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HER3 signaling is regulated through a multitude of redundant mechanisms in HER2-driven tumor cells

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Synopsis

HER2-amplified tumors are characterized by constitutive signaling via the HER2-HER3 co-receptor complex. While phosphorylation activity is driven entirely by the HER2 kinase, signal volume generated by the complex is under control of HER3 and a large capacity to increase its signaling output accounts for the resiliency of the HER2-HER3 tumor driver and accounts for the limited efficacies of anti-cancer drugs designed to target it. Here we describe deeper insights into the dynamic nature of HER3 signaling. Signaling output by HER3 is under several modes of regulation including transcriptional, post-transcriptional, translational, post-translational, and localizational control. These redundant mechanisms can each increase HER3 signaling output and are engaged in various degrees depending on how the HER3-PI3K-Akt-mTor signaling network is disturbed. The highly dynamic nature of HER3 expression and signaling, and the plurality of downstream elements and redundant mechanisms that function to ensure HER3 signaling throughput identify HER3 as a major signaling hub in HER2-amplified cancers and a highly resourceful guardian of tumorigenic signaling in these tumors.

Keywords

HER3; ErbB3; HER2

Introduction

The human epidermal growth factor receptor (HER) family of tyrosine kinase receptors consist of four members, EGFR, HER2, HER3, and HER4. Abnormalities of this family underly the pathogenesis of a variety of human cancers. Through transcriptional overexpression, gene amplification, mutational activation, or autocrine loop activation, the overactive functions of HER family members are implicated in the pathogenesis of a variety of subtypes of human cancers, designating them as proto-oncogenes [1, 2]. These include the amplification and overexpression of HER2 in breast cancers [3], or the mutational activation of EGFR in non-small cell lung cancers and glioblastomas [4, 5], and possibly the mutational activation of HER4 in melanomas [6]. The third member HER3 is not designated as a proto-oncogene, and its amplification or mutational alteration has not been described in human tumors. This may be due to the lack of significant catalytic function in the HER3 kinase domain, a distinguishing characteristic that sets it apart from the other HER family

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Authors contribution

This work was conceived by M.M.M. and designed by D.N.A, M.M.M., and A.G.. The experiments were performed by D.N.A., N.S., and L.L.. The data was prepared by D.N.A. and M.M.M..

members [7–9]. The lack of catalytic function however does not diminish its importance in signaling. These receptors function predominantly through heterodimerization and their tumorigenic signaling functions frequently involve their family member partners [10]. In particular, HER3 has emerged as a favorite interaction partner in tumorigenic signaling. While HER3 lacks catalytic function, its tumor promoting functions may involve the functions of its kinase domain as an allosteric activator of its partners, or the functions of its c-terminal signaling tail, in particular its ability to activate the PI3K/Akt signaling pathway [11, 12]. The HER3 c-terminal signaling tail is uniquely endowed with 6 consensus binding sites for the p85 subunit of PI3K and when phosphorylated by its HER family partners, HER3 is highly competent in the activation of the PI3K/Akt signaling pathway [13, 14].

The role of HER3 has been highlighted in a number of HER family-driven cancers. In HER2-amplified breast cancers, HER3 expression is required for tumorigenic growth and the pharmacologic suppression of HER2-amplified tumor growth requires the suppression of both HER2 and HER3 signaling functions [15–17]. In non-small cell lung cancers driven by mutationally activated EGFR, suppression of growth correlates best with the suppression of HER3 signaling [18].

While the role of HER3 as a collateral partner in some types of HER-driven cancers has been described for a number of years, its resiliency and resourcefulness at this function is only recently beginning to be appreciated. In particular, its role as a partner for HER2 in HER2-amplified cancers has been particularly revealing. While HER2 autophosphorylation is easily inhibited by tyrosine kinase inhibitor (TKI) treatment of HER2-amplified tumors, the inhibition of HER3 phosphorylation by HER2 is not so easily inhibited. This is due to a rapid compensatory restoration of HER3 signaling that functions to preserve tumorigenic HER2-HER3 signaling in the face of continued TKI therapy, an effect that considerably undermines the anti-tumor efficacy of HER2-directed TKIs [17, 19].

The resiliency in HER3 signaling forms a major barrier to the effective therapy of cancers driven in part through the collateral functions of HER3. In this study we undertook to better understand the mechanisms that regulate HER3 signaling output in a HER2-amplified cancer cell model. We report here that HER3 signaling is highly regulated through a variety of mechanisms in a reciprocal link with its downstream signaling throughput. The upregulation of its signaling output can be triggered through increased transcription, translation, localization, or phosphorylation, and through redundant mechanisms engaged by different downstream elements. These data reveal the highly regulated nature of HER3 and its role as a dynamic signaling hub linking HER family oncogenes with downstream PI3K/Akt pathway signaling.

Experimental

Cell lines and reagents used

SKBr3 cells were obtained from American Type Culture Collection and cultured at 37C, 5% CO2 in DMEM/HamF12 media supplemented with 10% fetal bovine serum, penicillin, streptomycin, and L-glutamine. Sodium orthovanadate, and cycloheximide were purchased from Sigma. Lapatinib, gefitinib, and erlotinib tablets were purchased from the pharmacy and the drugs purified as described before [17, 19]. BEZ235 was obtained from Novartis, and rapamycin was obtained from Cell Signaling. PP242 and DG2 were synthesized and provided by Morri Feldman and Kevan Shokat as described [20, 21]. All pharmaceutical drugs were re-constituted in DMSO. For NO donor experiments, cells were treated with or without lapatinib (200nM) for 48 hours. 8 hours prior to harvest, Diethylenetriamine/nitric oxide adduct (DETA/NO) (Sigma) was added to the cells at 1mM final concentration. For NO inhibitors, Nw-Nitro-L-arginine methyl ester hydrochloride (L-NAME), Carboxy-PTIO

potassium salt (PTIO), S-Methylisothiourea hemisulfate salt (S-MITU) were added to the cells for 24hrs.

