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## Escape from HER family tyrosine kinase inhibitor therapy by the kinase inactive HER3

Natalia V. Sergina<sup>1</sup>, Megan Rausch<sup>1</sup>, Donghui Wang<sup>1</sup>, Jimmy Blair<sup>2</sup>, Byron Hann<sup>1</sup>, Kevan M. Shokat<sup>2</sup>, and Mark M. Moasser<sup>1</sup>

<sup>1</sup> Dept. of Medicine, University of California, San Francisco

<sup>2</sup> Dept of Cellular and Molecular Pharmacology, University of California, San Francisco

### Abstract

Oncogenic tyrosine kinases have proven to be promising targets for the development of highly effective anticancer drugs. However HER family tyrosine kinase inhibitors (TKIs) show only limited activity against HER2-driven cancers despite effective inhibition of EGFR and HER2 *in vivo*<sup>1–8</sup>. The reasons for this are unclear. Signaling in *trans* is a key feature of this multimember family and the critically important PI3K/Akt pathway is driven predominantly through transphosphorylation of the kinase-inactive HER3<sup>9,10</sup>. We report that HER3 and consequently PI3K/Akt signaling evade inhibition by current HER family TKIs *in vitro* and in tumors *in vivo*. This is due to a compensatory shift in HER3 phosphorylation-dephosphorylation equilibrium driven by increased membrane HER3 expression driving the phosphorylation reaction and reduced HER3 phosphatase activity impeding the dephosphorylation reaction. These compensatory changes are driven by Akt mediated negative feedback signaling. Although HER3 is not a direct target of TKIs, HER3 substrate resistance undermines their efficacy and has thus far gone undetected. The experimental abrogation of HER3 resistance by siRNA knockdown restores potent pro-apoptotic effects to otherwise cytostatic HER TKIs, re-affirming the oncogene-addicted nature of HER2-driven tumors and the therapeutic promise of this oncoprotein target. However, since HER3 signaling is buffered against an incomplete inhibition of HER2 kinase, much more potent TKIs or combination strategies are required to effectively silence oncogenic HER2 signaling. The biologic marker to guide HER TKIs should be the transphosphorylation of HER3.

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Selective inhibitors of Abl tyrosine kinase (TK) are effective in putting nearly all patients with bcr-abl driven leukemia in chronic phase into complete remission<sup>11</sup>. This proof of concept sheds new hope for the treatment of other TK-driven cancers. Another important TK family is the human epidermal growth factor receptor (HER) family consisting of EGFR, HER2, HER3, and HER4. A subset of breast cancers are driven by overactive EGFR

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Correspondence: Mark M. Moasser, Box 0875, University of California, San Francisco, San Francisco, CA 94143-0875, Tel: 415-476-0158, Fax: 415-502-5665, mmoasser@medicine.ucsf.edu.

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or HER2 TKs and an abundance of data from *in vitro* and mouse models suggests that continued activity of these TKs drives cancer progression<sup>12–14</sup>. While the complexities of this multi-member TK family are not yet fully understood, their oncogenic signaling functions should, in theory, be amenable to silencing by TK inhibitors (TKIs). Several orally bioavailable HER family selective TKIs are in preclinical and clinical development. Although in *in vitro* biochemical assays these agents differ in their relative activities against individual HER kinase family members, in cell-based assays they are effective at inhibiting both EGFR and HER2 and equally effective at suppressing the growth of EGFR and HER2 driven tumor cells<sup>15–19</sup>. They are also effective at inhibiting EGFR and HER2 phosphorylation in patients tissues and tumors<sup>5–8</sup>. But these agents show very limited clinical anti-tumor activity<sup>1–5</sup>. Their clinical development to this point has been driven largely by the detection of modest delays in tumor progression. The failure to reverse cancer progression despite an apparent inhibition of HER kinase function has created an enigma in the concept of TKI therapy of cancer that we have been exploring.

