AKT Inhibition Relieves Feedback Suppression of Receptor Tyrosine Kinase Expression and Activity

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SUMMARY

Activation of the PI3K-AKT pathway in tumors is modulated by negative feedback, including mTORC1-mediated inhibition of upstream signaling. We now show that AKT inhibition induces the expression and phosphorylation of multiple receptor tyrosine kinases (RTKs). In a wide spectrum of tumor types, inhibition of AKT induces a conserved set of RTKs, including HER3, IGF-1R, and insulin receptor. This is in part due to mTORC1 inhibition and in part secondary to a FOXO-dependent activation of receptor expression. PI3K-AKT inhibitors relieve this feedback and activate RTK signaling; this may attenuate their antitumor activity. Consistent with this model, we find that, in tumors in which AKT suppresses HER3 expression, combined inhibition of AKT and HER kinase activity is more effective than either alone.

INTRODUCTION

The phosphatidylinositol 3-kinase (PI3K)—protein kinase B (PKB/AKT)—mammalian target of rapamycin complex 1 (mTORC1) kinase cascade transmits signals from ligand-stimulated receptor tyrosine kinases (RTKs) to effector molecules that control metabolism, proliferation, size, survival, and motility (Guertin and Sabatini, 2007; Vivanco and Sawyers, 2002). In cancer this pathway is frequently hyperactivated as a result of: (1) activation of RTKs by mutation (epidermal growth factor receptor) or gene amplification (HER2), (2) activating mutations of components of the pathway such as PI3K or AKT, and (3) deletion or decreased function of tumor suppressors such as the PIP3 phosphatase, phosphatase and tensin homolog (PTEN) (Hynes and Lane, 2005; Vivanco and Sawyers, 2002). Such lesions are extremely common in cancer.

Tumors with PTEN or PIK3CA mutations or HER2 amplification have been shown to be dependent on PI3K-AKT-mTOR

signaling for maintenance of the transformed phenotype and hypersensitive to inhibition of its components. This has led to a major effort to develop inhibitors of PI3K, AKT, mTOR, and other components of the pathway (Courtney et al., 2010; Workman et al., 2010). Analogs of the natural product rapamycin, an inhibitor of the mTORC1 complex, were among the first inhibitors of the PI3K pathway to be used for the treatment of cancer. Rapamycin does effectively inhibit mTORC1 signaling; however, it also relieves mTORC1-dependent feedback inhibition of IGF1 receptor (IGF-1R) signaling. This results in activation of PI3K-AKT signaling and enhanced phosphorylation of non-mTORC1 targets of AKT, such as the FOXO family of transcription factors (Haruta et al., 2000; O'Reilly et al., 2006).

Physiologic activation of signaling is regulated by feedback inhibition of components of the network and is a feature of both normal and oncogene-transformed cells. Relief of this feedback might be a common response to anticancer drugs and could attenuate the therapeutic response (Courtois-Cox et al.,

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Significance

Constitutive feedback inhibition of upstream signaling pathways is a fundamental feature of tumors with activated oncoproteins. AKT activation leads to feedback by inhibiting the nuclear localization of FOXO and other transcription factors and activating mTOR kinase. The antitumor effects of inhibitors of oncoproteins may be attenuated by relief of feedback. In tumors with activation of PI3K-AKT signaling, AKT inhibitors activate receptor signaling and expression by activating FOXO and inhibiting mTORC1. In contrast, rapamycin inhibits mTORC1 but activates AKT. Thus, inhibitors of different components of the activated pathway will relieve different aspects of negative feedback. Effective therapy may require combining signaling inhibitors with inhibitors of the specific pathways they induce.

2006; O'Reilly et al., 2006). We reasoned that specific inhibitors of other components of the PI3K-AKT pathway would reactivate different aspects of the network. To examine the role played by AKT in the feedback regulation of activated mitogenic signal transduction, we employed a specific, allosteric AKT inhibitor and examined its effect on the expression and phosphorylation of the RTKs that commonly stimulate these signaling pathways (DeFeo-Jones et al., 2005; Lindsley et al., 2005; She et al., 2008).

RESULTS

AKT Inhibition Is Associated with HER3 Induction

We used selective inhibitors to determine whether AKT mediates feedback inhibition of PI3K signaling in tumors in which it is dys-regulated. AKTi-1/2 and AKTi-1/2/3 are PH-domain dependent, non-ATP-competitive inhibitors that potently inhibit AKT1 and AKT2, and not other AGC kinases, with AKTi-1/2/3 having greater potency against AKT3 (Barnett et al., 2005; Lindsley et al., 2005). The drugs share similar potency against activated AKT in the HER2-amplified breast cancer cell line BT-474, as seen in Figure S1 (available online) (She et al., 2008). The drugs prevent binding of AKT to the plasma membrane and, thus, its phosphorylation by PDK1, so, unlike ATP-competitive inhibitors, they block rather than activate AKT phosphorylation (Figure 1A) (Okuzumi et al., 2009).

Breast cancers with HER2 amplification have elevated levels of AKT and ERK phosphorylation and are dependent on the former, but not the latter for maintenance of the transformed phenotype (Basso et al., 2002; Hermanto et al., 2001; She et al., 2008; Solit et al., 2006). In these tumors, PI3K-AKT signaling is HER2 dependent and predominantly driven by HER2-HER3 heterodimers (Holbro et al., 2003; Yakes et al., 2002). HER3 is a kinase-defective member of the HER kinase family that contains six tyrosines in its intracellular domain that, when phosphorylated, act as high-affinity docking sites for the p85 subunit of PI3K. In breast cancers, overexpressed HER2 dimerizes with and phosphorylates HER3, which binds to and activates PI3K (Holbro et al., 2003).

AKT inhibitors block AKT signaling in these cells and cause growth arrest and apoptosis (She et al., 2008). We asked whether activation of AKT feedback regulates the HER kinase family members that drive the pathway. In Figure 1A we determined the effect of AKT inhibition on HER kinase expression and phosphorylation in BT474, a breast cancer cell line with HER2 amplification. Treatment of BT-474 cells with 1 μM AKTi-1/2 inhibits phosphorylation of AKT and its substrate, PRAS40, by 1 hr after drug is added. At 24 hr, AKT phosphorylation begins to rise but remains significantly inhibited at 72 hr. AKT inhibition causes a marked change in the expression and phosphorylation of HER kinase family members. The expression of EGFR, HER3, and HER4 is induced beginning 2–4 hr after drug addition and reaches a maximum approximately 24 hr after drug exposure (1.6-, 4.1-, and 2.5-fold induction). The already high levels of HER2 expression do not change appreciably. There is a concomitant 4-fold induction of the tyrosine phosphorylation of HER3 at both the Y1197 and Y1289 sites, whereas phosphorylation of EGFR (Y1068), HER2 (Y1221), and HER4 (Y1284) declines over this period (0.4-, 0.5-, and 0.7-fold). Of the HER family receptors, only HER3 expression and phosphorylation are coordinately up-regulated in these cells. Inhibition of the PI3K-AKT pathway using an ATP competitive AKT kinase inhibitor or a PI3K inhibitor also results in upregulation of HER3 and P-HER3 expression (Figure S1). Moreover, interfering RNA against AKT1, AKT2, and AKT3 results in induction of HER3 expression and phosphorylation 72 hr after transfection (Figure 1B). HER3 levels in the plasma membrane increase, and this is accompanied by increased HER3 heterodimerization with HER2 and association with the p85 subunit of PI3K (Figure S1). These data suggest that PI3K-AKT activation in response to HER2 amplification and overexpression is associated with an AKT-dependent downregulation of HER family protein expression, which is relieved by the AKT inhibitor. Although AKT and ERK signaling are both driven by HER2 amplification, feedback regulation of receptor expression is mediated by the former, but not the latter. Selective inhibition of ERK phosphorylation with the MEK inhibitor, PD325901, has no effect on HER2 or HER3 expression or phosphorylation in tumor cells with HER2 amplification (Figure 1C).

Induction of HER3 expression in response to AKT inhibition does not require serum factors. Serum starvation of HER2-amplified breast cancer cells is associated with increased HER3 expression, and increased phosphorylation of HER2, HER3, and AKT (Figure 1D). In these cells, 1 μM AKTi-1/2 led to marked induction of HER3 expression, but no further induction of HER3 phosphorylation. The results distinguish induction of phosphorylation of the receptor from induction of expression. Whereas AKT inhibition can further induce HER3 expression in the absence of serum factors, it does not affect HER3 phosphorylation.

Induction of AKT Activation Reduces HER3 Expression

These results suggest that in HER2-amplified breast cancer cells, PI3K activation may induce an AKT-dependent feedback inhibition of HER3 expression and activation. To assess the effects of inducing AKT activation, BT-474 cells were treated with DMSO or 100 nM AKTi-1/2/3 in serum containing media for 12 hr (lanes 2 and 7, Figure 2A) and then the compound was washed out, and fresh medium was added (Figure 2A). Re-activation of AKT phosphorylation occurred 4 hr after the drug was removed and was sustained. This was associated with decreased HER3 expression and phosphorylation 8 hr after drug washout. Levels of HER3 and phosphorylated HER3 declined to near steady-state levels. The data suggest that AKT activation negatively regulates HER3 expression and phosphorylation in HER2-amplified breast cancer cells and that inhibition of AKT relieves this feedback.