Western blotting

Cell lysates were prepared using modified RIPA buffer supplemented with protease and phosphatase inhibitors. Western blots were performed using antibodies purchased from SantaCruz Biotechnologies (HER3, actin, HA), Cell Signaling (pS6, p4EBP1Th36/47, p-eNOS). The custom made anti-pY1289-HER3 was previously described [17].

Plasmid and miRNA expression

pGL3-HER3 promoter construct was a kind gift from Dr. Frederick Domann (University of Iowa) and has been described [22]. CMV-Renilla construct is from Promega. The HER3 3'UTR construct was obtained from Genecopeia. The pBABE-eIF4E and pBABE-4EBP1 constructs were a kind gift from Dr. Davide Ruggero. miR106b MIRIDIAN mimic was obtained from Thermo Fisher. The constructs were transfected into SKBr3 cells using Lipofectamine 2000 and standard protocols.

Luciferase Reporter Assays

Cells transfected with the firefly-luciferase constructs or the CMV-Renilla-luciferase constructs were seeded into 96 well plates 24 hours post-transfection. After the cells had attached the cells were switched to media containing lapatinib (200nM) or DMSO (mock) for 48 hours. Luciferase activity was measured using standard protocols described in the Dual Glo Luciferase system (Promega). Reporter assays were performed on triplicate wells of cells for luciferase activity on the 3'UTR or promoter construct of HER3 as well as the normalizing control for renilla for mock or drug treated cells for the indicated number of hours. Measurement from each well for the luciferase readout was normalized against the renilla readout. Ratios for the drug-treated and mock-treated cells were obtained against the average for the mock treated normalized readout. Average ratio and S.E. was determined on the triplicate ratio's obtained (n=3). Student's t-test were performed on the triplicate ratio's using two-tailed unequal variance parameters.

Quantitative Reverse transcription-PCR

Cells were treated with lapatinib (200nM), erlotinib (5uM), or rapamycin (25nM) for the indicated times. Total cellular RNA was isolated using the Qiagen RNeasy kits following the manufacturer's instructions. Reverse transcription and PCR amplification of HER3 was performed using primers and methods that were previously described [17]. QPCR assays were performed in triplicates for HER3 and the housekeeping gene GAPDH from cDNA prepared from mock or drug treated cells for the indicated number of hours. We used the ddCT methodology to obtain the ratios after normalizing to GAPDH. The ratio's were obtained against the average for the mock treated readout. Average ratio and S.E. was determined on the triplicate ratio's obtained (n=3). Student's t-test were performed on the triplicate ratio's using two-tailed unequal variance parameters.

For the miRNA quantification total RNA was isolated using the Qiagen RNeasy plus kits. Expression of specific mature miRNAs was confirmed by real-time polymerase chain reaction (PCR) analysis using a TaqMan Human MicroRNA Assay kit (Applied Biosystems, Foster City, CA) Samples were analyzed in duplicate and CT values normalized to RNU48 expression.

Surface labeling

Cells were washed with ice cold PBS (pH 8.0) and then were incubated for 45 minutes with 0.5mg/ml of EZ-Link Sulfo-NHS-Biotin (Thermo Scientific) at 4C. Cells were washed with PBS (pH 8.0) and lysed with mRIPA (supplemented with protease and phosphatase inhibitors). Biotinylated proteins were pulled down by adding streptavidin conjugated agarose (Sigma). The agarose beads, after washes in mRIPA, were subjected to western blotting.

Software

The 5'UTR of HER3 was analyzed using Genebee (http://www.genebee.msu.su)

Transcription factors binding to the promoter region of HER3 used in this publication was analyzed using MATINSPECTOR (http://www.genomatix.de) and PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo) software.

Statistical analyses

Student's t-tests were performed using two-tailed tests with unequal variance to obtain p-values.

Results

Diversity in HER3 compensation in HER2-amplified cancer cells

We studied how different HER2-amplified cancer cells respond to the inhibition of HER2. Treatment of a panel of 7 HER2-amplified cancer cells with 200nM lapatinib shows a diversity of responses. While lapatinib completely inhibits HER3 phosphorylation in the immediate timeframe (1 hr), all except for one restore HER3 phosphorylation at the new steady state (figure 1A, Supplementary Figure 1). In HCC1954 cells, the inhibition of HER3 signaling is complete and durable. This is because of redundancy in receptor tyrosine kinase overexpression. These tumor cells have overexpression of both HER2 and c-MET, and downstream Akt signaling is driven by both HER2 and c-MET [23, 24]. As such, the inhibition of HER2 alone fails to inactivate downstream Akt signaling and induce the negative feedback effects that would lead to restoration of HER3 phosphorylation. HER3 signaling is restored in the other six cell types, but through apparent different mechanisms. In some, the restoration of HER3 signaling is concomitant with a marked increase in HER3 protein expression (SkBr3,N87) while in others there is only minimal increase in expression but a marked increase in membrane re-localization (MDA-453,OE19)(figure 1B). In some cells HER3 expression is induced in a rapid timeframe (OE19), whereas in most others it requires longer latencies. These differences suggest that there are a plurality of mechanisms by which HER3 signaling can be restored in HER2 amplified cancer cells. This may be either due to different signaling circuitries operating in different cancer cell lines, or it may reflect the presence of redundant mechanisms regulating HER3 signaling and which can respond to diminished signaling throughput such as seen with drug therapy. To study the latter hypothesis, we selected SkBr3 cells for more in-depth analysis of whether there are in fact a plurality of mechanisms present that can upregulated HER3 signaling.