It is through heterodimerization and transphosphorylation that the HER family performs its signaling functions. Importantly, downstream PI3K/Akt pathway signaling is predominantly mediated through the transphosphorylation of the kinase-inactive member HER3<sup>9,10</sup>. We have previously reported that sensitivity to HER family TKI therapy correlates with the inhibition of PI3K/Akt pathway signaling<sup>15,20</sup>. We and others have also reported that failure to inhibit PI3K/Akt signaling leads to TK inhibitor resistance<sup>20–22</sup>. In contrast to reports from *in vitro* models, Akt activity is not inhibited in most patients on HER TKI therapy<sup>5,6,8</sup>. This discordancy has led us to look more closely at the inhibition of PI3K/Akt signaling.

To investigate this discrepancy, we studied the durability of Akt inhibition by TKI with surprising results. Although as previously reported, gefitinib inhibits Akt signaling in HER2-driven cancer cells, this inhibition is not durable. Akt signaling resumes after a transient inhibition despite continued drug therapy (figures 1A,B). In light of this finding, we looked at the broader HER family signaling activities over a period of 96 hours following continuous exposure of BT474 breast cancer cells to gefitinib at concentrations that nonselectively inhibit EGFR and HER2. TKI treatment effects a sustained inhibition of EGFR and HER2 phosphorylation and a durable inhibition of downstream MAPK and JNK pathway signaling (figure 1A). However phosphorylation of the kinase-inactive family member HER3 is merely transient. HER3 signaling resumes and persists despite continued drug exposure and effective suppression of EGFR and HER2 (figure 1A,B). The reactivation of HER3 signaling explains the reactivation of Akt signaling since HER3 is the principal HER family member that binds PI3K and drives Akt signaling<sup>9,10</sup>. TKI-refractory Akt signaling remains sensitive to PI3K inhibitors as expected (not shown). These time-dependent findings are not due to drug degradation since the drug is replenished daily in these studies and HER3/Akt signaling resumes despite repeatedly refreshing drug supply up to and beyond the point of resumption of Akt signaling (not shown). There is no significant expression of HER4 before or after drug treatment in these cells (data not shown). These findings are not unique to BT474 and SkBr3 cells and have been confirmed in other HER2 overexpressing breast cancer cells including MDA-453, AU565, MDA-361, HCC1954

(supplementary figure 1). These findings are not unique to gefitinib and are seen with other HER TKIs including agents with *in vitro* selectivity profiles favoring EGFR or HER2, such as erlotinib or AG825 (figure 1C,D). These findings are not artifacts of the *in vitro* models either. Treatment of mice bearing various HER2-driven xenograft tumors with gefitinib similarly fails to durably suppress HER3 and Akt signaling, despite a transient suppression (figure 1E, and supplementary figure 2). This is not due to ineffective drug biodistribution, since in these models gefitinib was dosed three times higher than doses known to achieve sustained xenograft tumor concentrations above 2–4 $\mu$ M and averaging 6–10 $\mu$ M<sup>23</sup>. Since we had previously established that inactivation of PI3K/Akt signaling is mechanistically linked to HER family TK inhibitor sensitivity in HER family driven cancers, we felt that the failure of these drugs to durably inactivate PI3K/Akt signaling is entirely consistent with their limited clinical activities. Therefore we set out to study the molecular basis by which HER3 evades TKI therapy.

TKI-refractory HER3 phosphorylation is due to HER2, since it can be suppressed by anti-HER2 siRNA transfection (figure 2A). Although cross talk between receptor families can occur, we have found no evidence for non-HER family TKs mediating TKI refractory HER3 phosphorylation. The reactivation of HER3 signaling is not associated with the induction of any new TKs and remains resistant to the broad spectrum kinase inhibitor staurosporine (not shown). This apparent desensitization of HER3 signaling to TKIs is due to a forward shift in the equilibrium of the HER3 phosphorylation-dephosphorylation reactions establishing a new steady state HER3 phosphorylation despite significant inhibition of HER2 kinase and autophosphorylation activity by TKIs. This forward shift becomes clearly evident in the form of HER3 and Akt superphosphorylation when drug inhibition is withdrawn during the new steady state (figure 2B). HER3 phosphorylation remains suppressible by TKI in the new steady state, but much higher concentrations are required to completely dephosphorylate HER3 since the uninhibited HER3 phosphorylation state is significantly higher in the new steady state (figure 2C, compare left and right). Therefore drug-refractory HER3 phosphorylation is due to resistance at the level of the substrate HER3, and is driven by residual HER2 kinase activity. Similar characteristics apply to the more potent irreversible TKIs<sup>24</sup>. The irreversible pan-HER family TKI PD168393, when used at partially or near-maximal inhibitory concentrations, induces a similar desensitization of HER3 to continued drug therapy (figure 2D, 0.1–0.2 $\mu$ M doses). However, at fully inactivating concentrations both reversible (figure 2C, 40 $\mu$ M dose) and irreversible TKI (Figure 2E) can durably suppress HER3 and Akt signaling.

