK-ERK signaling

As all *NIC*$^{+}$ *PIK3CA*^{H1047R} primary mammary tumors showed regression shortly after transgene *PIK3CA*^{H1047R} deinduction, we further determined whether prolonged inactivation of *PIK3CA*^{H1047R} leads to sustained regression of *NIC*$^{+}$ *PIK3CA*^{H1047R} mammary tumors. To this end, we continuously monitored the regressing tumors in mice maintained without induction of transgene *PIK3CA*^{H1047R} expression. Strikingly, up to 58% (35/62) of tumors regressed to a non-palpable state, whereas the remaining tumors (42%, 27/62) showed partial regression following doxycycline withdrawal (Figures 3a and b). Nevertheless, regardless of the degree of initial tumor response, all these tumors eventually resumed growth. Since expression of HA-tagged p110αH1047R was no longer detectable in these recurrent tumors (Supplementary Figure 12), it is unlikely that re-expression of *PIK3CA*^{H1047R} accounts for resumed tumor growth. However, as NIC mice also developed mammary tumors, albeit with a much longer latency (Figure 1a), we cannot exclude the possibility that some of these seemingly recurring tumors in *NIC/iPIK3CA*^{H1047R} mice were independently caused by constitutive expression of the transgene *NIC* (activated Her2).

As the *NIC*$^{+}$ *PIK3CA*^{H1047R} tumors resumed growth in a *PIK3CA*^{H1047R}-expression independent manner, we further explored the mechanism that accounts for regained tumor growth. We found that while PI3K activation resulted in attenuated RTK signaling in primary tumors coexpressing *NIC* and *PIK3CA*^{H1047R} (Figure 2c), the abundance of Her2/Her3 proteins and their activity were restored in the *PIK3CA*^{H1047R} deinduced recurrent tumors and comparable to those seen in the control *NIC*$^{+}$ tumors (Figure 5a and

Supplementary Figure 13). This result complements recent studies that show inactivation of PI3K/AKT signaling relieves feedback suppression of receptor tyrosine kinase expression and activity [23, 24, 28, 29], and thus provides a molecular basis for the rational therapy targeting both PI3K/AKT and their upstream RTKs.

HER2/HER3 activation also has the capacity to signal through the MAPK pathway.[2, 18] When compared to the NIC^{+} tumors, $NIC^{+}PIK3CA^{H1047R}$ primary tumors expressed similar levels of phospho-ERK (p42/44), a marker of activated Mapk signaling (Figure 2a). Interestingly, while removal of $PIK3CA^{H1047R}$ expression did not have an immediate impact on Mapk signaling in the tumors at initial stages of regression (Figure 3b), the phosphorylation levels of p42/p44 Mapk proteins substantially increased in a fraction of recurrent tumors following prolonged inactivation of $PIK3CA^{H1047R}$ (Figure 5b). Of note, the phosphorylation level of p42/p44 MAPK in these recurrent tumors was also remarkably higher than that in the control NIC mammary tumors (Figures 2a and 5b). Thus, the compensatory hyperactivation of Mapk signaling clearly distinguished the fraction of bona fide recurrent tumors from those arising from constitutive expression of transgene *NIC* in the $NIC/iPIK3CA^{H1047R}$ model or other molecular mechanisms. Together, these results suggest that $PIK3CA^{H1047R}$-initiated mammary tumors may evade PI3K inhibition by an adaptive resistance mechanism through compensatory activation of Mapk signaling.

MEK inhibition potentiates the anti-tumor activity of lapatinib

The prevalence of the PI3K pathway activation in human cancers has led to an intense effort to develop inhibitors targeting PI3 kinases and to conduct clinical trials of such inhibitors in the treatment of malignancies, including HER2 positive breast cancer (*clinicaltrials.gov*). Our data suggest that the compensatory activation of MAPK signaling pathways may allow Her2 positive mammary tumor cells to escape from PI3K inhibition and thus limit the therapeutic effect of PI3K inhibitors. Indeed, recurrent tumor transplants that had aberrant activation of Her2/Her3 and Mapk signaling did not respond to treatment with the PI3K inhibitor GDC-0941 (Supplementary Figure 14). MAP kinase signaling is one of the major downstream effectors of HER receptor tyrosine kinases.[2] To determine if the compensatory activation of the Mapk pathway following PI3K inhibition occurs via HER receptor activation, we treated transplants of recurrent tumors with lapatinib. Indeed, HER2 inhibition by lapatinib resulted in attenuated tumor growth with reduced phospho-p42/p44 Mapk (Figures 6a and b).

However, a substantial pERK retained after lapatinib treatment, suggesting that there is additional signaling event contributes to the Mapk hyperactivation observed in these tumors. Since EGFR activation is a frequent event that activates the MAPK in tumors, we next examined whether EGFR signaling may account for the MEK/ERK hyperactivation in these recurrent tumors. We isolated primary cells from recurrent tumors with high abundance of p-MEK/ERK and treated them with EGFR inhibitor Gefinitib or EGFR/HER2 inhibitor Lapatinib, either as a single agent or in combination for 24 hours. Indeed, Gefitinib as a single agent markedly reduced pERK signaling and additional use of Gefitinib nearly completely abolished pMEK/pERK signals (Supplementary Figure 15). Furthermore, we showed that lapatinib or Gefitinib treatment alone induced Parp cleavage and the

combination of these two drugs resulted in a more substantial increase in cleaved Parp indicative of apoptotic response (Supplementary Figure 15). Together, these results indicated that in addition to restored Her2/Her3 signaling, activated EGFR signaling likely contributes to the hyperactivation of MEK/ERK signaling and survival of these recurrent tumors.