The Phosphorylation of Multiple RTKs Is Induced by AKT Inhibition

Multiple RTKs activate PI3K-AKT signaling in normal and tumor cells. In particular, many of the metabolic effects and other consequences of stimulation of the insulin and IGF-1Rs are mediated via induction of PI3K-AKT signaling. We used an anti-phosphotyrosine receptor antibody array to assess whether RTKs other than HER3 were induced in response to AKT inhibition (Figure 2B). These RTK arrays detect phosphorylation of 42 RTKs (Stommel et al., 2007). In BT-474 cells, HER2 and HER3 were the most prominent phosphorylated RTKs detected
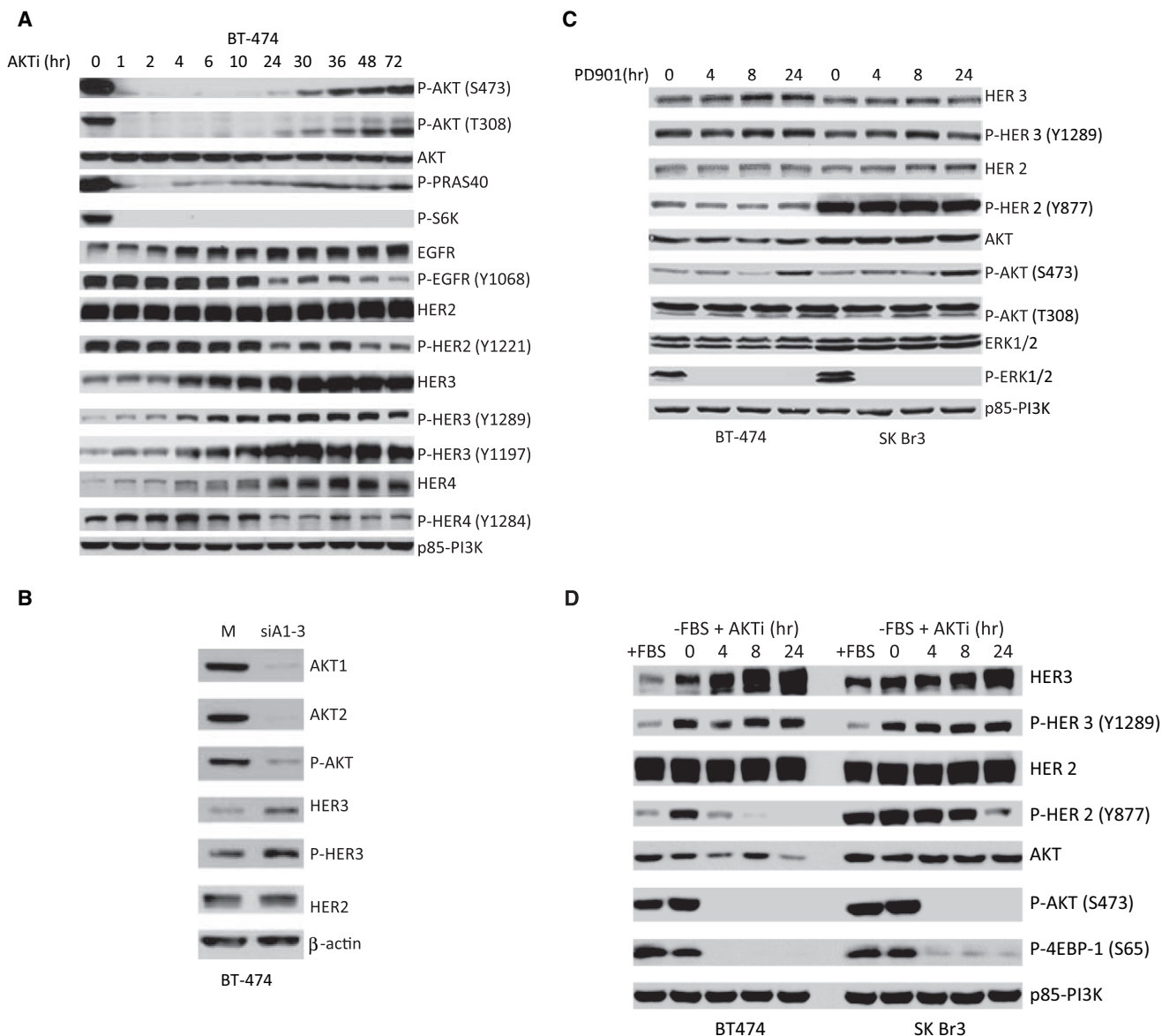
A

B

C

D

Figure 1. AKT Inhibition Promotes HER3 Expression and Phosphorylation

(A) BT474 cells were treated with AKTi-1/2 (1 μM) and collected at indicated times. AKT inhibition as measured by loss of S473 phosphorylation, along with AKT targets S6K and PRAS40, is associated with increases in HER3 and P-HER3.

(B) BT-474 cells treated with siRNAs against AKT1, AKT2, and AKT3 (AKT3 was not detectable) for 72 hr were collected and lysates immunoblotted, demonstrating that loss of AKT expression is associated with increased expression of RTKs.

(C) The HER2-amplified cancer cell lines BT-474 and SK-BR3 were treated with the MEK inhibitor PD325901 (50 nM). Inhibition of ERK1/2 phosphorylation was not associated with an induction of HER3 or P-HER3.

(D) BT-474 and SK-BR3 cell lines were serum starved for 12 hr followed by treatment with 1 μM AKTi. The induction of HER3 expression occurred upon AKT inhibition despite the lack of serum. See also Figure S1.

(first set of panels, 50 μg loaded). Twenty-four hours after exposure to the AKT inhibitor, P-HER2 was not significantly changed, whereas P-HER3 was strongly induced. Removing the anti-P-HER2 and anti-P-HER3 dots from the array and increasing the amount of loaded protein (250 μg) increased the sensitivity of the assay. This allowed detection of multiple other receptors in the untreated control. The phosphorylation of a group of RTKs (HER3, FLT-3, HER4, EphA7, MSPR, insulin receptor [IR],

EphA1, IGF-1R, FGFR3, and FGFR1) was induced at least 3-fold 24 hr after inhibition of AKT. The induction of the total tyrosine phosphorylation of HER4, in which 19 P-TYR sites have been identified, contrasts with the loss of phosphorylation of the Y1284 site on HER4 in Figure 1A (Kaushansky et al., 2008). Overall, the data suggest that the phosphorylation of a group of receptors is feedback inhibited in response to PI3K-AKT activation and induced when AKT is inhibited.
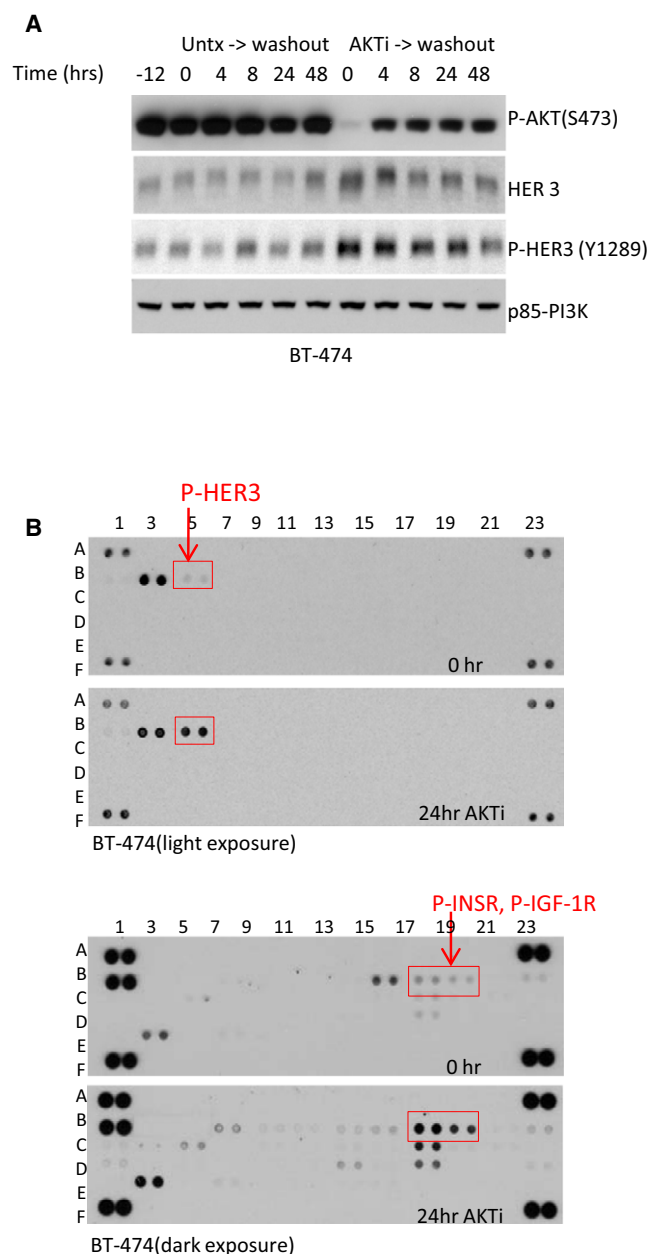
Figure 2. Activation of AKT Represses HER3, Whereas Inhibition Induces Several Phosphorylated RTKs

(A) BT-474 cells (at time = −12) were treated with AKTi-1/2/3 (100 nM) or DMSO for 12 hr (until t = 0). Cells were then washed four times with PBS, placed in fresh media, and collected at indicated times after (t = 4, 8, 24, and 48 hr). Immunoblots demonstrate that loss of HER3 and P-HER3 correlated with activation of AKT.

(B) BT474 cells were left untreated (0 hr) or treated with AKTi-1/2/3 (1 μM) for 24 hr and lysates applied to Phospho-RTK arrays. Spots are in duplicate, and each pair corresponds to a specific P-RTK. Top set of blots correspond to 50 μg lysates, whereas bottom two correspond to 250 μg lysates with HER2 and HER3 dot blots excised from the membrane. Phospho-HER3 corresponds to the doublet at B5 and shows increased expression with prolonged treatment with the AKT inhibitor.