HER3 can be transcriptionally induced

Treatment of HER2-driven SKBr3 breast cancer cells with lapatinib increases HER3 transcript levels significantly within 48 hours of treatment (Figure 2a and Suppl. Figure S2). The increase in HER3 transcript is also observed with erlotinib, indicating it is not a drug specific mechanism but a drug class effect (Figure 2b and Suppl. Figure S2). To determine whether the increase in HER3 transcript is driven by increased promoter activity, we

assayed the activity of a luciferase reporter driven by the HER3 promoter. This reveals that TKI treatment induces an ~4-fold increase in HER3 promoter activity in response to TKI treatment (Figure 2c and Suppl. Figure S3). This suggests that the TKI-induced increase in HER3 transcript is driven at least in part through increased transcription activity. Feedback signaling to HER3 transcription is mediated through FOXO transcription factors [25, 26]. Analysis of the 1118 base pair promoter region included in our HER3 promoter construct using the PROMO database finds no FOXO binding sites within this sequence.

HER3 transcript stability can be induced by miRNA downregulation

The levels of mRNA transcripts can also be regulated by miRNAs which destabilize transcripts by targeting sequences within the 3'UTR of transcripts. To determine whether the 3'UTR of HER3 can mediate drug-induced increase in HER3 transcripts, we assayed the activity of the luciferase coding sequence fused to the HER3 3'UTR. Lapatinib treatment of SKBr3 cells transfected with the HER3 3'UTR reporter construct showed a 2-fold increase in the firefly-luciferase activity compared to mock treated cells (Figure 3a and Suppl. Figure S4), suggesting that the drug-induced upregulation of HER3 expression may be due to a decrease in HER3-targeting miRNAs.

In a screen of miRNAs expressed in SkBr3 cells, miR106b emerged as a miRNA that is downregulated following lapatinib treatment. qPCR analysis confirmed that miR106b is downregulated more than 5-fold within 48 hours of lapatinib treatment (Figure 3b and Suppl. Figure S5). The suppression of HER3 expression by miR106b was confirmed in a transient transfection assay. Transfection of SkBr3 cells with a miR106b mimic decreased HER3 expression compared to mock transfected cells (Figure 3c). Furthermore, if the downregulation of miR106b expression is prevented by exogenous expression of miR106b, the lapatinib-induced upregulation of HER3 expression is attenuated (figure 3d).

HER3 expression can be induced through increased protein translation

The upregulation of HER3 is not just seen when its own signaling is disrupted, such as with TKI treatment. Rather, the expression and signaling activity of HER3 is reciprocally linked in a robust feedback signaling loop with downstream PI3K/Akt signaling activity in HER2-amplified cancer cells. This was previously shown through the negative and positive perturbation of downstream signaling using downstream inhibitors or an activated Akt construct [17]. Although transcriptional and miRNA mechanisms and can be induced to upregulate HER3 expression when HER2-HER3-driven cancer cells are treated with HER-targeting TKIs, different mechanisms can be induced when the signaling throughput is disturbed at downstream targets. Rapamycin is an allosteric inhibitor of mTorc1 and inhibits some but not all the functions of mTorc1. Rapamycin treatment induces a compensatory increase in HER3 protein expression in SKBr3 cells (Figure 4a). This is accompanied by the complete inhibition of the mTorc1 substrate p70S6K1 (seen through dephosphorylation of its substrate S6), but a compensatory increase in phosphorylation of another mTorc1 substrate 4EBP1 at Thr37/46 (Figure 4a). In contrast to the TKI-mediated upregulation of HER3, the rapamycin-induced upregulation of HER3 is not transcriptionally mediated (figure 4b). The inhibition of S6K has been shown to induce a compensatory increase in upstream signaling activity in other cellular contexts [27–29]. To determine whether the rapamycin-induced upregulation of HER3 is mediated through an S6K feedback signaling mechanism, we determined whether a similar induction of HER3 can be seen through directly targeting S6K. Direct inhibition of p70S6K1 with the S6K inhibitor DG2 does not induce an upregulation of HER3 expression (figure 4c). Therefore the rapamycin-induced upregulation of HER3 expression in these cells is not mediated through an S6K-dependent feedback signaling mechanism. The increase in HER3 observed with rapamycin is not due

to a decrease in HER3 protein turnover since HER3 expression is not increased or prolonged by rapamycin in the absence of new protein synthesis (Figure 4d).

Since rapamycin induces a compensatory upregulation of 4EBP1 phosphorylation, we thought that the upregulation of HER3 protein expression may be mediated through increased protein translation driven by increased 4EBP1 phosphorylation. HER3 has a highly structured 5'UTR with a free energy of -61.8 kkal/mol (figure 4e, shown in comparison to actin with a free energy of -14.1 kkal/mol) rendering it more dependent on eIF4E, which is negatively regulated by 4EBP1 (Figure 4f). We interrogated the role of 4EBP-1/eIF4E function in the regulation of HER3 expression through transient transfection. The overexpression of eiF4E increases HER3 expression and conversely, overexpression of 4EBP1 decreases HER3 levels (Figure 4g, 4h). These data show that HER3 protein expression can be upregulated in response to drug inhibition through a translational mechanism involving the regulation of 5'cap binding complexes.