We also determined if dual blockade of Mapk and Her2 activation would be an effective strategy to treat these tumors. The combined use of MEK162, a MEK inhibitor currently under clinical development, with HER kinase inhibitor lapatinib, almost completely abolished Mapk signaling as evidenced by diminished phospho-Erk levels (Figure 6b). Furthermore, we found that the combination treatment, but not either single agent alone, resulted in sustained tumor regression (Figure 6a). In line with this, we observed a significant increase in anti-proliferative and pro-apoptotic activity in the tumors treated with MEK162 in combination with Lapatinib, and to a lesser extent as a single agent, when compared to the vehicle-treated tumors (Figures 6c and d).

Discussion

PIK3CA mutations and *HER2* amplification/overexpression frequently co-occur in human breast cancer and are associated with poor prognosis. To understand the role of *PIK3CA* mutations in the initiation and maintenance of this subtype of breast cancers, we generated a complex mouse model of breast cancer driven by concomitant Her2 activation and $PIK3CA^{H1047R}$ expression. We showed that induction of $PIK3CA^{H1047R}$ expression in mouse mammary glands expressing an activated *Her2/Neu* resulted in significantly accelerated mammary tumorigenesis with enhanced metastatic potential as compared to *NIC*-driven tumors. The latency of $NIC^{+}PIK3CA^{H1047R}$ mammary tumors is also significantly shorter than that of previously reported $PIK3CA^{H1047R}$-driven mammary tumors,[15] indicating the more aggressive nature of $NIC^{+}PIK3CA^{H1047R}$ mammary tumors.

A previous study by Hanker et al also generated a genetic mouse model of HER2/PIK3CA^{H1047R} breast cancer.[27] In both Hanker's and our models, the PIK3CA^{H1047R} transgene is of human origin and its expression is doxcycyline-inducible. The major difference between the two models is that Hanker's model expresses human wild-type *HER2* gene whereas our model expresses a constitutively activated rat *Her2/neu* mutant,[30] In addition, Hanker's model is maintained on a high fat diet to achieve faster tumor formation initiated by wild-type HER2 expression whereas our model is maintained on a regular diet. In both studies, Her2 and mutant PI3K strongly cooperated to promote mammary gland hyperplasia, tumor growth and metastasis. Specifically, in Hanker's model, inducible expression of transgene $PIK3CA^{H1047R}$ accelerates HER2-driven mammary tumorigenesis and metastatic progression, alters the intrinsic phenotypes of HER2-overexpressing tumors (from luminal to claudin-low subtype of breast cancer with characteristics of epithelial-to-mesenchymal transition and stem cell markers), and generates resistance to combinations of anti-HER2 therapies. Of note, unlike the highly heterogeneous histology of Hanker's HER2/$PIK3CA^{H1047R}$ tumors, our $NIC^{+}PIK3CA^{H1047R}$ tumors are homogeneous, solid adenocarcinomas. Given the important role of PI3K in glucose and lipid metabolism, it remains to be investigated if the high-fat diet may contribute to the heterogeneous histology and altered intrinsic phenotypes of HER2/ $PIK3CA^{H1047R}$ tumors in Hanker's model.

Co-existing PIK3CA mutation and HER2 amplification/overexpression have been frequently found in human breast cancer. However, the relative timing of the emergence of HER2 and PIK3CA alterations in breast tumorigenesis remains unclear. HER2 overexpression or PIK3CA mutation, as single events, have been previously shown to induce mammary tumor formation in a number of genetically engineered mouse models, suggesting both genetic alterations have the potential to be an early oncogenic driver in tumorigenesis. While a previous study reported that PIK3CA mutations may be an early event, [31] recent findings by Stachler et al suggested that PIK3CA mutations may be a relatively late event. [32] It is likely that, in patients, either order of occurrence for these two events could have occurred. In our model, inducible expression of PIK3CA-H1047R resulted in expedited mammary tumor formation in a Her2(NIC)-altered/overexpressed setting with the histology of solid adenocarcinoma, recapitulating, at least in part, some aspects of a type of human breast cancer in which PIK3CA mutation emerges after HER2 alteration/ overexpression. Future studies using genetic mouse models of breast cancer will be necessary to directly compare the phenotypic consequences brought by the expression of PIK3CA mutation and HER2 alteration in different temporal appearance.

Recent work using a *Kras* mouse model of lung cancer underscored the notion that blocking tumor initiation is not necessarily equivalent to treating a cancer that is already established.[33] Taking advantage of the inducible mouse model established in our study, we investigated the role of the oncogenic *PIK3CA* in the maintenance of $NIC^{+}PIK3CA^{H1047R}$-driven mammary tumors. Remarkably, these primary mammary tumors are highly dependent on oncogenic PI3K signaling as evidenced by their exquisite initial response to inactivation of the single oncogene $PIK3CA^{H1047R}$, providing a rationale for the use of $PIK3CA^{H1047R}$-specific inhibitors in this genetic context. Given the essential roles of p110α in cellular physiology,[34] inhibitors that specifically target the mutant forms are attractive therapeutic agents that would avoid the toxicity, such as disturbed insulin signaling, caused by inhibition of the wild-type form.[12, 13]

We also showed that Her2 positive primary mammary tumors expressing *PIK3CA* mutation are intrinsically resistant to the HER2 kinase inhibitor lapatinib. These results are not unexpected, as analysis of these primary tumors revealed not only strong PI3K/AKT activation, but also significantly attenuated Her2/Her3 expression and phosphorylation. These findings are consistent with, and provide a mechanistic explanation for, the clinical observation that HER2 positive cancers with co-existing *PIK3CA* mutations are relatively resistant to lapatinib and other HER2-targeted therapies.[10, 20–22, 35] Together with our finding that Her2 positive breast cancers with co-existing $PIK3CA^{H1047R}$ are primarily vulnerable to PI3K inhibition, these results further corroborate the rationale for using PI3K targeted therapy in combination with HER2-directed agents as a potential first-line treatment of Her2 positive breast cancer harboring *PIK3CA* activating mutations.