Activated RTKs Are Induced in Many Tumor Models

In HER2-amplified tumor cells, overexpression of HER2 dysregulates PI3K-AKT signaling by promoting HER2-HER3 heterodimer formation. Our data suggest that AKT activation feedback downregulates HER3 and a set of other RTKs in these cells. We asked whether this is a general phenomenon or peculiar to tumor cells in which HER2 drives PI3K-AKT signaling. We used the phospho-RTK arrays to determine if the effects of AKT inhibition on receptor phosphorylation were shared among different cancer models (Figure 3A; Figure S2). Two additional HER2-amplified cell lines (SK-BR3 and MDA-MB-453) were evaluated. When the results were quantified and the mean of the 3 cell lines taken, 12 receptors (HER3, IGF-1R, IR, HER2, HER4, MSPR, SCFR, EphA1, EphA7, FLT-3, ROR1, and RET) had an induction greater than 3-fold after 24 hr of AKT inhibition. P-HER3 was highly induced in all three of these models. These results were compared with six tumor cell lines lacking HER2 amplification and representing four different cell lineages (LNCAP, prostate; IGROV-1, ovarian; MDA-MB-231, breast; and NCI-H292, NCI-H3255, and NCI-H1975, lung). In these six cell lines, nine receptors had average inductions above 3-fold (HER3, IGF-1R, IR, MER, MET, RET, ROR1, TIE-2, and EphA7). Comparing the HER2 and non-HER2 lists, we noted overlap of IGF-1R, IR, HER3, EphA7, and RET, which were significantly induced (>3-fold mean induction) in the majority of these cell lines. We evaluated the effect of AKT inhibition in a non-transformed, breast epithelial cell line, MCF10A, and found that only IGF-1R, IR, and PDGFRa were induced after AKT inhibition. Figure 3B lists the receptors in the ten cell lines for which average phosphorylation was induced at least 3-fold. The number of cell lines in which each receptor was induced and the average degree of induction are shown. IGF-1R, IR, HER3, EphA7, and RET were induced in the majority of models, whereas HER4, EphA1, ROR1, and MET phosphorylations were induced in a subset. We conclude that inhibition of AKT induces the phosphorylation of a group of RTKs, and induction is not confined to a particular type of tumor or to tumors in which signaling is driven by HER2.

AKT Inhibition Induces the Expression of the HER3, IGF1R, and IRs

In BT-474 cells, AKT inhibition caused a parallel and equivalent induction of HER3 phosphorylation and expression, consistent with the possibility that the former is due to the latter. We evaluated whether induction of HER3, IR, and IGF-1R phosphorylation could be explained by induction in the expression of these proteins. In six out of seven cell lines without HER2 amplification, AKT inhibition caused increased levels of HER3 protein (Figure 3C). Increases in IR were detected in five out of seven of these cell lines, and IGF-1R was detected and induced in six out of seven cell lines. In contrast, RET protein was detected and induced in only two out of the seven cell lines, and HER4 protein was not induced in any of the seven (Figure 3C). The induction in HER3, IGF-1R, and IR protein occurred in the majority of tested models and was not restricted to a specific tumor lineage. Moreover, in the non-transformed model, MCF10A, upregulation in the protein levels of HER3, IR, and IGF-1R was also observed. It was notable that induction of phosphorylated IGF-1R and IR, but not P-HER3, was seen in this model, which may be related to the comparably low levels
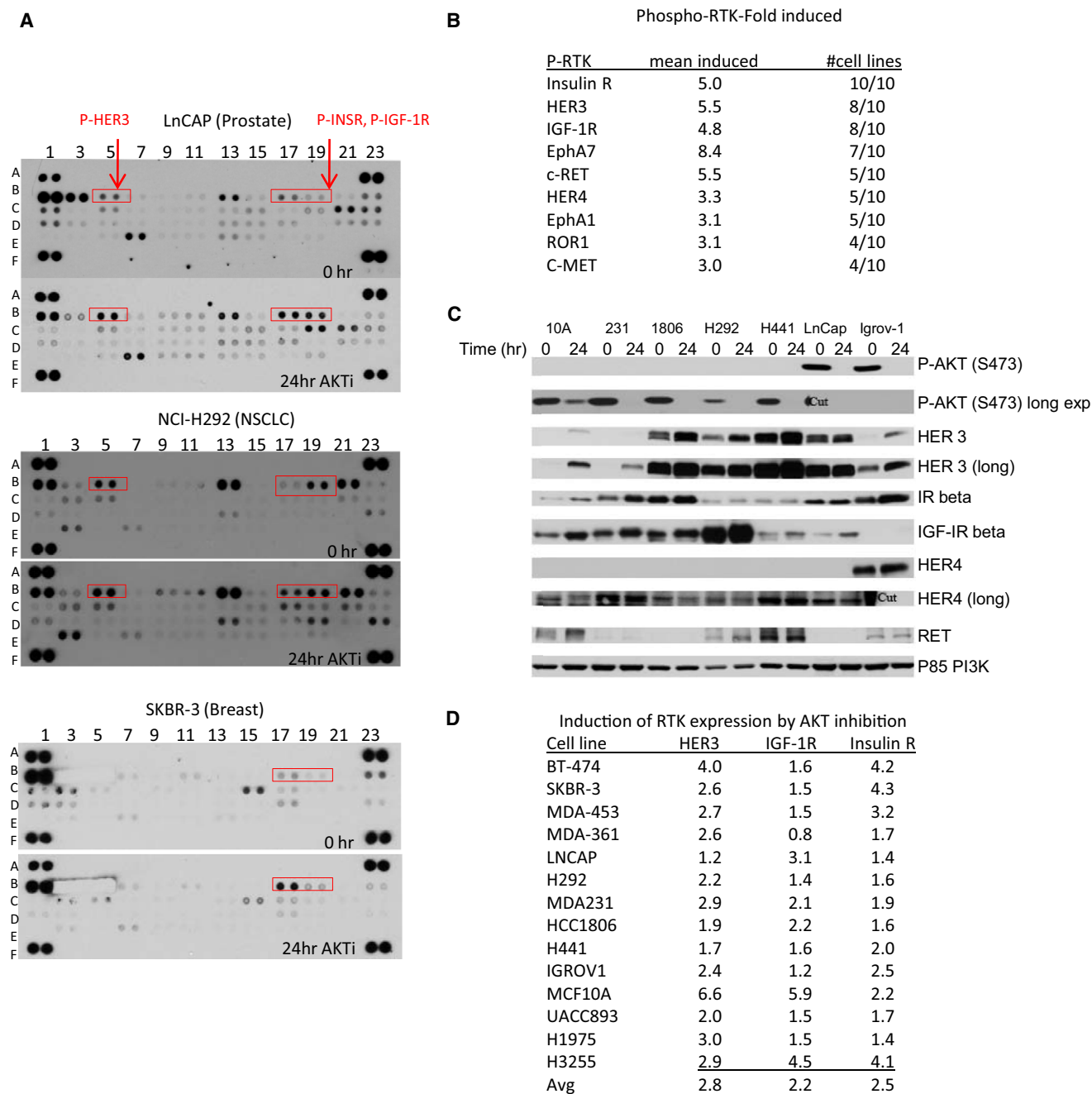
B Phospho-RTK-Fold induced

P-RTK	mean induced	#cell lines
Insulin R	5.0	10/10
HER3	5.5	8/10
IGF-1R	4.8	8/10
EphA7	8.4	7/10
c-RET	5.5	5/10
HER4	3.3	5/10
EphA1	3.1	5/10
ROR1	3.1	4/10
C-MET	3.0	4/10

D Induction of RTK expression by AKT inhibition

Cell line	HER3	IGF-1R	Insulin R
BT-474	4.0	1.6	4.2
SKBR-3	2.6	1.5	4.3
MDA-453	2.7	1.5	3.2
MDA-361	2.6	0.8	1.7
LNCAP	1.2	3.1	1.4
H292	2.2	1.4	1.6
MDA231	2.9	2.1	1.9
HCC1806	1.9	2.2	1.6
H441	1.7	1.6	2.0
IGROV1	2.4	1.2	2.5
MCF10A	6.6	5.9	2.2
UACC893	2.0	1.5	1.7
H1975	3.0	1.5	1.4
H3255	2.9	4.5	4.1
Avg	2.8	2.2	2.5

Figure 3. RTKs Expression and Phosphorylation Are Induced by AKT Inhibition in Multiple Tumor Types

(A) Shown are immunoblots from representative Phospho-RTK arrays from cancer cell lines treated with 1 μM AKTi-1/2/3 (24 hr) compared to untreated (0 hr), demonstrating that phosphorylation of several receptors is induced with inhibitor treatment. The HER2 and HER3 dot blots were excised from the membrane in the case of SKBR-3 cell line shown here. See Supplemental Experimental Procedures for list of receptors corresponding to labels (e.g., B5 = HER3) and Figure S2 for other cell lines tested.

(B) Listed are the receptors in which average tyrosine phosphorylation (ten cell lines) induced >2.9-fold after 1 μM AKTi-1/2/3 (24 hr), as measured by densitometry on P-RTK arrays. Mean induction and the number of cell lines with an induction (>1.3) are listed (cell lines: BT-474, H292, MCF10A, SKBR3, MDA231, MDA453, LNCAP, H3255, H1975, IGROV1).

(C) A panel of seven non-HER2 amplified cell lines was treated with 1 μM AKTi-1/2/3, and immunoblots demonstrate induction of the expression of several RTKs.

(D) Listed is the fold induction of protein expression of HER3, IR, and IGF-1R after AKT inhibition calculated by densitometry from immunoblots. See also Table S1.

of activated HER kinases in this cell. The average induction in protein level of these three receptors across these cell lines and additional HER2-amplified or EGFR mutant cancer cells was quantified by densitometry. All three receptors are commonly induced (HER3, 13/14; IGF-1R, 12/14; IR, 14/14), with average inductions of HER3 2.8-fold, IGF-1R 2.2-fold, and IR 2.5-fold.

Induction of expression of HER3, IGF1R, or IR was almost always associated with increased phosphorylation (in 25 out of 28 instances). However, in most cases, induction of phosphorylation was out of proportion to induction of expression (Table S1). Therefore, the induction of phosphorylation of these proteins can only be partially explained by increased protein expression. Nevertheless, the data suggest that the expression of a common set of receptors is upregulated among cell lines, and this correlates with the induction of their phosphorylation in response to AKT inhibition.