HER3 signaling can be induced without an induction of expression

Since rapamycin treatment induces a compensatory increase in 4EBP1-mediated HER3 protein translation, the complete inhibition of mTorc1 functions with a mTor kinase inhibitor should avert this mechanism of HER3 upregulation. Treatment of SkBr3 cells with the mTor kinase inhibitors PP242 or BEZ235 does inhibit all mTorc1 functions including the inhibition of both S6 and 4EBP1 phosphorylation (figure 5a). Consequently there is no upregulation of HER3 protein expression with these mTor kinase inhibitors (figure 5a). Despite this, HER3 signaling activity is yet upregulated without an increase in its total expression (figure 5a). The addition of an mTor kinase inhibitor also blocks the lapatinib-induced upregulation of HER3 expression as shown previously [17]. The upregulation of HER3 signaling activity occurs within an hour of mTor kinase inhibitor treatment. The increase in HER3 phosphorylation induced by mTor kinase inhibitors is not due to an increase in membrane localization of HER3, as there is no increase in plasma membrane-bound HER3 following treatment with these inhibitors (Figure 5b). The increase in HER3 phosphorylation is likely mediated through decreased dephosphorylation, likely through an inhibition of phosphatases that regulate HER3. We have previously shown that this can occur through an inhibition of HER3 tyrosine phosphatases by increased reactive oxygen species [19]. Alternatively, HER3 phosphorylation could be influenced by changes in protein tyrosine phosphatase expression and/or activity. The dephosphorylation of HER3 in SkBr3 cells is a rate-limiting step in HER3 signaling and potentially subject to regulation by feedback signaling mechanisms. This is evident in the sodium vanadate treatment of these cells, which inhibits PTPs and induces an increase in HER3 phosphorylation (figure 5c). The specific PTPs that regulate HER3 are not currently known.

HER3 signaling can be induced by membrane trafficking

Another potential means of modulation of HER3 signaling is through the regulation of its membrane localization. HER3 protein is found in cytoplasmic and membrane pools, but HER3 signaling in HER2-amplified cancer cells is generated through HER2-HER3 interactions occurring at the plasma membrane. As such, colocalization with membrane HER2 is potentially subject to regulation by membrane trafficking of HER3.

Since HER3 signaling in HER2-amplified tumors is tightly coupled to Akt activity, its membrane expression may be linked to trafficking mechanisms known to be regulated by Akt. Akt is capable of either positive or negative regulation of protein translocation to the membrane. One mechanism for the negative regulation of membrane translocation is through the regulation of nitric oxide (NO) signaling (figure 6a). Akt phosphorylates and activates nitric oxide synthase (NOS) leading to increased NO production [30–32] .NO is

known to inhibit vesicular trafficking and inhibit exocytosis through the nitrosylation of N-ethylmaleimide-sensitive factor (NSF) [33, 34]. NSF is a cytoplasmic protein that through its ATPase activity dissassembles vesicular SNARE complexes leading to membrane fusion and exocytosis [35, 36]. Therefore Akt can negatively regulate membrane trafficking through NO signaling. This contrasts with other mechanisms whereby Akt can positively regulate membrane trafficking, such as seen in the insulin-induced membrane localization of GLUT4 [37, 38].

The negative regulation of HER3 localization by NO signaling is apparent in experiments designed to interfere with NO signaling. Treatment of SkBr3 cells with the NOS inhibitors L-nitroarginine methyl ester (L-NAME) or s-methylisothiourea (S-MITU) or the NO scavenger carboxy-phenyl-dihydrotetramethyl-imidazolyl-oxy-oxide (cPTIO) induces an increase in HER3 plasma membrane expression without an increase in total cellular HER3 protein expression (figure 6b). The TKI-induced upregulation of total cellular HER3 expression is accompanied by a parallel increase in membrane HER3 expression. This increase in membrane HER3 induced by TKI treatment can be inhibited by NO donor treatment without reducing total cellular HER3 expression (figure 6c). The effects of TKI treatment on Akt activity are transient and the compensatory upregulation of HER3 eventually restores Akt activity [17, 19]. These effects are also seen in the Akt substrate eNOS, which is similarly dephosphorylated and inhibited following TKI treatment, and its phosphorylation partially restored with prolonged TKI therapy at the new steady state (figure 6d).

Discussion

The role of HER3 as a signaling partner for EGFR and HER2 has been known for many years. Its signaling functions are regulated through its ligand-mediated conformational activation, leading to dimerization with its kinase-competent partners and consequent phosphorylation of its c-terminal signaling tail [11, 12]. In physiologic signaling, HER3 can initiate and regulate the signaling process by virtue of its direct recognition of and activation by ligand stimuli.

HER3 is important in tumorigenic signaling as well, although it does not appear to initiate the signal generation in these scenarios. Its particular importance as a collateral partner in HER2-induced tumorigenesis has also been recognized for a number of years. Although HER3 is not transforming by itself, it is synergistically transforming with HER2 [39]. Furthermore, the functions of HER3 are required for tumorigenic growth in HER2-amplified cancers as shown in knockdown experiments [15, 16]. Since HER3 is catalytically inactive, its functions in HER2-driven tumorigenic signaling had been presumed to be a slave function, transmitting signals generated by HER2 and dictated entirely by the activities of the HER2 kinase. This paradigm was dismissed when it became apparent that the inhibition of HER2 kinase activity in HER2-driven cancer cells fails to silence HER3 signaling, revealing that HER3 signaling activity is not entirely dictated by its partner HER2 [19]. The signaling activity of HER3 is tightly coupled in a reciprocal relationship with downstream PI3K/Akt signaling in a way that ensures that signaling throughput is maintained despite inhibition of HER2 functions by inhibitors [17]. As such, the expression of HER3 is a potential point of impact in tumorigenic signaling, and in drug resistance.