The present study clearly sets an example of how an inducible GEM model of breast cancer can be used to recapitulate naturally occurring therapeutic responses in human tumors. Our results reveal that prolonged exposure to PI3K inactivation leads to restored Her2/Her3 expression with concomitant signaling reactivation, and consequently a switch of oncogene dependency from PI3K to MAPK activation that contributes to tumor relapse. Meanwhile,

we found that ERK activation is not an immediate event that can be detected shortly after transgene *PIK3CA*^{H1047R} deinduction and was found in a subset of regrowing tumors that had experienced initial tumor regression as a result of oncogene inactivation. Of note, this delay in ERK activation has been previously reported in *in vitro* and *in vivo* xenograft studies of HER2 positive breast cancers with PI3K/mTOR inhibition by treatment with the dual kinase inhibitor BEZ235.[24] Together, these findings could have important implications for the time at which it is appropriate to monitor ERK activation during clinical trials of PI3K inhibitor treatment in HER2 positive breast cancer. We further demonstrated that this compensatory hyperactivation of ERK confers adaptive resistance to PI3K inactivation in *PIK3CA*^{H1047R} and Her2 initiated mammary tumors, suggesting the utility of combination therapy targeting both MEK and HER2 in the treatment of recurrent tumors that have evaded PI3K inhibition. While the observed compensatory activation of ERK represents one mechanism by which Her2 positive breast cancer evades PI3K inhibition, alternative molecular mechanisms that contribute to the regained growth will be explored in our future studies. Further understanding the oncogenic rewiring of cellular signaling pathways associated with resistance to PI3K inhibition will help us define a rational combination therapy that targets both dominant oncogenic PI3K signaling as well as secondary pathways of tumor escape in HER2-directed cancer therapy.

Materials and Methods

Transgenic mice

TetO-PIK3CA^{H1047R}[15, 25], *MMTV-rtTA(MTB)* mice [36] and *MMTV-NIC* mice [25] were in the FVB/N background. *NIC/iPIK3CA*^{H1047R} mice were administered doxycycline (2mg/ml) in their drinking water. All animals were housed and treated in accordance with protocols approved by the Institutional Animal Care and Use Committees of Dana-Farber Cancer Institute and Harvard Medical School.

Histology and Immunohistochemistry

Tumors were fixed in formalin overnight before paraffin embedding. Paraffin blocks were sectioned and stained with H&E at the Dana-Farber/Harvard Cancer Center Rodent Histopathology Core. Immunohistochemistry was carried out using the antibodies Ki67 (Vector), FoxO1, p-Akt (Ser473) (Invitrogen), p-S6rp and p-Erk (Cell Signaling Technology). TUNEL assays were conducted using the ApoTag Plus Peroxidase *in situ* TUNEL Apoptosis Kit (Millipore) according to the manufacturer's instructions.

Tumor grafting and in vivo treatment studies

Tumor grafts were established in immunodeficient nude mice. GDC-0941 and Lapatinib (MedChemexpress) were dissolved in 0.5% methylcellulose/0.2% Tween 80. MEK162 (MedChemexpress) were dissolved in 1% carboxymethyl cellulose/0.5%Tween 80. Tumor volumes were measured twice a week with calipers and calculated according to the following formula: tumor volume = (length × width^2)/2. Tumors were harvested for further analysis 4 hours after the last dose on day three of treatment.

Western blot analysis and immunoprecipitation

Tumors were homogenized in ice-cold RIPA buffer (Sigma) containing protease inhibitor cocktail (Roche) and phosphatase inhibitor (Roche). For tumor lysates, standard western blotting was conducted as described [15] using antibodies against p-AKT (Ser473), p-AKT(Thr308), p-FoxO1 (Thr24)/FoxO3a(Thr32), p-ERK(1/2) (Thr202/Tyr 204), ERK, pMEK(Ser217/Ser221), MEK (Cell Signaling Technology), Her2 and Her3 (Santa Cruz Technology) and vinculin (Sigma-Aldrich). For immunoprecipitation assay, anti-phosphotyrosine antibody (clone 4G10) agarose conjugate (EMD Millipore) was used. Immunofluorescently labeled antibody to mouse IgG (Rockland Immunochemicals) and antibody to rabbit IgG (Molecular Probes) were used to visualize western blots on an Odyssey scanner (Li-Cor). MK2206 and Gefitinib were purchased from MedChemexpress and Selleck, respectively.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNAs from mammary tumors or normal mammary tissues were isolated using an Allprep DNA/RNA Mini Kit (Qiagen) and used to synthesize cDNAs with a High Capacity RNA-to-cDNA kit (Applied Biosystems). Quantitative PCR was performed with Taqman gene expression assays or Power SYBR Green PCR master mix (Applied Biosystems). Human *PIK3CA*^{H1047R}, mouse *Her2/Neu*, *Her3* and *Gapdh* (as an endogenous control) were amplified with commercially designed Taqman gene expression assays (Applied Biosystems). *NIC* was amplified with primers sequences, Forward, 5′CAGGACAATGTCGCCGCCTCC3′, Reverse, 5′ CAGAGCTGAGGGTTCCCACGG3′. Quantitative expression data were acquired and analyzed with a 7300 Real-time PCR System (Applied Biosystems).