HER-Kinase Dependence of Induced P-RTKs
In most of the tumor cells we examined, the induction of HER3 phosphorylation was the most prominent effect of AKT inhibition. We used selective HER1/2 kinase inhibitors to determine whether induction of phosphorylation of receptors was HER-kinase dependent. Lapatinib, a HER2/EGFR inhibitor that potently blocks HER2 kinase, was used to address this question in breast cancer cells with HER2 amplification (Karaman et al., 2008). Gefitinib, which preferentially inhibits EGFR kinase activity, was used in H292, a non-small cell lung cancer cell line with overexpression of wild-type EGFR. We compared the effects of the AKTi-1/2/3, lapatinib, or the combination on the induction of receptor phosphorylation in the HER2-amplified breast cancer cell (BT-474) (Figure 4A). After 24 hr exposure to AKTi-1/2/3 (1 μM, second panel), phosphorylation of HER3, IGF-1R, IR, and several other receptors was induced. When the HER1/2 kinase inhibitor (1 μM) was combined with the AKT inhibitor, levels of phosphorylated HER3, IGF-1R, and IR were repressed below basal levels. Treatment with lapatinib alone blocked the phosphorylation of all the assessed RTKs below steady-state levels. The effect of the HER kinase inhibitors is unlikely to be due to direct inhibition of IGF1R or IR kinases, to which they do not bind avidly (Karaman et al., 2008). Moreover, these inhibitors do not suppress IGF1R or IR phosphorylation in H1975, in which the T790M mutant EGFR is resistant to gefitinib (Figure S3). These data suggest that induction of IGF1R and IR phosphorylation is HER2 dependent in breast cancers in which this receptor is amplified.

We evaluated the HER-kinase dependence of induction of receptor phosphorylation in a non-HER2-dependent model, NCI-H292, as well. AKT inhibition resulted in induction of phosphorylated HER3, IGF-1R, IR, and several other receptors, including FGFR1 and EphA7 (Figures 4B; Figure S3). In this case the AKTi induction of phosphorylation of some but not all of the RTKs could be suppressed by the HER1/2 inhibitor gefitinib (5 μM). For instance, phosphorylation of HER3 and IR is suppressed below basal levels by the combination, and induction of P-EphA7 is significantly weaker. In contrast, induction of P-FGFR1 is unaffected by gefitinib, and IGF-1R phosphorylation is supra-induced by the combination. The data suggest that in cells in which HER2 is not the dominant RTK, the HER-kinase dependence of induction of receptor phosphorylation is variable.

Induction of Receptor Expression by AKT Inhibitors Is Not Dependent on HER Kinase Activity
AKT inhibition leads to a parallel induction of the expression and phosphorylation of IGF-1R, IR, and HER3. We asked whether the induction of expression of these RTKs is dependent upon HER

kinase activity as well. BT-474 cells were treated for 8 or 24 hr with 1 μM AKTi-1/2/3, 1 μM lapatinib, or the combination (Figure 4C). As expected, both inhibitors block AKT phosphorylation (P-S473). The induction of HER3 and IGF-1R/IR phosphorylation (antibody detects both receptors) by the AKTi was completely blocked by lapatinib. In contrast the induction of IGF-1R, IR, and HER3 protein expression was unaffected by the addition of lapatinib. Similarly, the AKTi-mediated induction of HER3 phosphorylation but not protein expression was blocked by lapatinib in the H292 lung cancer cell line (Figure 4D). These data show that the upregulation of receptor expression caused by AKT inhibition is not dependent on HER kinase activity, whereas the induction of phosphorylation is.

Inhibition of AKT but Not mTORC1 Induces HER3, IGF-1R, and IR Expression
The mTORC1 complex is one of the primary downstream targets of AKT signaling. Direct inhibition of mTORC1 with rapamycin blocks S6Kinase-dependent feedback inhibition of IRS-1 expression and activates IGF-1R signaling (O'Reilly et al., 2006). We asked whether induction of receptor expression in response to AKT inhibition was mediated by mTORC1 inhibition. The effects of HER2, AKT, and mTORC1 inhibition on HER kinase expression and signaling were examined in BT474 cells (Figure 5A). Cells were exposed to AKTi-1/2, lapatinib, or rapamycin for up to 24 hr. Each inhibitor causes potent inhibition of its respective target (P-HER2 for lapatinib, P-S6K for rapamycin, and P-AKT for AKTi-1/2) at 8 and 24 hr. Rapamycin and AKTi-1/2 each induces HER3 phosphorylation, although the effect of the latter is more marked (Figures 5A and 5B; Figure S4). Both AKTi-1/2 and lapatinib inhibit AKT phosphorylation and induce HER3 expression. In contrast, rapamycin does not induce expression, only phosphorylation. Thus, AKT and mTORC1 both regulate the feedback inactivation of HER kinase signaling. However, mTORC1 affects HER3 phosphorylation, but not expression. AKT, but not mTORC1, is responsible for the feedback regulation of HER3 expression.

We compared the effects of rapamycin and AKTi-1/2 on the phosphorylation of RTKs in BT474 (Figure 5B). After 24 hr of treatment, the effects of the two drugs were markedly different. Rapamycin induced HER3 phosphorylation more than 4-fold (2.5-fold less than the AKTi-1/2) and had no effect on IR or IGF1-R phosphorylation. Several other receptors (FLT-3, HER4, MSPR, EphA1, and EphA7) whose phosphorylation was induced by AKT inhibition were either less affected or unaffected by rapamycin. In contrast, rapamycin induced the phosphorylation of two receptors (MER and MuSK) more potently than AKT inhibition did. These findings suggest that inhibition of AKT and mTORC1 relieves different aspects of PI3K-induced feedback.

AKT Inhibition Upregulates RTK Transcript Levels
Because levels of HER3, IGF-1R, and IR protein increase after AKT inhibition, we determined whether the mRNAs encoding these receptors were affected as well. We compared the effects of AKT inhibition and mTORC1 inhibition on HER2, HER3, IGF-1R, IR, RET, and MET mRNA levels. IR, HER3, and IGF-1R mRNA levels were all induced between 2.5- and 5-fold after exposure of cells to either the AKT inhibitor or lapatinib,, but not to rapamycin (Figure 6A). Rapamycin induced only minor
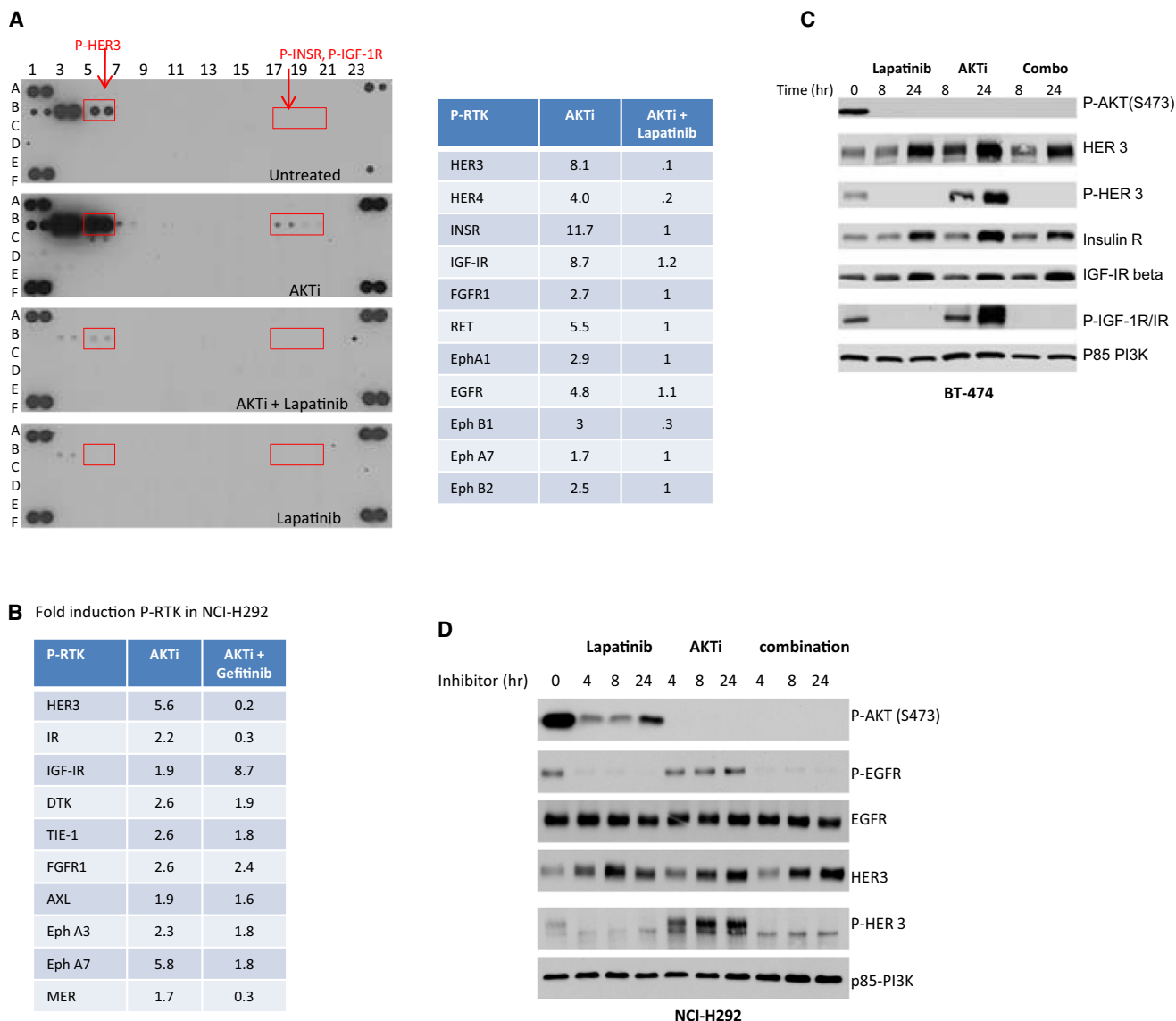
A