The biological consequence of drug-refractory HER3 and Akt signaling is tumor cell survival. In fact the anti-proliferative activity of TKIs is reversible and tumor cells resume proliferative growth after drug withdrawal. If drug-refractory HER3 signaling is averted by anti-HER3 siRNA, TKI treatment of HER2-driven cancer cells leads to apoptotic cell death (figures 2F, 2G, and supplemental figure 4). This is the expected outcome of effective oncoprotein inactivation and recapitulates the apoptotic fate of oncogene withdrawal seen in reversible transgenic models of HER2 tumorigenesis<sup>14</sup>. Sustained inhibition of HER3 signaling using TKIs at their fully inactivating doses (from figures 2C,2D,2E) also leads to apoptotic tumor cell death not seen with doses that allow HER3 escape (figure 2H).

The TKI-induced forward shift in the HER3 phosphorylation-dephosphorylation steady state is due to increased HER3 substrate concentration driving the forward reaction, and decreased phosphatase activity impeding the reverse reaction. Increased HER3 substrate concentration occurs through a significant increase in HER3 expression at the plasma membrane where the phosphorylation reaction occurs (figure 3A,B). Unlike HER2 which is predominantly localized to the plasma membrane, the HER3 pool is largely within intracellular compartments with some membrane expression<sup>25</sup>. The TKI-induced forward shift in HER3 steady state phosphorylation is driven by HER3 relocalization to the plasma membrane and can be suppressed by inhibitors of vesicular trafficking (figure 3C,3D). The HER3 dephosphorylation rate is also slowed after 48 hours of TKI exposure (figure 3E). The retarded HER3 dephosphorylation rate may be due to reduced access to cytosolic protein tyrosine phosphatases (PTPs) due to altered endocytic trafficking, or it may be due to inhibition of PTPs. In support of the latter, TKI therapy leads to increased cellular reactive oxygen species (ROS) (figure 3F) which are known to inhibit PTPs and are emerging as an important regulator of PTP activity<sup>26,27</sup>. Consistent with this, drug-refractory HER3 signaling can be suppressed by concomitant treatment with certain anti-oxidants (figure 3G).

The changes in steady state HER3 signaling that evolve with TKI treatment are driven by the loss of Akt signaling and likely involve Akt-mediated negative feedback signaling. Consistent with this, HER3 signaling does not escape TKI treatment when a constitutively active Akt is transfected (figure 4A). Conversely, inhibition of Akt signaling by a PI3K inhibitor leads to a compensatory increase in HER3 phosphorylation (figure 4B). Complete inactivation of HER2 kinase with high doses of TKIs induces the maximum feedback signaling and HER3 redistribution, however due to the complete inactivation of HER2 kinase HER3 signaling cannot be restored and the feedback loop fails to rescue Akt activity.

## Discussion

Since standard preclinical models have notoriously overestimated the clinical potential of HER TKIs, we challenged the traditional approach to evaluating TKIs in these models. Traditionally, signaling inhibitors are thought to have a continuous suppressive effect through rapid and sustained inhibition of their direct molecular targets and downstream signaling events. This notion of drug therapy may be too simplistic. Clearly, continuous exposure to a growth factor stimulus does not produce continuous high output downstream signaling. Rather, it leads to a sequelae of signaling events, programmed by negative and positive feedback signaling, until establishment of a new steady state in the presence of continued stimulus. We find here that continuous exposure to TKIs similarly leads to a sequence of signaling events that manifest over time, until a new steady state is reached. With this new perspective, we report that HER3/PI3K/Akt signaling is not effectively inhibited by current TKIs. In particular, the allocation of kinase and signaling functions to different members within the HER family allows the signaling substrate HER3 to restore signaling activity despite significant inhibition of HER2 kinase, in effect buffering HER3/PI3K/Akt signaling against an incomplete loss of HER2 kinase function. This inherent signal buffering capacity allows tumor cells to evade the pro-apoptotic effects of TKIs resulting in a significant loss of their anti-tumor activity. The highly effective treatment of HER-driven cancers may require drugs with much higher potency or drugs that completely