Statistical analyses

The survival of mice was analyzed by Kaplan-Meier *log-rank* test. Quantitative results were analyzed by two-tailed unpaired Student's *t* test. $P < 0.05$ was considered statistically significant.

In vivo bioluminescence imaging

We anesthetized mice with ketamine and xylazine and administered d-luciferin (Promega) intraperitoneally to monitor luciferase gene expression *in vivo*. We analyzed images with KODAK Molecular Imaging Software (version 4.5.0b6 SE).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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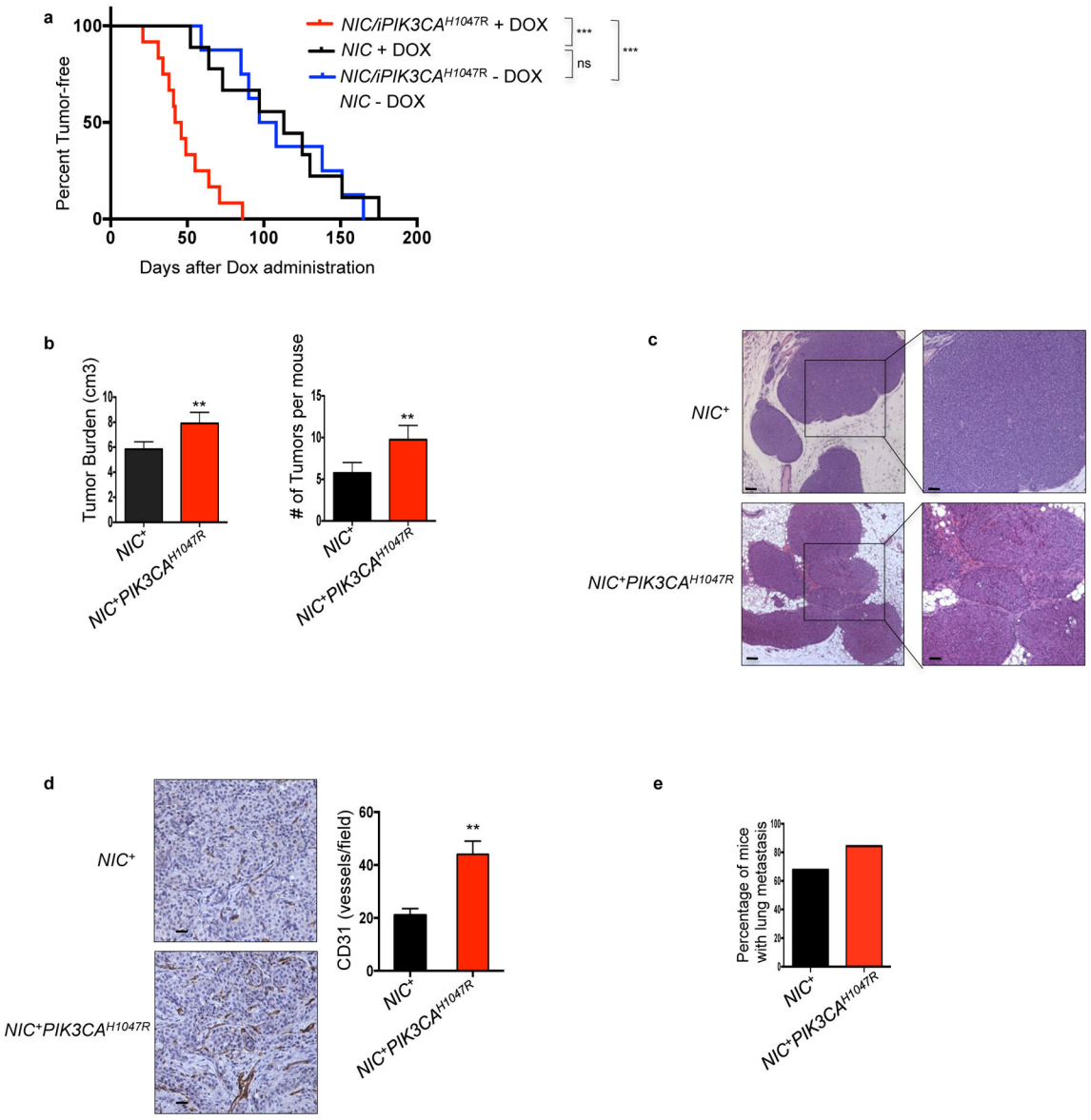

Figure 1. Mutant *PIK3CA* cooperates with Her2 to accelerate mammary tumor formation

(**a**) Kaplan-Meier curve of tumor-free survival of mouse cohorts of the indicated genotypes. T_{50} represents median tumor-free survival days after doxycycline administration. T_{50} for *NIC/iPIK3CA*^{H1047R} mice (on DOX, n=26), 44 days; (on T_{50} for *NIC* mice (on DOX, n=11), 113 days; T_{50} for *NIC* mice (without DOX, n=14) and *NIC/iPIK3CA*^{H1047R} mice (without DOX, n=6), 102 days; ****P < 0.0005*, *log rank test*. ns, no significance. (**b**) Average tumor burden (left panel) and numbers of tumors (right panel) per mouse at study end point (6 weeks post-palpation of the first tumor in each mouse). Data are means ± s.e.m. (n=7 per genotype). ** *P<0.01*, Student's *t* test. Representative H&E-stained (**c**) and CD31-stained (**d**) sections of primary mammary tumors from *NIC/iPIK3CA*^{H1047R} mice and *NIC* mice following doxycycline treatment for 1 month and 3 months, respectively. Quantification of $CD31^+$ blood vessels/field in tumors as indicated in (**d**). Data are means ±

s.e.m. (n= 7 tumors per genotype). $^{**}P < 0.01$, Student's *t* test. Scale bars, 50 μm (**c,** left panels; **d**, both panels), 25 μm (**c,** right panels). (**e**) Percentage of tumor-bearing mice with lung metastases from the indicated genotypes.