P-RTK	AKTi	AKTi + Lapatinib
HER3	8.1	.1
HER4	4.0	.2
INSR	11.7	1
IGF-IR	8.7	1.2
FGFR1	2.7	1
RET	5.5	1
EphA1	2.9	1
EGFR	4.8	1.1
Eph B1	3	.3
Eph A7	1.7	1
Eph B2	2.5	1

C

B Fold induction P-RTK in NCI-H292

P-RTK	AKTi	AKTi + Gefitinib
HER3	5.6	0.2
IR	2.2	0.3
IGF-IR	1.9	8.7
DTK	2.6	1.9
TIE-1	2.6	1.8
FGFR1	2.6	2.4
AXL	1.9	1.6
Eph A3	2.3	1.8
Eph A7	5.8	1.8
MER	1.7	0.3

D

Figure 4. HER Kinase Inhibition Does Not Block AKTi-Stimulated RTK Expression but Can Alleviate AKTi Induction of P-RTKs
(A) BT-474 cells were treated with DMSO, 1 μM AKTi-1/2/3, 1 μM lapatinib, or the combination. Immunoblots of P-RTK arrays were quantified by densitometry. The effect of the HER1/2 kinase inhibitor upon the induction caused by AKT inhibition is shown with the induced expression of most P-RTKs blocked in the combination treatment.
(B) NCI-H292 cells were treated with DMSO, 1 μM AKTi-1/2/3, 5 μM gefitinib, or the combination. The effect of the HER kinase inhibitor upon the induction caused by the AKTi is shown with the induced expression of some P-RTKs blocked in the combination treatment.
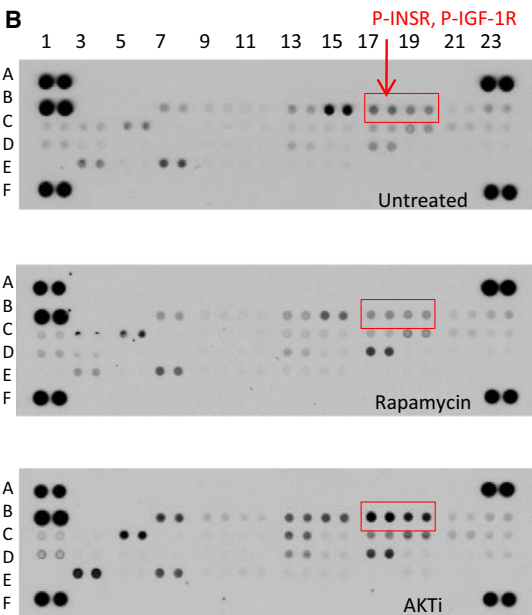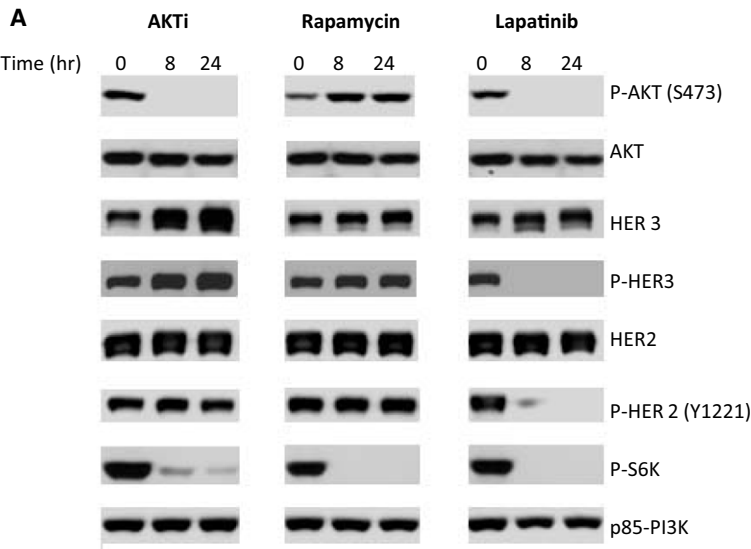(C) BT-474 cells were treated with DMSO, 1 μM AKTi-1/2/3, 1 μM lapatinib, or the combination and collected at 8 and 24 hr posttreatment. Immunoblotting of lysates demonstrates AKTi or lapatinib, or the combination induces HER3, IGF-1R, and IR expression, but phosphorylation of these receptors is blocked by lapatinib treatment.
(D) NCI-H292 cells were treated with 1 μM lapatinib, 1 μM AKTi-1/2/3, or the combination and collected at 4, 8, or 24 hr posttreatment, and lysates were immunoblotted showing induction of HER3 and P-HER3 in response to AKT inhibition and blockade of HER3 phosphorylation by lapatinib. See also Figure S3.

changes in mRNA expression. Expression of HER2, RET, and MET mRNAs was not induced by any of the inhibitors. The results confirm that relief of feedback inhibition of RTK signaling in response to AKT inhibition cannot be explained by inhibition of mTORC1 alone. Feedback inhibition of mitogenic signaling in response to AKT activation must be mediated by other targets as well.

FOXO Transcription Factors Mediate Induction of RTK Expression
FOXO transcription factors regulate a number of genes involved in cell survival and longevity and are inhibited when phosphorylated by AKT (Kenyon et al., 1993; Kops et al., 1999; Myatt and Lam, 2007). The *Drosophila* IR has been demonstrated to be dependent upon the activity of the FOXO transcription factor

A

B

P-INSR, P-IGF-1R

Figure 5. Inhibition of AKT but Not mTORC1 Promotes the Expression and Phosphorylation of Several RTKs

(A) BT-474 cells were treated with 1 μM AKTi-1/2, 50 nM rapamycin, or 500 nM lapatinib and collected at indicated times. Loss of AKT S473 phosphorylation is associated with an induction in HER3 protein levels, whereas treatment with rapamycin is not associated with significantly increased levels of HER3.

(B) BT474 cells were treated with 1 μM AKTi-1/2 or 50 nM rapamycin and lysates applied to Phospho-RTK arrays. The HER2 and HER3 dot blots were excised from the membranes shown here. Densitometry was performed to calculate fold induction over untreated cells. All receptors induced >1.9-fold are listed. See also Figure S4.

Fold induction P-RTK in BT-474

P-RTK	AKTi	Rapa
HER3	12.1	4.6
HER4	3.2	1.0
INSR	2.4	0.5
IGF-IR	2.3	0.6
FLT-3	6.1	1.2
MSPR	2.9	1.5
Mer	1.7	2.0
Eph A1	2.4	0.7
Eph A7	3.1	0.5
MuSK	1.3	2.0
MET	0.2	1.0

ing HER3, IGF1R, and IR induction by 64%, 90%, and 69%. Knocking down FOXO1 or FOXO4 had less effect, diminishing HER3 and IR inductions by <20%. However, combined knockdown of all three isoforms was most potent, diminishing HER3, IGF1R, and IR induction by 73%, 91%, and 80% of that in the control cells. Knockdown of FOXO isoforms had comparable effects on receptor protein expression in BT-474 cells and the nontransformed MCF10A cell line (Figure 6D; Figure S5). Moreover, the induction of phosphorylation of HER3, IGF-1R, and IR was significantly reduced in cells in which FOXOs were silenced (Figure S5). Induction of P-HER3 was reduced nearly 3-fold, whereas inductions of P-IGF-1R and P-IR did not occur. The incomplete suppression of induction of HER3 phosphorylation in cells in which FOXO expression was knocked down is consistent with the modest induction of HER3 phosphorylation by rapamycin (Figure 5). This suggests that the increase in RTK phosphorylation in response to AKT inhibition is due both to a FOXO-dependent increase in receptor expression and to increased phosphorylation in response to TORC1 inhibition. These data show that activation of FOXOs in response to AKT inhibition is required for the upregulation of RTK expression.