inactivate HER kinase function. Irreversible TKIs, although more potent, are subject to similar limitations. Due to their reactive groups and reduced selectivity, many irreversible agents cannot be delivered at completely inactivating doses. Future highly selective irreversible inhibitors may turn out to be more effective. Until highly specific and fully inactivating drugs can be designed, combination treatment strategies designed to undermine the resiliency of HER family signaling may offer the most promising approach in the near future. In addition, inhibition of autophosphorylation activity deceptively overstates the efficacy of TKIs and is a poor *in vivo* biologic marker. The true biological marker of efficacy to guide future therapies should be HER3 transphosphorylation.

The signal buffering capacity endowed by the separation of kinase and signaling functions to different family members in the HER kinase family attests to an evolutionary advantage conferred by the loss of catalytic activity in the HER3 protein kinase. This can shed light on why approximately 10% of the human kinome appears to be catalytically inactive<sup>28</sup>.

## Brief Methods

### Cell culture and reagents

PD168393 was synthesized as previously described<sup>29</sup>. Commercially available gefitinib and erlotinib were purified for *in vitro* use. Reagent sources are detailed in supplementary materials. For immunofluorescence studies, cells grown on fibronectin coated cover slips were treated as indicated, fixed in 4% paraformaldehyde, permeabilized, and stained with the indicated primary antibodies and FITC conjugated secondary antibodies. Cells were visualized using a Zeiss Axioplan 2 fluorescence imaging microscope.

### Apoptosis

Cells were seeded at 300,000–500,000 per well in 12-well or 6-well clusters. Apoptotic cells were identified and quantified by analysis of Annexin V binding using the Annexin V-FITC Apoptosis Detection Kit (Calbiochem) according to the manufacturer's instructions, or by their sub-G1 DNA content and quantified by FACS analysis as previously described<sup>30</sup>. All experimental arms were done in duplicate and displayed as averages with standard of deviation error bars.

### Transfections

Cells were seeded at a density of 300,000 cells per well in 12-well plates and transfected the following day. For siRNA transfections 100–300nmol of siRNA (Dharmacon) was premixed with Lipofectemine2000 in Opti-MEM media and then added to each well. For plasmid transfections, 2 ug of plasmid DNA was premixed with Lipofectamine2000 in Opti-MEM media and added to wells for 6 hours.

### Cell surface biotinylation

Cells were chilled on ice and rinsed twice with ice-cold PBS. Freshly prepared sulfo-NHS-SS-biotin was added to the final concentration of 0.5 mg/ml in PBS. Following 45min incubation at 4°C cells were lysed for immunoprecipitation.

## Reactive Oxidation Species Assay

Cells were rinsed twice with PBS and incubated with 10  $\mu$ M of freshly prepared H<sub>2</sub>DCFDA in phenol-red free media for 45 min at 37°C. Cells were then trypsinized and ROS levels were detected by flow cytometry.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