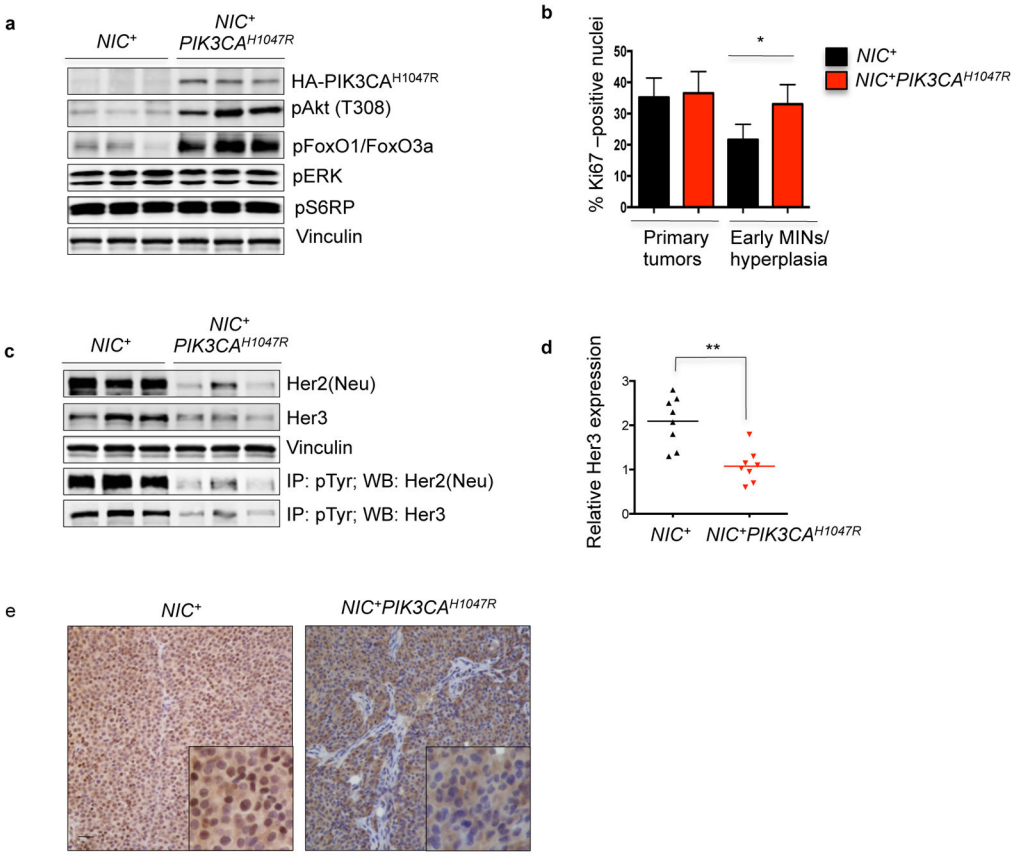

Figure 2. Inducible expression of mutant *PIK3CA* resulted in activation of PI3K/AKT signaling but attenuation of Her2/Her3 signaling

(**a**) Western blot analysis. Tumor lysates were prepared from mice of indicated genotypes at study end point. Vinculin was used as a loading control. Note, all mice were treated with doxycycline. (**b**) Quantitative analysis of Ki67-positive nuclei from primary mammary tumors and early MINs/hyperplasia of indicated genotypes. 8 fields per tumor slide were scored and depicted as the percentage of Ki67 positive nuclei ± s.e.m (n= 6 slides for each category as indicated). $*P < 0.01$, Student's *t* test. (**c**) Western blot analysis. Tumor lysates were prepared from mice of indicated genotypes at study end point. p-Tyr immunoprecipitation was performed on 500μg of tumor lysates followed by immunoblotting with Her2 or Her3 antibodies as indicated. (**d**) Quantitative reverse transcriptase PCR analysis of mRNA levels of Her3 in mice of indicated genotypes. Mouse *Gapdh* was used as an endogenous control. Results are shown as mean ± SD of triplicate experiments. $**P < 0.001$, Student's *t* test. (**e**) Representative immunohistochemical stained sections of FoxO proteins in primary tumors from mice of indicated genotypes. Scale bars, 50 μm.