FOXO protein binding to the HER3, IGF-1R, and IR promoters and whether binding is altered in response to AKT inhibition were examined by chromatin immunoprecipitation (ChIP) assays (Figure 6C). BT-474 cells were treated with AKT inhibitor, fixed with formalin, and the total chromatin was collected. Immunoprecipitation with a combination of antibodies against FOXO1, FOXO3, and FOXO4 resulted in enhanced amplification of the RTK 5′UTR over input chromatin as measured by RT-PCR. Exposure of cells to the AKT inhibitor resulted in an enhancement of RTK amplification (HER3, 4.3-fold; IGF-1R, 2.4-fold, INSR,
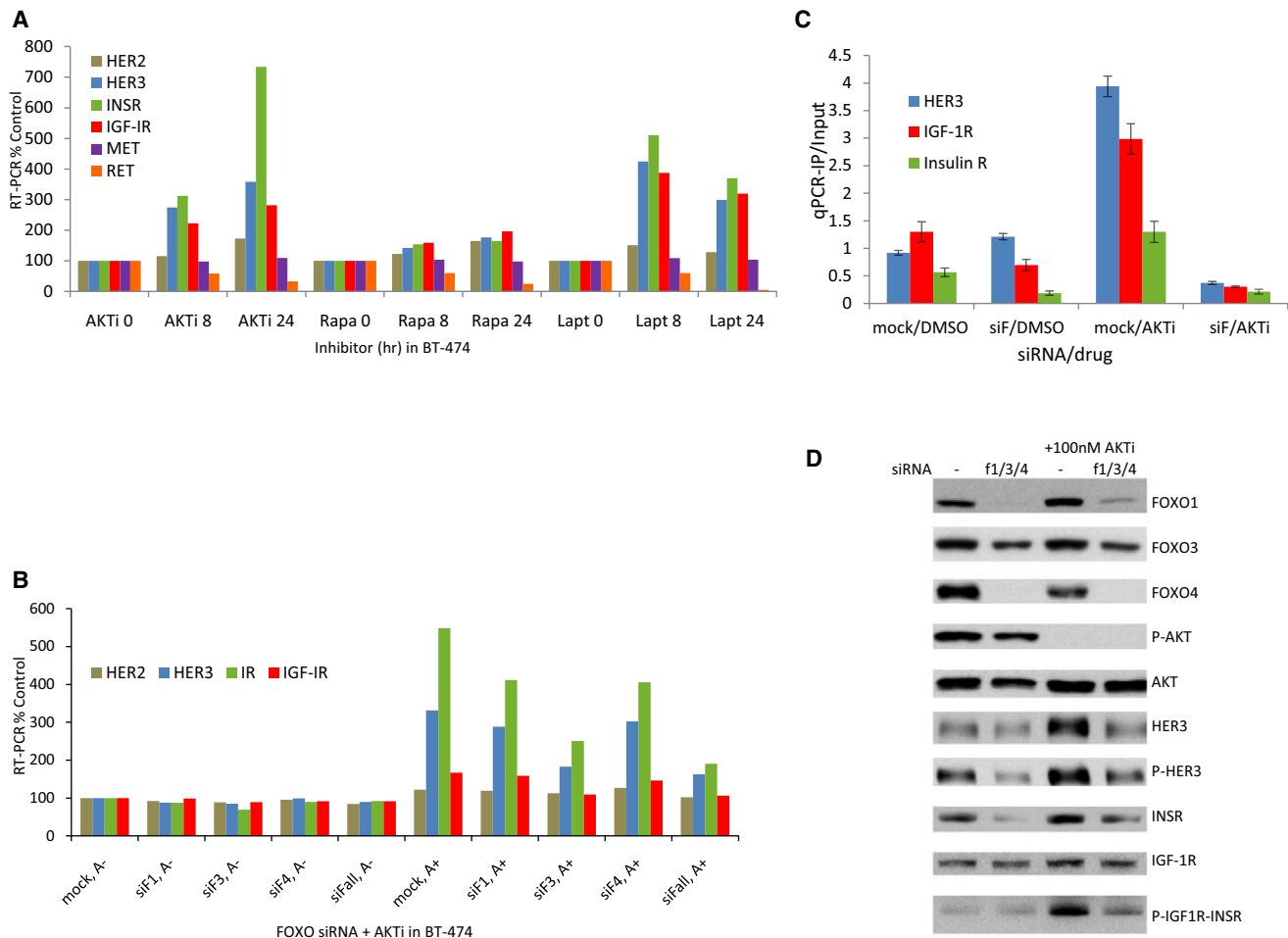
for its expression (Ayala et al., 1999; Puig and Tjian, 2005). Because inhibition of the PI3K-AKT pathway causes dephosphorylation of FOXO proteins and prevents their nuclear translocation, we investigated whether mammalian FOXO transcription factors may be involved in the induction of the RTKs by the AKT inhibitor (Myatt and Lam, 2007). FOXO1, 3, and 4 were depleted individually and in combination using specific, small-interfering RNAs, and the effect on basal and induced levels of receptors was examined (Figure 6B). In BT-474 cells, knockdown of these factors alone or in combination had little effect on steady-state levels of HER3, IGF-1R, or IR mRNA expression. However, the induction of receptor mRNA by the AKT inhibitor was markedly diminished by FOXO knockdown. Of the three isoforms, inhibition of FOXO3 expression had the most potent effect, diminish-

Figure 6. AKT but Not mTORC1 Inhibition Induces RTK RNA Expression in a FOXO-Dependent Manner

(A) BT-474 cells treated with 1 μM AKTi-1/2, 50 nM rapamycin, or 500 nM lapatinib, and RT-PCR was performed using reverse-transcribed cellular RNA. Fluorescence was normalized to housekeeping gene expression (β-actin and GAPDH) and is displayed as the percentage of the value for untreated lysate. Expression of HER3, IGF-IR, and IR was induced by effective suppression of activated AKT but minimally with rapamycin treatment.

(B) BT-474 cells were treated with interfering RNAs against FOXO1 (siF1), FOXO3 (siF3), FOXO4 (siF4), FOXO1 + 3 + 4 (siFall), or mock (M) with or without AKTi-1/2/3 (1 μM). Cells were collected 72 hr after siRNA transfection (24 hr after AKTi), and RT-PCR was performed with the indicated probes. Inhibition of AKT is associated with enhanced expression of the HER3, IR, and IGF-1R RNAs. RNA induction is suppressed by FOXO1/3/4 knockdown.

(C) BT-474 cells were treated with FOXO1/3/4 siRNAs or mock for 48 hr followed by treatment with AKTi-1/2/3 (1 μM) or DMSO for 24 hr followed by formalin fixation and isolation of chromatin. Immunoprecipitation was performed using antibodies against FOXO 1, 3, and 4, and RT-PCR was done with oligonucleotides complementary to a sequence 1 kb 5′ to the HER3, INSR, and IGF-1R start sites. Expression was normalized to the non-immunoprecipitated (input) fraction and reported as fold enrichment (± SEM) and demonstrated increased binding of FOXO proteins to the RTK 5′ UTRs upon AKT inhibition.

(D) BT-474 cells were treated as in (C) and collected for immunoblotting, demonstrating that knockdown of FOXO proteins associated with diminished induction of the RTKs. See also Figure S5.

2.3-fold), whereas inhibition of FOXO protein expression with siRNA abolished this effect. These data suggest that activation of FOXO proteins is directly responsible for induction of RTK transcription.

Combined Inhibition of AKT and HER1/2 Kinases Is Effective In Vivo

The prevalence of AKT activation in human tumors has led to a widespread effort to develop inhibitors of the pathway. Relief of feedback inhibition of signaling by these inhibitors may limit their therapeutic effects. The induction of HER3 expression in response to the AKTi-1/2/3 in BT-474 cells is associated with increased HER3 binding to PI3K and to HER2, as shown

by co-immunoprecipitation and crosslinking experiments in Figure S1.

We used HER kinase inhibitors to ask whether blocking induction of HER family kinase activity enhances the antitumor efficacy of AKT inhibitors in vivo. We investigated the effects of the combination in two models in which AKT inhibition induces HER3: BT474, in which HER2 is amplified (Figure 7A), and H292 non-small cell lung cancer, in which wild-type EGFR is overexpressed (Figure 7B). In both models, AKT inhibition induces HER3 and IGF1R/IR phosphorylation (Figures 7C and 7D).

In the BT474 model, chronic administration of the AKT inhibitor completely inhibits tumor growth but does not cause regression (Figure 7A). In H292 the AKT inhibitor causes a marginal decrease
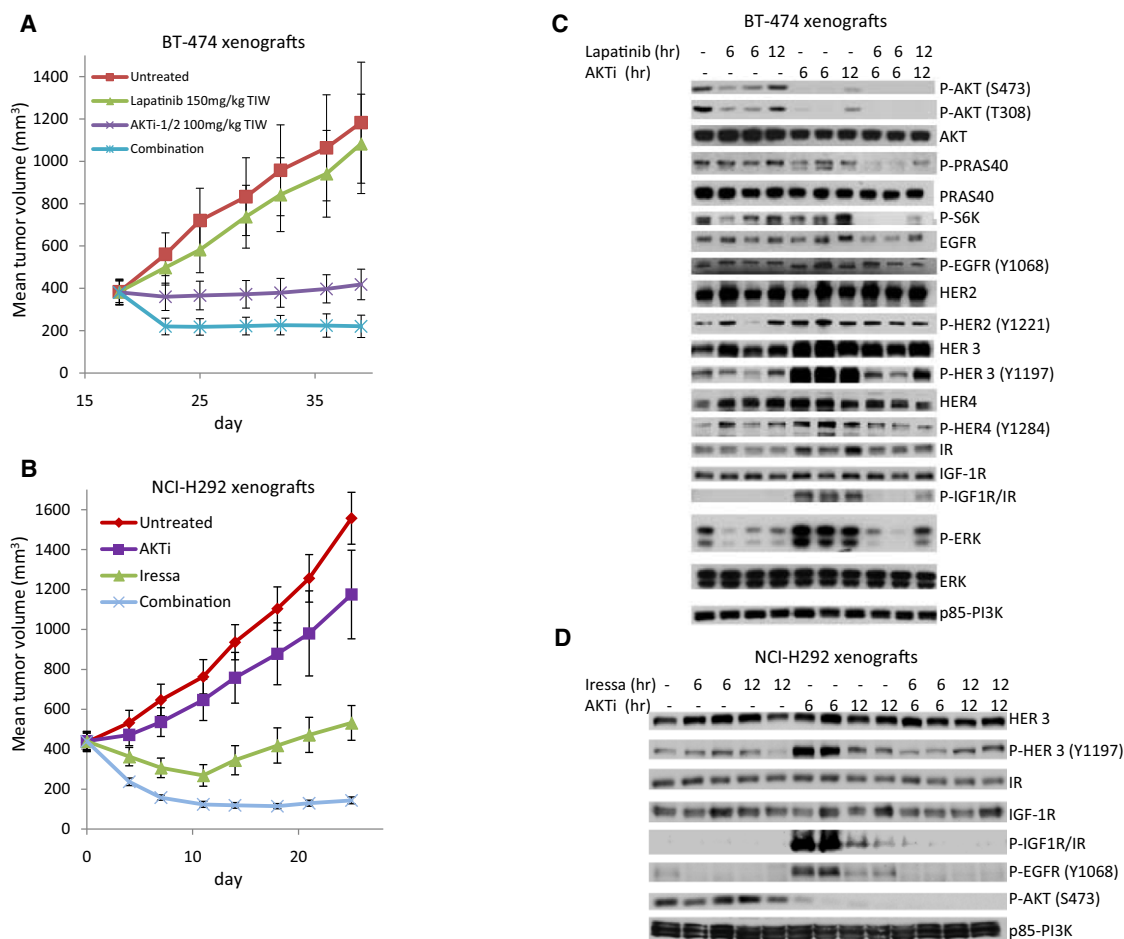
Figure 7. HER1/2 Kinase Inhibition Improves Antitumor Efficacy When Given in Combination with the AKTi

(A) Mice bearing BT-474 tumors were randomized to (1) no treatment, (2) lapatinib 150 mg/kg three times/week, (3) AKTi-1/2 100 mg/kg three times/week, or (4) combination of (2) and (3) and tumor size measured two times/week with the combination treatment demonstrating superior antitumor efficacy compared to the single agents.