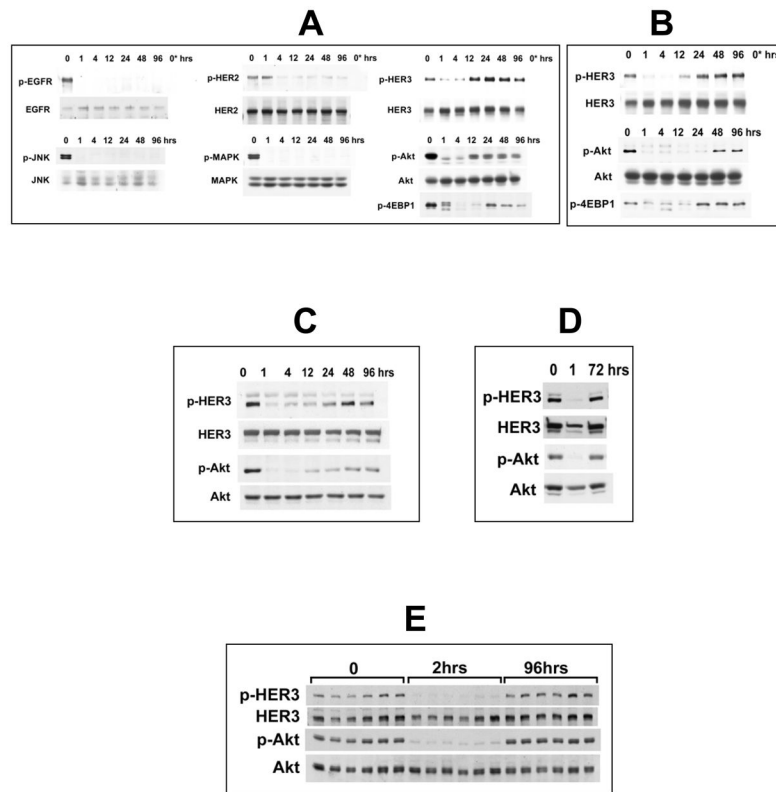
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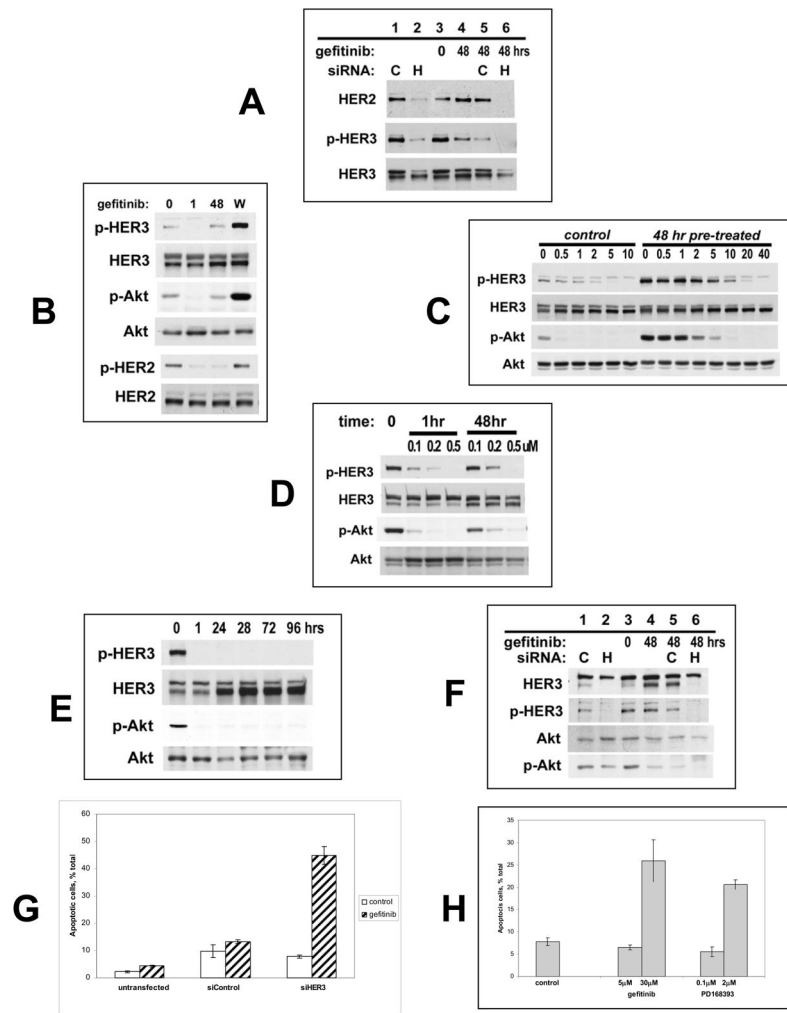
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**Figure 1. HER TK inhibitors fail to induce sustained inhibition of HER3 signaling in HER2-driven breast cancer cells**