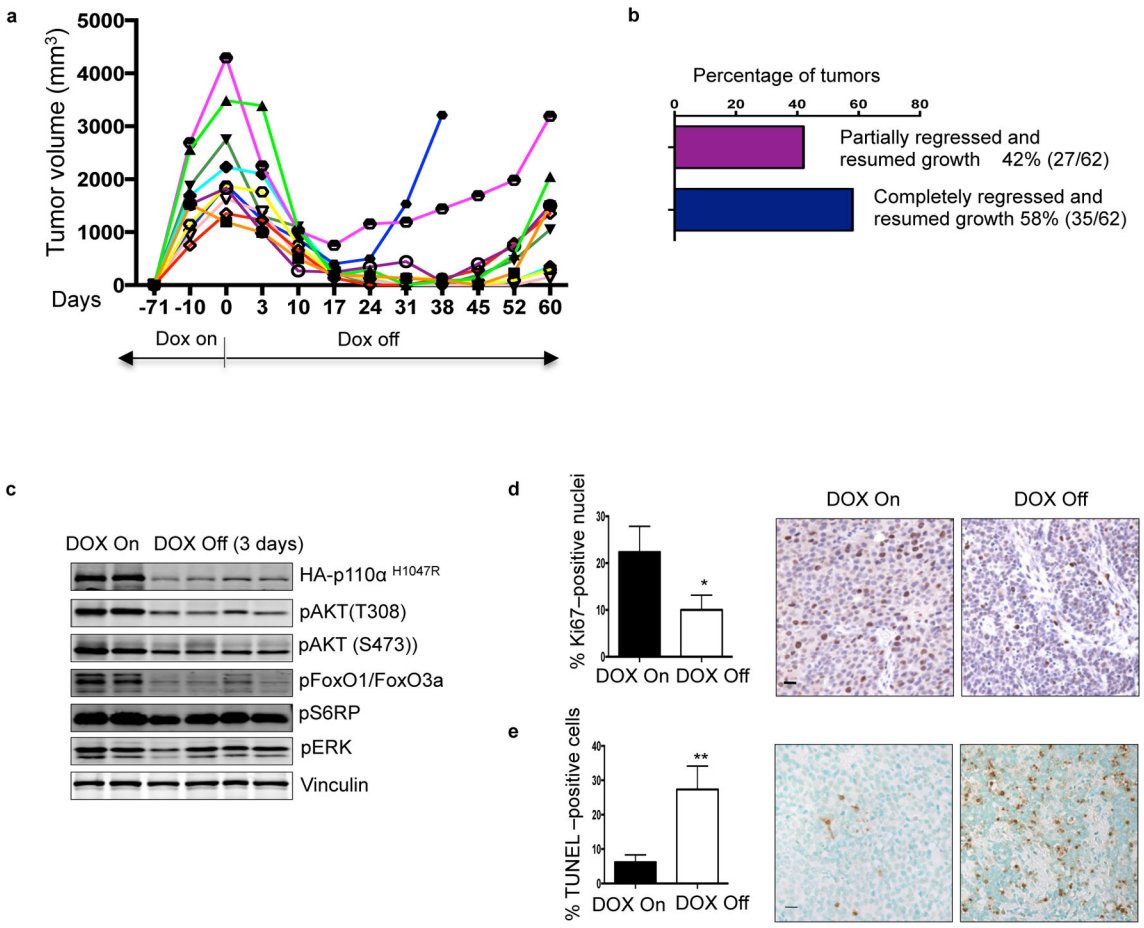

Figure 3. Primary mammary tumors rapidly regressed in response to PIK3CA^{H1047R} shut-off

(**a**) Representative tumor growth curves showing responses of primary mammary tumors in *NIC/iPIK3CA^{H1047R}* mice with (Dox on) or without doxycycline (Dox off) treatment as indicated. (**b**) Summary of tumor responses to doxycycline withdrawal. (**c**) Western blot analyses of HA-p110α^{H1047R}, p-Akt, pFoxO1/3a, p-S6RP and p-ERK in primary tumors as indicated. On DOX, on doxycycline; Off DOX, doxycycline withdrawal for 3 days. Vinculin was used as a loading control. (**d**) Representative H&E-stained sections of mammary tumors isolated from NIC/iPIK3CA^{H1047R} mice before and after DOX withdrawal for 3 days. Representative images of immunohistochemistry for Ki67 (**d**) or TUNEL (**e**) carried out on tumors isolated from *NIC/iPIK3CA^{H1047R}* mice maintained on doxycycline (DOX on) or 3 days after doxycycline withdrawal (DOX off). Scale bars, 25 μm. Data are means ± s.e.m. (n = 7). * $P < 0.001$, ** $P < 0.0001$ (Student's t test). Scale bars, 25 μm.