(B) Mice bearing NCI-H292 tumors were randomized to (1) no treatment, (2) gefitinib (Iressa) 150 mg/kg three times/week, (3) AKTi-1/2/3 100 mg/kg three times/week, or (4) combination of (2) and (3) and tumor size measured by vernier calipers two times/week with the combination treatment demonstrating superior antitumor effects to the single agents. The results in (A) and (B) are presented as the mean tumor volume ± SEM (n = 5 mice/group).

(C) Mice (n = 12) bearing BT-474 tumors were randomized to no treatment, treatment with a single dose of lapatinib 150 mg/kg, AKTi-1/2/3 100 mg/kg, or the combination and collected at 6 and 12 hr posttreatment. Immunoblotting of tumor lysates demonstrates that AKT inhibition induces P-HER3 and P-IGF-1R/INSR in vivo, and this is attenuated by lapatinib coadministration. Note, two mice treated for the 12 hr time points lacked adequate tumor tissue at retrieval and were omitted.

(D) Mice bearing NCI-H292 tumors were treated with a single dose of gefitinib 150 mg/kg, AKTi-1/2/3 100 mg/kg, or the combination and collected at 6 and 12 hr posttreatment. Immunoblotting of lysates demonstrates that AKT inhibition induces P-HER3 and P-IGF-1R/INSR, and this is attenuated by gefitinib coadministration. See also Figure S6.

in growth rate (Figure 7B). In order to determine whether inhibition of feedback induction of HER kinases increases the therapeutic effects of the AKT inhibitor, we used low doses of HER kinase inhibitors that affect baseline HER3 phosphorylation only minimally but do prevent induction of HER3/IGF1R/IR phosphorylation in response to the AKT inhibitor (Figures 7C and 7D). Although lapatinib given at a more frequent schedule has significant antitumor effects in this model, at this schedule the lapatinib had little effect. However, it significantly altered the response to the AKT inhibitor, and the combination caused partial tumor regression. Maximal doses of the AKT inhibitor alone cause growth arrest without regression in this model.

In the H292 model the AKT inhibitor did not significantly block tumor growth. However, the addition of the EGFR kinase inhibitor to the AKTi-1/2/3 caused significant and persistent tumor regression (Figure 7B). Iressa by itself did significantly inhibit tumor growth, but as previously reported, the effects were transient and significantly less than the combination (Buck et al., 2008). Thus, doses of HER kinase inhibitor sufficient to prevent relief of feedback inhibition of HER3 by the AKT inhibitor sensitize tumors to this drug. In keeping with these data, we find that prevention of RTK induction with an Hsp90 inhibitor also sensitizes tumors to AKT inhibitors and causes profound tumor regression in the BT474 model (Figure S6).

DISCUSSION

It is increasingly clear that dysregulation of mitogenic signaling by constitutively activated oncoproteins in cancer cells drives high levels of feedback inhibition of the signaling network (Courtois-Cox et al., 2006; O'Reilly et al., 2006; Pratilas et al., 2009). This may have important phenotypic consequences in the transformed cell. Hyperactivation of signaling by the oncoprotein may depend on its relative insensitivity to negative feedback or to other mutations that inactivate elements of the feedback machinery. Anticancer drugs that inhibit oncoprotein function will relieve this negative feedback and, thus, reactivate multiple signaling pathways that limit the extent and duration of the anticancer effects.

The PI3K-AKT signaling pathway is a central downstream effector of growth factor receptors and is often dysregulated in cancer. The insulin and IGF-1Rs exert many of their physiologic effects by activating PI3K, but mutation or overexpression of other receptors, such as HER2 in breast cancer, commonly dysregulates the pathway in tumors (Holbro et al., 2003; Kooijman et al., 1995). Activation of PI3K-AKT leads to activation of many downstream targets that together account for the proliferative, antiapoptotic, and metabolic effects of the pathway. Of these, the mTOR kinase has attracted much attention because of its central function in integrating nutrient and energy availability and growth signals in the regulation of cell proliferation and size. mTOR functions in two multiprotein complexes, mTORC1 and mTORC2 (Guertin and Sabatini, 2007). The natural product rapamycin is a specific inhibitor of mTORC1 and leads to dephosphorylation of its two most well-characterized substrates, S6 kinase and 4EBP1 (Brunn et al., 1997; von Manteuffel et al., 1997). It thereby inhibits cap-dependent translation and cell proliferation.

Experiments with rapamycin first revealed the extent and clinical implications of oncogene-induced feedback. Insulin signaling is feedback regulated in part by an mTOR/S6K-dependent phosphorylation and downregulation of the major IR substrate IRS1 (Haruta et al., 2000). Inhibition of mTOR with rapamycin relieves this feedback, activates insulin and IGF signaling, and thereby activates PI3K and ERK signaling (O'Reilly et al., 2006). This occurs in vivo in patients and likely decreases the therapeutic efficacy of the drug (Mellinghoff et al., 2005; O'Reilly et al., 2006).

PI3K and AKT regulate many processes besides mTORC1 activity. We reasoned that in tumor cells, mutational activation of the PI3K-AKT pathway would induce mTOR-independent feedback pathway as well. We used a selective, allosteric inhibitor of AKT to assess AKT-dependent feedback in breast tumor cells in which the pathway is driven by amplification of HER2. We found that inhibition of AKT in these cells induced the expression of HER3. There was a concomitant induction of HER3-HER2 heterodimers and a marked induction of HER3 phosphorylation.

The results are consistent with the idea that AKT activation causes feedback inhibition of HER kinase expression, especially of HER3, which when phosphorylated, docks with and activates PI3K. The induction of HER3 in response to AKT inhibition is associated with an increase in HER2-HER3 heterodimers and leads to increased HER3 phosphorylation. HER3 phosphorylation is blocked by the HER1/2 kinase inhibitor lapatinib, but the increase in HER3 expression is not. This finding suggests that the increase in HER3 expression is in large part responsible for the observed increase in phosphorylation.

HER3 expression is induced by inhibitors of PI3K or AKT or by knockdown of AKT. That induction of HER3 expression and phosphorylation in response to AKT inhibition represents release of AKT-dependent feedback inhibition of the pathway is supported by the downregulation of HER3 expression that occurs when the AKT inhibitor is washed out of cells. We used phospho-RTK arrays to ask whether AKT-induced negative feedback was confined to HER3 or involved other receptors as well. We found that, although HER3 induction was very prominent, the phosphorylation of multiple other receptors was induced as well. Induction of receptor phosphorylation was not confined to HER2-dependent breast cancers; it occurred in tumor cells derived from all lineages tested (breast, prostate, ovary, lung, melanoma). We identified a set of nine RTKs whose phosphorylation is commonly induced after AKT inhibition. Four of these (HER3, IGF1R, IR, and EphA7) responded in almost all cells tested.

Phosphorylated HER3 has a high capacity and affinity for PI3K, docking it to the membrane. The most obvious physiologic role of PI3K-AKT signaling is mediating the effects of the insulin and IGF-1Rs. It seems from our data that these three receptors are coordinately feedback downregulated by AKT when the pathway is activated. AKT inhibition induces the expression as well as the phosphorylation of HER3, IGF1R, and IRs. Induction of other kinases such as RET and HER4 is confined to phosphorylation; expression is typically unaffected. HER2 is the dominant activated kinase in breast cancers in which it is amplified, and in these tumors the induction of phosphorylation of the other RTKs is HER2 dependent. Lapatinib blocks their phosphorylation, but not the induction of expression of IGF1R and IR. Previous work by other labs has demonstrated that IGF1R and IR kinases are not antagonized by lapatinib at doses as high as 3 μM, and the lack of effect of lapatinib upon IGF1R and IR in non-HER2 driven models like H1975 (Figure S3) supports that the activity seen here is not due to direct inhibition of those kinases. Whether the HER-kinase dependence of induction of IGF-1R/IR phosphorylation represents transphosphorylation of these kinases by HER2 or a HER2-dependent activation of autophosphorylation is under investigation. Activation of IGF1-R and IR by AKT inhibition does involve both induction of expression and HER kinase-dependent phosphorylation of these kinases. In the non-small cell lung cancer model H292, the induction of phosphorylation of some RTKs like HER3 is HER kinase inhibitor sensitive. Others, such as FGFR and IGF1R, are insensitive. It is clear that activation of AKT in tumors induces a complex and broad pattern of feedback inhibition of RTKs that is relieved by inhibition of AKT.