In this study, we show that the expression of HER3 can be increased through a multitude of redundant mechanisms in HER2-amplified cancers, all of which ultimately function to preserve tumorigenic signaling downstream of HER3. The specific mechanism or mechanisms engaged to increase HER3 signaling output depend on the specific manner in which the network topology is disturbed and the specific upstream or downstream signaling

proteins that are targeted by drugs. The plurality of mechanisms that can be engaged to increase HER3 signaling output reveal the highly dynamic nature of this HER family member, and attest to its role as a critical signaling hub that links its kinase-competent HER partners with the important cellular functions regulated by the Akt/mTor network.

One mode of induction of HER3 expression is through increased transcriptional activity. We show here that HER3 promoter activity is indeed induced in response to HER TKI treatment. This at least in part, accounts for increased HER3 mRNA transcripts seen with HER TKI therapy. The transcriptional regulation of HER3 has been shown to be in part regulated through FOXO1 and FOXO3a transcription factors through binding sites >2.5kb upstream of the transcription start site [25, 40]. The HER3 promoter used in our experiments encompasses only 1.1Kb of upstream sequences, excluding the putative FOXO1 and FOXO3 binding sites, and revealing a role for additional transcription factors in the transcriptional induction of HER3 following TKI treatment. Transcription factors described to regulate HER3 via more proximal elements include AP-2, ZNF217, and CtBP2 [22, 41].

Another mode of induction of HER3 expression is through the downregulation of repressive miRNAs. miRNAs can interfere with expression through destabilization of the mRNA transcript or suppression of its translation. miRNAs typically act through target sequences in the 3'UTR of mRNA transcripts, and the fact that the 3'UTR of HER3 confers lapatinib-inducibility to the luciferase coding sequences suggests a component of regulation mediated through miRNAs. From a miRNA microarray screen we identified miR106b levels as a HER3-targeting miRNA species that is reduced upon TKI treatment. The role of miR106b was confirmed more specifically as shown in figures 3b,c. Many other miRNAs may regulate HER3 expression as well. In particular miR205 has been described to regulate HER3 and miR125a, and miR125b have been described to regulate both HER2 and HER3 [42, 43]. But we specifically looked for TKI-induced changes in miRNA expression, and the one we have been able to confirm to be affected by TKI treatment is miR106b.

Another mode of regulation of HER3 is through increased translation. We previously showed that an activated Akt construct represses HER3 protein levels without an associated downregulation of HER3 mRNA transcripts [17]. However the induction of HER3 transcription was suppressed by the activated Akt construct. This suggested that Akt can negatively regulate HER3 expression through both transcriptional and post-transcriptional mechanisms. We show here that rapamycin also induces an increase in HER3 protein expression without an induction of HER3 transcript levels, in agreement with previous reports [25]. The mechanism of translational upregulation induced by rapamycin is through the regulation of cap-binding proteins. While rapamycin effectively inhibits mTorc1 phosphorylation of p70S6K, it fails to inhibit the mTorc1 phosphorylation of 4EBP1, and in fact induces a compensatory increase in 4EBP1 phosphorylation [44]. We show that the induction of HER3 protein expression is specifically a consequence of the induction of 4EBP1, not the inhibition of S6K. The data from lapatinib treatment are also consistent with this, since lapatinib treatment does effectively inhibit S6 phosphorylation, yet the induction of HER3 in this scenario is mediated predominantly through mechanisms other than translational upregulation. While in other cellular contexts, a negative feedback signaling loop puts IRS-1 expression under feedback from S6K and regulates insulin sensitivity [27–29], the circuitry is different in these HER2-amplified tumor cells. The 5'UTR of HER3 is highly structured with multiple hairpins and loops typical of transcripts highly regulated by 5'cap binding proteins.

Although in HER2-amplified tumor cells the level of HER3 phosphorylation and signaling can be regulated through its level of expression or through its membrane localization, its signaling activity can also be regulated directly through regulation of its phosphorylation.

This is most apparent upon treatment with mTor kinase inhibitors which suppress the total cellular or membrane upregulation of HER3 expression, yet they still elicit a compensatory upregulation of HER3 phosphorylation. This is highly unlikely to be due to phosphorylation by tyrosine kinases outside the HER family, as we have previously interrogated this hypothesis exhaustively with no evidence to support it [17,19]. The observed increase in HER3 phosphorylation seen with mTor kinase inhibitors is likely mediated through a downregulation of HER3 dephosphorylation. This could be either through mechanisms specific for certain protein tyrosine phosphatases (PTPs), or it could be through a more general inhibition of PTPs through an increase in reactive oxydative species as is seen following TKI treatment of these cells. It is difficult to speculate on these mechanisms, since the specific PTPs that regulate the phosphorylation of HER3 are not yet known.

Another mode of regulation of HER3 is through the regulation of its membrane localization. In particular, its membrane expression has been shown to be upregulated by the transmembrane mucin MUC4 [45]. We didn't observe drug-induced changes in MUC4, but it remains possible that some drugs can elicit such a compensatory cellular response. But we did observe Akt-driven nitric oxide mediated membrane trafficking as one of the compensatory mechanisms induced by drug treatment.