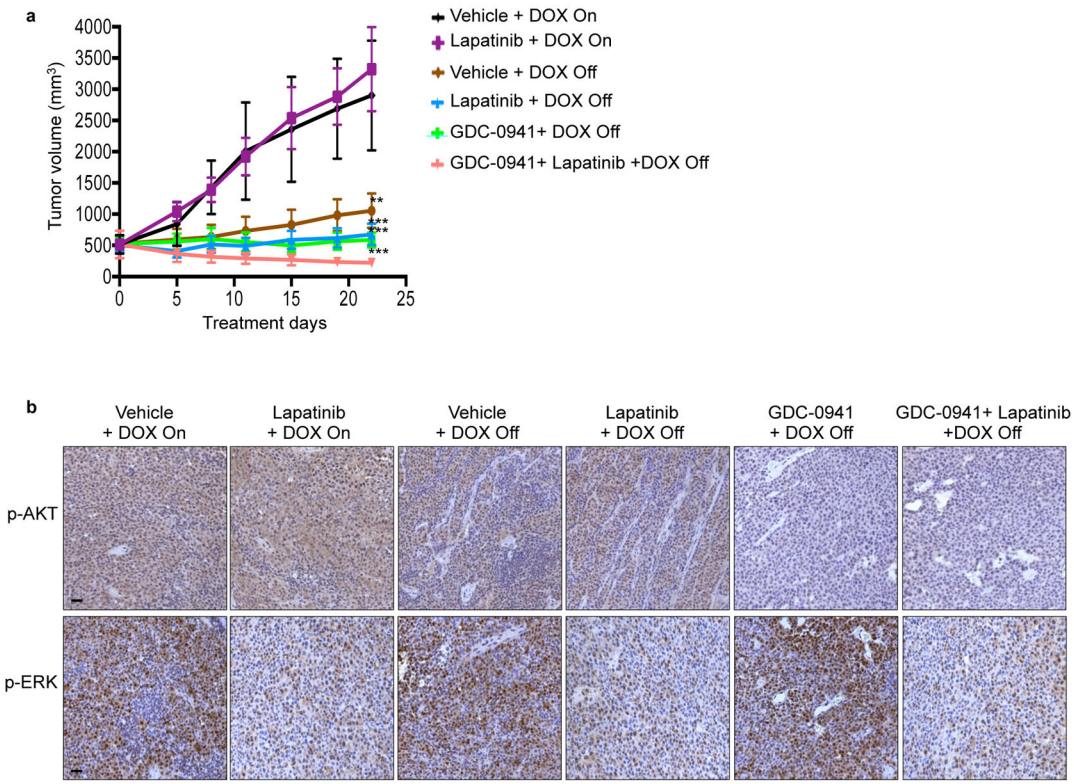

Figure 4. PI3K activation confers intrinsic resistance to Her2 inhibition by lapatinib

(**a**) Responses of tumor transplants to treatment as indicated. Data are means ± s.e.m. (n = 10 tumors /treatment group). $^{**}P < 0.001$, $^{***}P < 0.0001$ (Student's t test). Transplants of $NIC^{+}PIK3CA^{H1047R}$ primary mammary tumors were first established in athymic nude mice maintained on doxycycline. Treatment started when tumor transplants reached 500 mm^3. DOX On, on doxycycline; DOX Off, doxycycline withdrawal. Lapatinib, 100mg/kg/day, p.o; GDC-0941, 120mg/kg/ day, p.o. (**b**) Representative images of immunohistochemistry for p-AKT (S473) and p-ERK carried out on transplanted tumors treated as indicated. Four hours after their last dose on day three of treatment, the animals were sacrificed. Scale bars, 50μm.

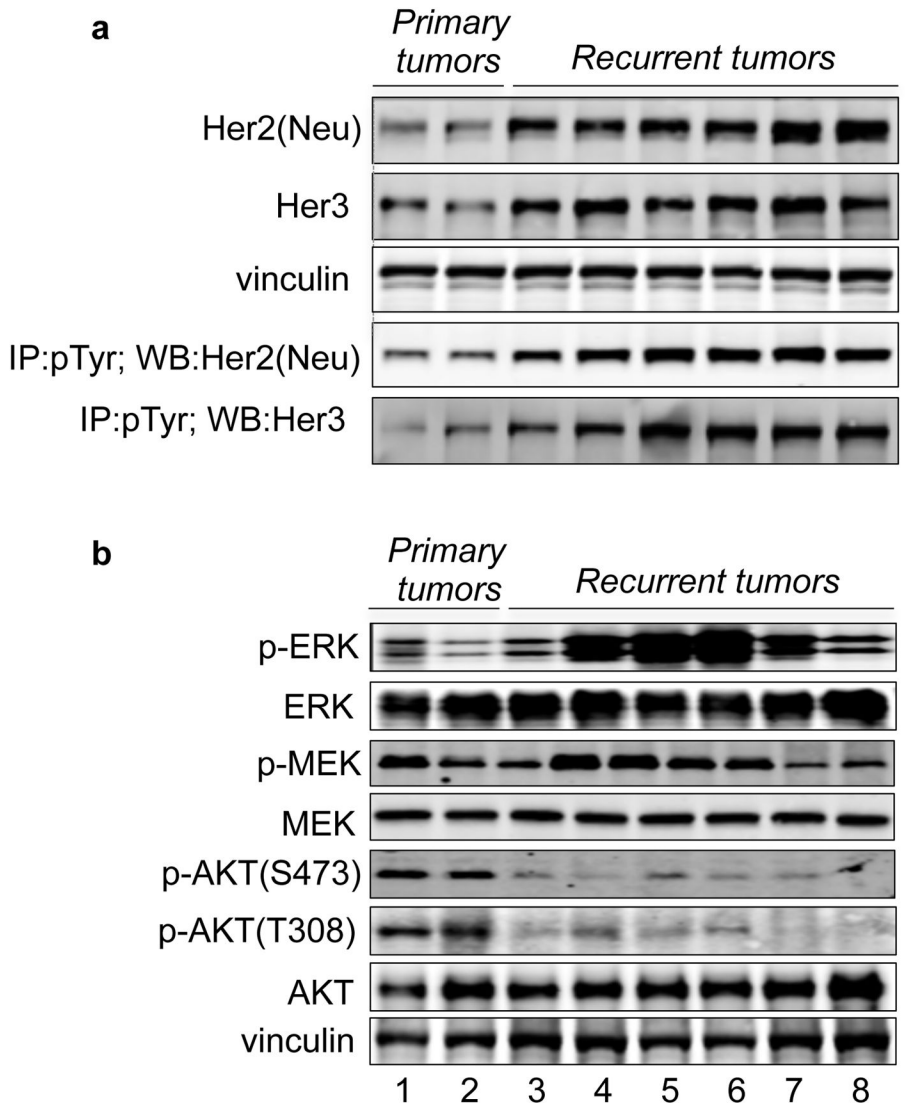

Figure 5. Prolonged inhibition of PI3K induces hyperactivation of MEK-ERK signaling

(**a**) Western blot analyses of Her2 and Her3 proteins in the recurrent tumors from *NIC/iPIK3CA*^{H1047R} mice maintained without doxycycline compared to the primary tumors from *NIC/iPIK3CA H1047R* mice maintained on doxycycline. phospho-tyrosine immunoprecipitation was performed on 500μg of tumor lysates followed by immunoblotting with Her2 or Her3 antibodies as indicated. (**b**) Western blot analyses of p-ERK/ERK, pMEK/MEK and p-AKT/AKT in tumors as described above. Vinculin was used as a loading control.