TORC1 inhibition by rapamycin has also been shown to activate signaling, and less-selective PI3K inhibitors that target both mTORC1 and PI3K have been shown to induce HER3 expression (Amin et al., 2010; Sergina et al., 2007). We asked whether AKT inhibition activated signaling via inhibition of mTORC1. Rapamycin partially reproduced the effects of AKT inhibition, inducing the phosphorylation of HER3 along with several other RTKs. However, induction of HER3 was considerably weaker than that observed with AKT inhibition, and the
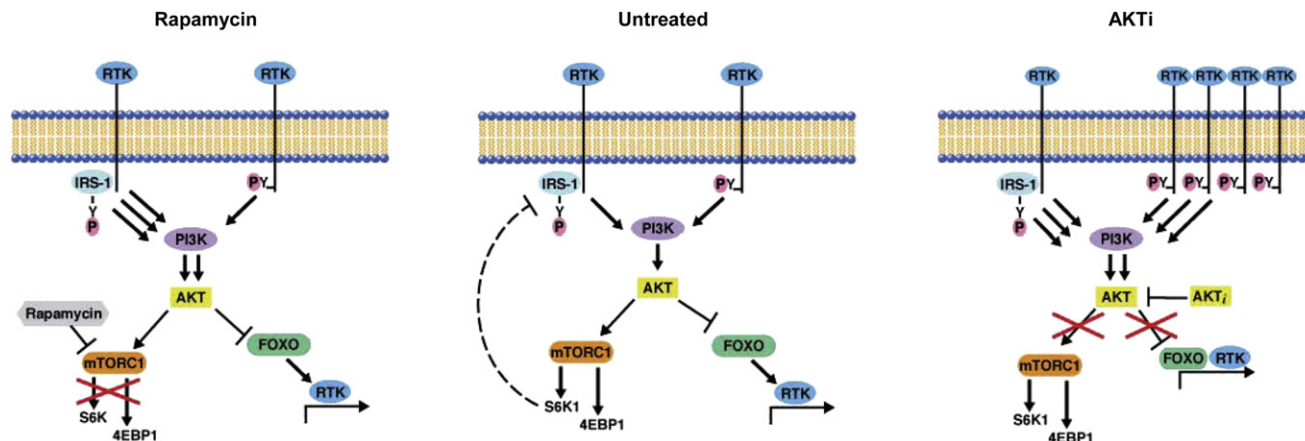
Figure 8. AKT Inhibition and mTORC1 Inhibition Relieve Feedback Inhibition at Unique Nodes of Oncogenic Growth Factor Signaling Pathways

Depicted in middle panel is the steady-state feedback program elicited by activation of AKT and/or mTORC1. Receptor activation stimulates PI3K-AKT activity, which inhibits FOXO-mediated activation of RTK transcription. Receptor stimulation also activates mTORC1, which directs inhibitory feedback upon the expression of IRS-1, thereby attenuating PI3K-AKT activity. In the left panel, rapamycin blocks mTORC1 action, relieving the feedback inhibition upon IRS-1 and causing hyperactivation of PI3K-AKT signaling. In the right panel, AKT inhibition blocks both AKT and the downstream mTORC1. The consequence is to relieve mTORC1 feedback but also to activate FOXO-driven transcription of RTKs and stimulate PI3K-AKT activity through higher RTK expression and activity.

phosphorylation of most of the RTKs induced by AKT inhibition was unaffected by rapamycin. The differences between the effects of the AKT inhibitor and rapamycin suggest that there are AKT-regulated feedback pathways that are not mediated by TORC1.

A clue to the nature of these pathways came from studies on the mechanism of induction of expression of HER3, IGF1R, and IR. AKT inhibition results in marked induction of the mRNAs encoding these receptors, whereas rapamycin has either no or marginal effects. AKT has been shown to phosphorylate the FOXO family of transcription factors and thereby prevent their nuclear translocation, thus inhibiting their function (Brunet et al., 1999). We show that AKT inhibition recruits FOXO proteins to the HER3 promoter and that FOXO1/3/4 knockdown with siRNA suppresses the induction of IGF1-R/IR/HER3 expression and phosphorylation. We note that the knockdown of FOXO proteins has little effect on the basal expression of the RTKs. We postulate that in these cells with activated PI3K/AKT signaling, FOXOs are effectively inhibited, and expression of HER3, IGF1R, and IR is dependent on other factors in this state. However, AKT inhibition results in activation of these transcription factors, enabling them to promote RTK expression. Thus, we conclude that AKT regulates the expression of these receptors by inhibiting FOXO-dependent transcription.

We propose the following model based on our current understanding to explain the regulation of PI3K-AKT signaling by negative feedback in tumors and how it is affected by targeted drugs (Figure 8). Receptor activation of PI3K-AKT causes AKT-dependent phosphorylation of FOXO proteins, which downregulate the expression of some of the receptors that are tightly coupled to PI3K, including HER3, IGF1R, and IR. In addition, AKT activation leads to activation of TORC1 and S6K, which feedback inhibits IRS1 expression and other undefined regulators of receptor signaling. The result is down modulation of the signal.

Therapeutic inhibition of different components of the pathway reactivates feedback, but by mechanisms specific to the inhibited target (Figure 8). Thus, AKT inhibition will result in activation of FOXO-dependent transcription of receptors and inhibition of S6K-dependent inhibition of signaling with resultant activation of multiple receptors. The downstream effects of AKT will be suppressed, but other RTK-driven signaling pathways will be activated. In contrast, TORC1 inhibition blocks S6K-dependent feedback, activates IGF and HER kinases, but not their expression, and, thus, activates both AKT and ERK signaling.

These findings have important basic and therapeutic implications. The enhancement of signaling by autocrine activation or mutation of RTKs that activate PI3K-AKT signaling would be expected to be limited by negative feedback. Selection of oncogenes that encode proteins that overcome or are unresponsive to feedback would be favored (e.g., activating mutation in PI3K or loss of PTEN). All drugs that inhibit components of dysregulated mitogenic signaling pathways would be expected to relieve feedback inhibition of other components of the signaling network. This may reduce the antitumor effects of the drug but also ameliorate toxicity. Combined inhibition of the oncoprotein and key pathways reactivated by inhibition of negative feedback should have enhanced antitumor activity. This is consistent with our finding that the AKT inhibitor causes tumor regressions when combined with low doses of HER kinase or HSP90 inhibitors that prevent or attenuate induction of receptor phosphorylation. Whether effective inhibition of both PI3K-AKT signaling and feedback reactivated pathways will have an enhanced therapeutic index will have to be evaluated in clinical trials.

EXPERIMENTAL PROCEDURES

Reagents

AKTi-1/2 and AKTi-1/2/3 (MK-2206) were from Merck; both drugs were dissolved in DMSO for in vitro studies. For in vivo studies, AKTi-1/2 was dissolved

in 25% hydroxypropyl β-cyclodextrin (pH 4–5) and administered subcutaneously, whereas AKTi-1/2/3 was dissolved in 30% Capitisol and administered by oral gavage. A detailed list of other reagents utilized is provided in Supplemental Experimental Procedures.

Immunoblotting

Lysates from cells in culture were prepared by washing twice in cold PBS followed by lysis with either SDS-lysis buffer (50 mM Tris-HCl [pH 7.4], 2% SDS) or RIPA-lysis buffer supplemented with protease and phosphatase inhibitors (Pierce Chemical). For immunoprecipitations, cells were lysed with NP40-lysis buffer (50 mmol/l Tris [pH 7.4], 1% NP40, 150 mmol/l NaCl, 40 mmol/l NaF) supplemented with protease and phosphatase inhibitors (Pierce Chemical). For lysis in SDS, lysates were boiled for 5 min followed by brief sonication. Lysates were cleared by centrifugation at $14,000 \times g$ (10 min), and the supernatant was collected. Protein concentration of each sample was determined using the BCA kit (Pierce) per manufacturer's instructions. Twenty-five or 50 μg protein was loaded onto 6% or 10% SDS-PAGE minigels for immunoblotting. Further details on immunoprecipitation and crosslinking are provided in Supplemental Experimental Procedures.

RT-PCR

TaqMan reactions were done using an ABI 7500 real-time quantitative PCR system. For data analysis, raw counts were normalized to housekeeping gene average for the same time point and condition (ΔCt). Counts are reported as fold change relative to the untreated control ($2^{-\Delta\Delta Ct}$). A list of probes and reagents used is provided in the Supplemental Experimental Procedures.

ChIP Assays

ChIP assays were performed according to the manufacturer's instructions (MagnaChIP assay kit; Upstate Biotechnology). Approximately 1×10^7 cells were used for each immunoprecipitation. DNA was amplified by PCR using primer pairs against the HER3 5′ UTR [GPH003183(-)01A from SA Biosciences], IGF-1R 5′ UTR [GPH004604(-)02A)] and IR 5′ UTR [GPH019975(-)01A] and SYBR Green reaction mix (SA Biosciences), and product was quantified using an ABI 7500 real-time quantitative PCR system. Reactions were performed in triplicate, and the mean was normalized to input chromatin and reported as relative fold enrichment ± SEM.

Cell Lines

BT-474, SKBr-3, MDA-MB-453, MDA-MB-361, LNCAP, NCI-H292, MDA-MB-231, HCC-1806, NCI-H441, MCF10A, UACC-893, and NCI-H1975 were obtained from the American Type Culture Collection. H3255 cells were a gift from B. Johnson and P. Janne (Dana Farber Cancer Institute), and IGROV-1 cells were a gift from D. Spriggs (Memorial Sloan-Kettering Cancer Center). Details on medium conditions and use of cell lines in xenograft studies are found in Supplemental Experimental Procedures.

Animal Studies

Four to six-week-old nu/nu athymic BALB/c female mice were obtained from the NCI-Frederick Cancer Center and maintained in pressurized ventilated caging. All studies were performed in compliance with institutional guidelines under an IACUC approved protocol (Memorial Sloan-Kettering Cancer Center No. 09-05-009). For efficacy studies, mice with well-established tumors were selected and randomized approximately 14 days postimplantation (size >200 mm³); BT-474 xenograft tumors were established in nude mice by subcutaneously implanting 0.72 mg sustained release 17β-estradiol pellets with a 10 g trocar into one flank followed by injecting 1×10^7 cells suspended 1:1 (volume) with reconstituted basement membrane (Matrigel, Collaborative Research) on the opposite side 3 days afterward. Mice were treated with AKTi-1/2, AKTi-1/2/3, gefitinib, SNX5422, or lapatinib with the indicated doses. Tumor dimensions were measured with vernier calipers and tumor volumes calculated: $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2.

RTK Arrays

Human Phospho-RTK arrays were utilized according to manufacturer's instructions. Briefly, cells were washed with cold PBS and lysed in NP40 lysis buffer, and 50–250 μg of lysates was incubated with blocked membranes overnight. Membranes were subsequently washed and exposed to chemilumi-

nescent reagent and exposed to X-ray film. Quantification of pixels was performed by densitometry using Adobe CS2 and Fuji Film Multi Gauge software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at doi:10.1016/j.ccr.2010.10.031.

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