The functions of HER3 as a collateral partner in tumorigenic growth driven by its HER family partners is now well established, in particular in HER2-driven breast cancers where its functions are essential. And the tumor cell requirement for continued HER3 signaling is evident in the feedback signaling relationship we previously demonstrated between upstream HER3 signaling and downstream Akt signaling activities [17]. Determining the precise circuitry that protects HER3 against drug-induced loss of its signal can lead to ideas about more effective combination therapy approaches that can potentially disrupt cellular mechanisms involved in the compensatory upregulation of HER3. But we now find that the feedback to HER3 is not a simple circuit and can be engaged through a variety of mechanisms. There appears to be a multitude of redundant mechanisms regulating HER3 signaling in HER2-amplified tumors, which are rapidly inducible by drugs that disrupt the flow of signal along the HER3-PI3K-Akt-mTor signaling pathway. The highly dynamic nature of HER3 expression and signaling, and the plurality of downstream mechanisms ultimately regulating its signaling functions identify HER3 as a major signaling hub in HER2-amplified cancers. This only attests further to the central role of HER3 in HER2-driven tumorigenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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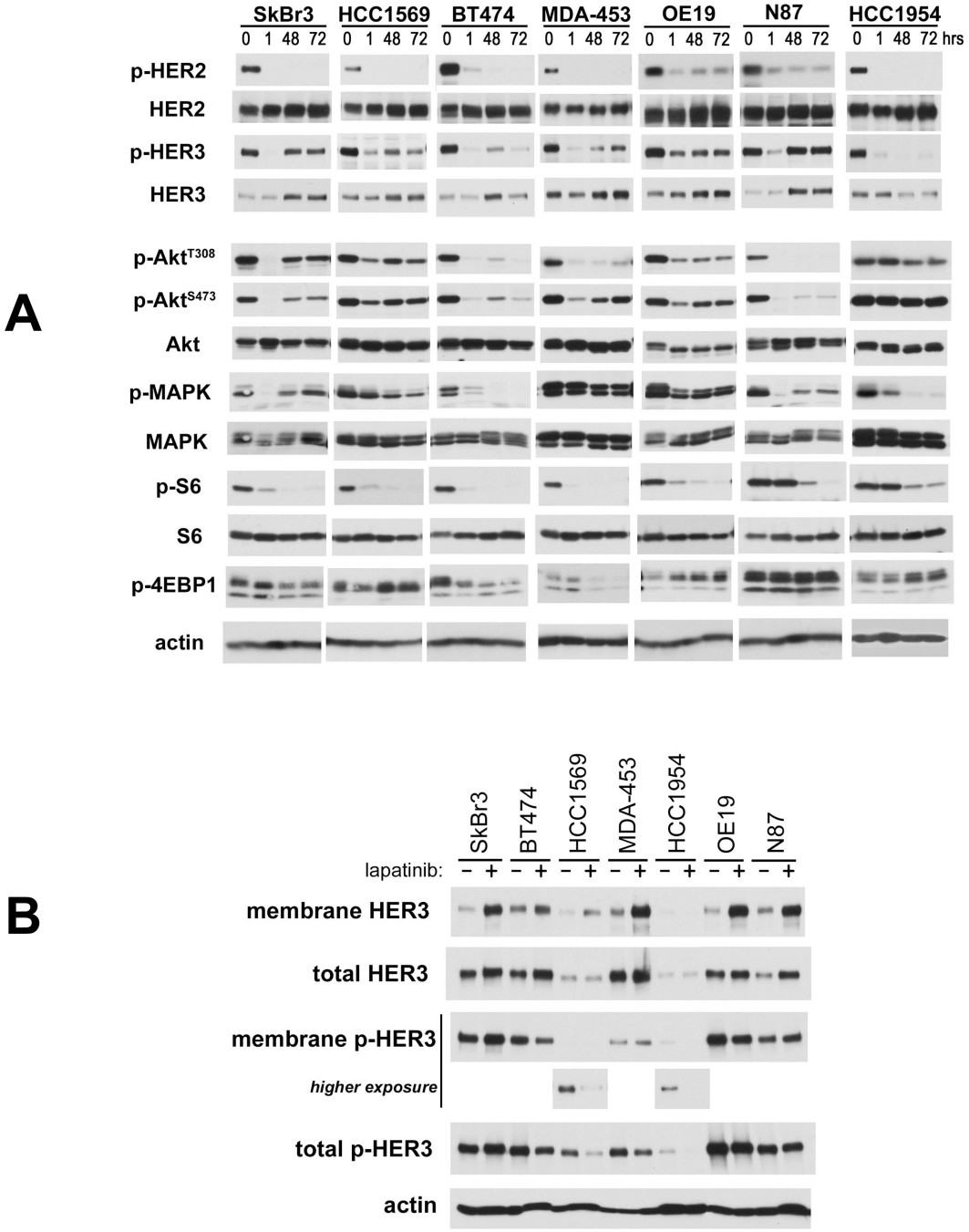


Figure 1. Response to TKI therapy in HER2-amplified breast cancer cell lines

A) A panel of HER2-amplified breast cancer cells were treated with 200nM lapatinib for up to 72 hours to detect the initial downregulation and subsequent upregulation of HER2-HER3 and downstream signaling. Immunoblots were performed using the indicated antibodies. B) The cell surface expression of HER3 (mHER3) was more specifically quantified by labeling cells with an impermeable biotinylation reagent and streptavidin pulldown beads as described in methods. Total HER3 expression is shown below it by simple immunoblotting of total cell lysates. The experiments include treatment with 200nM lapatinib or control for 48 hours.