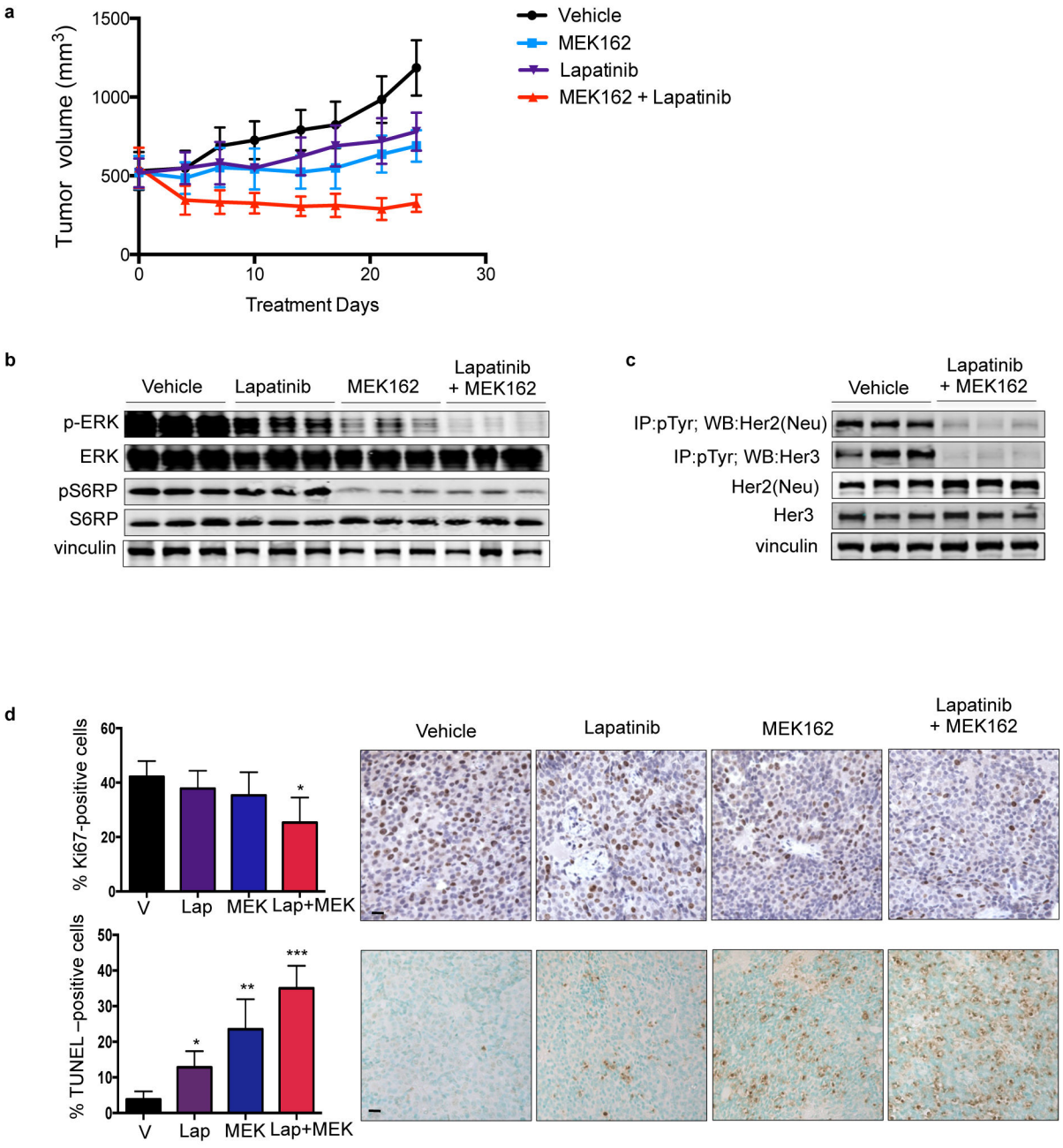

Figure 6. Aberrant activation of MEK signaling confers the regained growth of Her2 positive mammary tumors

(**a**) Responses of tumor transplants to treatment as indicated. Data are means ± s.e.m. (n = 10 tumors/treatment group). *$P < 0.001$ (Student's t test). Transplants of $NIC^{+}PIK3CA^{H1047R}$ primary mammary tumors with high p-ERK were established in immunodeficient nude mice maintained without doxycycline. Treatment started when tumor transplants reached 500 mm^3. MEK162, 30mg/kg/day, p.o; lapatinib, 100mg/kg/day, p.o. Western blot analyses of ERK signaling (**b**) and Her2/Her3 signaling (**c**) in tumor transplants from mice treated as indicated. Three hours after their dose on day four of treatment, the mice were sacrificed for analysis. Vinculin was used as a loading control. (**d**) Percentage of

Ki67 positive and TUNEL positive cells in tumor transplants isolated from mice treated as described in A. Data are means ± s.e.m. (n = 6 tumors/group). * P <0.01, ** P <0.001, *** P <0.0001, Student's t test. Scale bars, 25 μm.