(A) BT474 cells were treated with 5 μM gefitinib for the indicated times and assayed for expression and phosphorylation of the indicated proteins. Lane 0\* is an IgG immunoprecipitation control. (B) Data of interest shown from the identical experiment done in SKBr3 cells. (C) SKBr3 cells were treated with 5 μM erlotinib for the indicated times and lysates immunoblotted with the indicated antibodies. (D) SKBr3 cells were treated with 20 μM AG825 for the indicated times with drug being replenished 1 hr before the 72 hour harvest. (E) Mice bearing HCC1569 human breast cancer xenografts were treated with gefitinib at 150 mg/kg once daily. Animals were sacrificed at 2 hours after the first dose and 2 hours after the 4<sup>th</sup> dose (96 hours total treatment) and tumors rapidly harvested for immunoblotting with the indicated antibodies. The six lanes corresponding to each timepoint represent six different animals.





**Figure 2. Forward shift in HER3 phosphorylation-dephosphorylation equilibrium following extended HER TKI treatment**

(A) BT474 cells were transfected with anti-HER2 (H) or control (C) siRNA and harvested 64 hours after transfection (lanes 1,2). Additional arms were treated with 48 hours of gefitinib untransfected (lanes 3,4) or following siRNA transfection (lanes 5,6). (B) SKBr3 cells were treated with 5  $\mu$ M gefitinib for 0,1, or 48 hours. Arm W was treated for 48 hours, washed, and incubated in drug-free media for one more hour. (C) SKBr3 cells were treated with the indicated concentrations of gefitinib for one hour (left side). Additional arms were treated with 5  $\mu$ M gefitinib for 48 hours and subsequently treated with the indicated concentrations of gefitinib for one additional hour (right side). (D) SKBr3 cells were treated with the indicated concentrations and durations of PD168393. (E) SKBr3 cells were treated with 2  $\mu$ M PD168393 for the indicated times. (F) SKBr3 cells were transfected with anti-HER3 (H) or control (C) siRNA and harvested 4 days after transfection (lanes 1,2). Additional arms were treated with gefitinib or control in untransfected cells (lanes 3,4) or following siRNA transfection (lanes 5,6). (G) In parallel with figure F, SkBr3 cells were either left untransfected, or transfected with anti-HER3 or control siRNA followed by 5  $\mu$ M gefitinib or control for 48 hours. Apoptotic cells were identified by their subG1 DNA

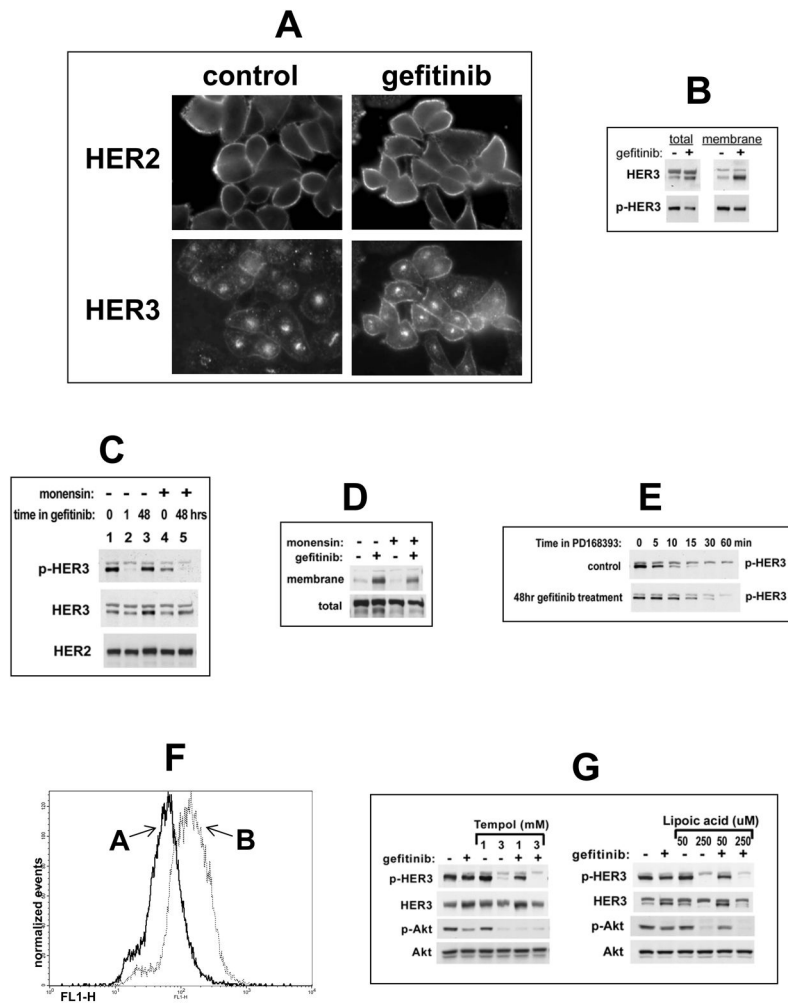
content. **(H)** SKBr3 cells were treated as indicated for 48 hours. Apoptotic cells were identified by Annexin V expression.