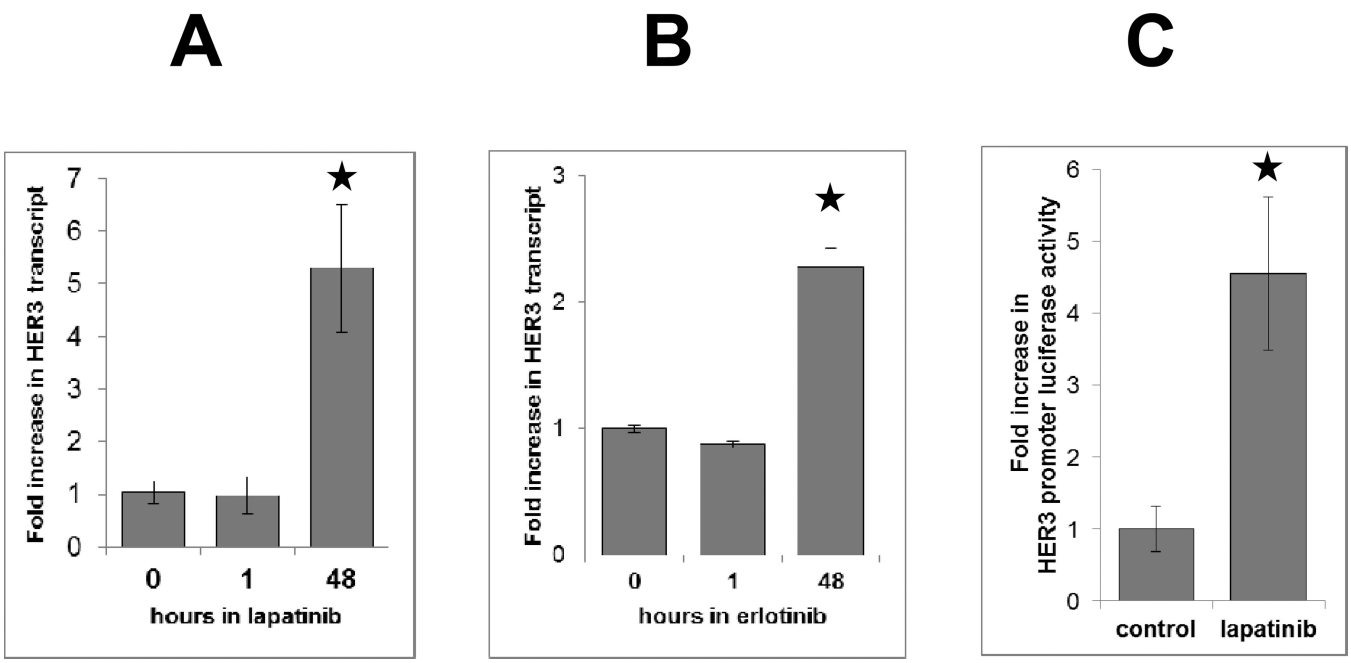


Figure 2. Induction of HER3 transcriptional activity

A,B) HER3 transcript levels were measured by QPCR after treatment of SkBr3 cells with lapatinib (200nM) or Erlotinib (5uM) for 0, 1 and 48 hours. Fold increase in HER3 transcript levels was measured compared to the 0 hour time point. C) A HER3 promoter-reporter assay was performed using the 1.1Kb HER3-pGL3 promoter construct driving a luciferase reporter, transfected into SKBr3 cells. Shown is the relative fold increase in the HER3 promoter activity upon treatment of the cells for 48 hours with lapatinib (200nM) compared to mock treatment. Results were normalized to a transfected construct reporting. Renilla luciferase activity driven by the constitutive CMV promoter. Shown are data from triplicate values of a representative experiment. * denotes $p<0.05$