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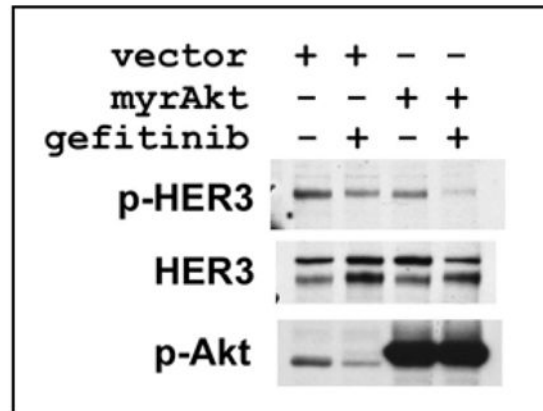
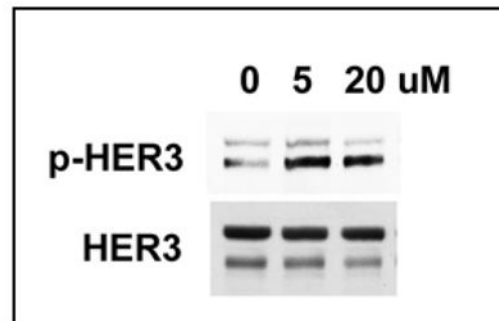
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**Figure 3. Mechanism of HER3 reactivation following extended HER TKI treatment**

(A) SKBr3 cells were treated with 5  $\mu$ M gefitinib or control for 48h and stained with anti-HER2 or anti-HER3 antibodies for immunofluorescence microscopy. (B) In addition, cell surface proteins in control or 48 hour pre-treated cells were biotinylated, precipitated, and immunoblotted as indicated. (C) Control or gefitinib pre-treated (5  $\mu$ M, 48h) SKBr3 cells were treated with 20  $\mu$ M monensin for the final 6h and analyzed by western blotting. (D) SkBr3 cells were treated with gefitinib for 48 hrs and with 20uM monensin for the final 6 hours. Membrane and total HER3 was immunoblotted from cell surface proteome pull-downs (above) or total lysates (below). (E) The dephosphorylation rate of p-HER3 following 48hrs of gefitinib treatment was determined immediately after initiation of fully inactivating concentrations of the irreversible HER TK inhibitor PD168393 (2  $\mu$ M). (F) Reactive oxygen species were quantified in control (A) or gefitinib pre-treated (B) (5  $\mu$ M, 48h) SKBr3 cells as described in methods. (G) SKBr3 cells were treated with 5 uM gefitinib or in combination with the indicated concentrations of the anti-oxidants Tempol or  $\alpha$ -lipoic acid for 48 hours.

**A****B****Figure 4. Akt regulates HER3 signaling via negative feedback signaling**

(**A**)  $2 \times 10^6$  SKBr3 cells were transiently transfected with 5  $\mu$ g of pcDNA3-myr-Akt plasmid or control pcDNA3 plasmid. The following day 5  $\mu$ M gefitinib was added for an additional 48h where indicated. Lysates were immunoblotted as indicated. (**B**) SKBr3 cells were treated with 5 or 20  $\mu$ M LY294002 for 8h and lysates immunoblotted as indicated.