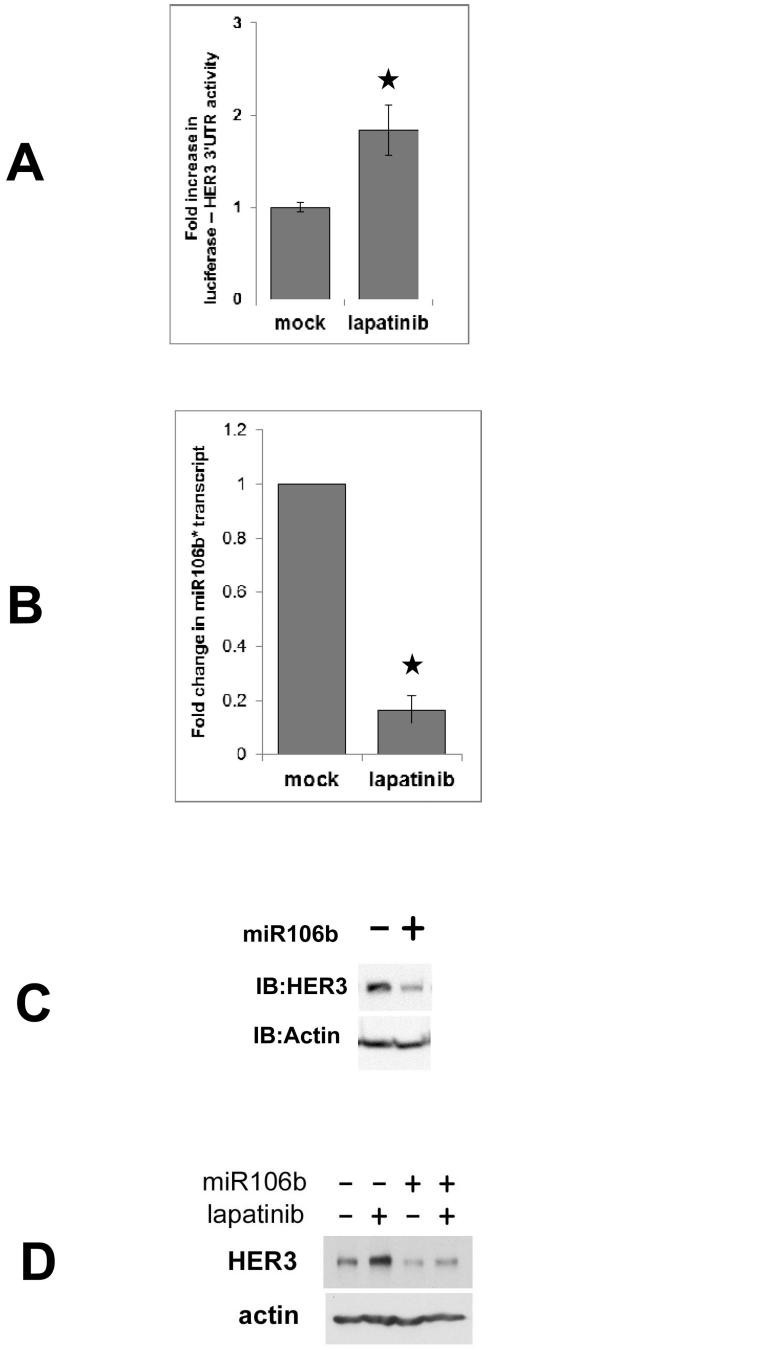
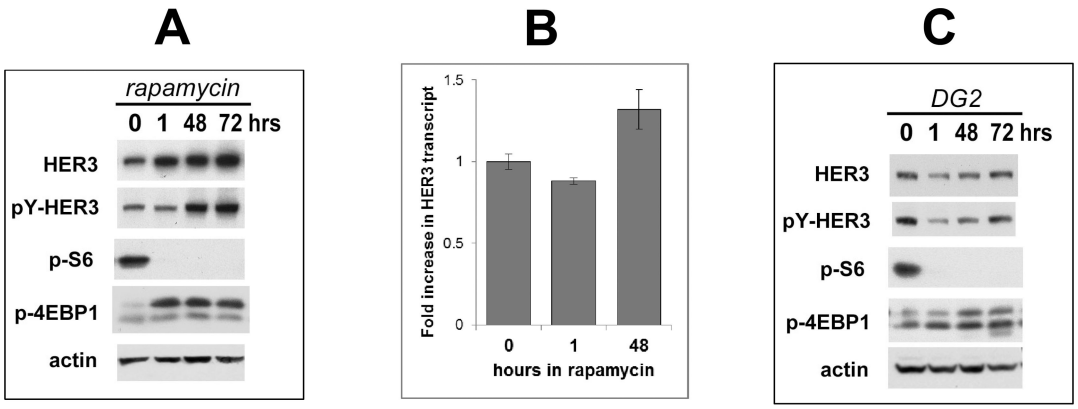
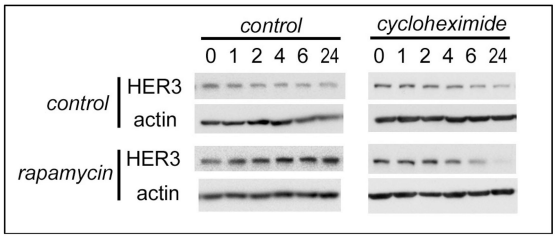


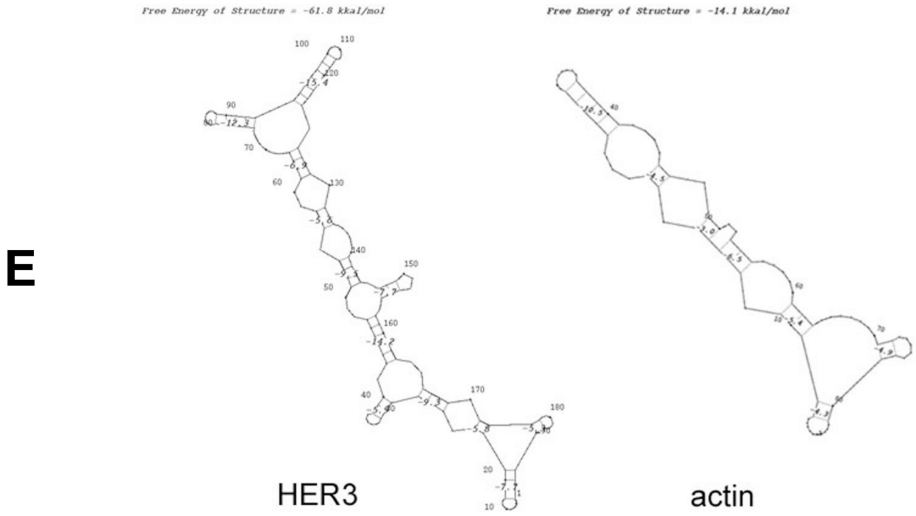
Figure 3. Induction of HER3 by miRNA de-repression

A) Luciferase reporter assays were performed using the firefly luciferase coding sequences fused to the 3'UTR of HER3. Shown here is the induction of luciferase activity in SkBr3 cells following 48 hours of lapatinib (200nM) treatment. Results are normalized to the constitutive activity of a transfected Renilla luciferase reporter to account for changes due to cell growth inhibition. B) Quantitative PCR was performed to ascertain the transcript levels of miR106b after treatment of SkBr3 cells for 48 hours with 200nM lapatinib. Shown is the fold change in miR106b transcript levels after lapatinib treatment compared to mock treatment. RNU48 was used as a normalization factor. Shown are data from triplicate values of a representative experiment. * denotes $p<0.05$ C) Western blot analysis of HER3 protein

levels after transfection of SKBR3 with a miR106b mimic, and compared with a mock transfection control. D) Western blot analysis of HER3 expression following transfection of SkBr3 cells with a miR106b mimic and 48 hours of lapatinib treatment.







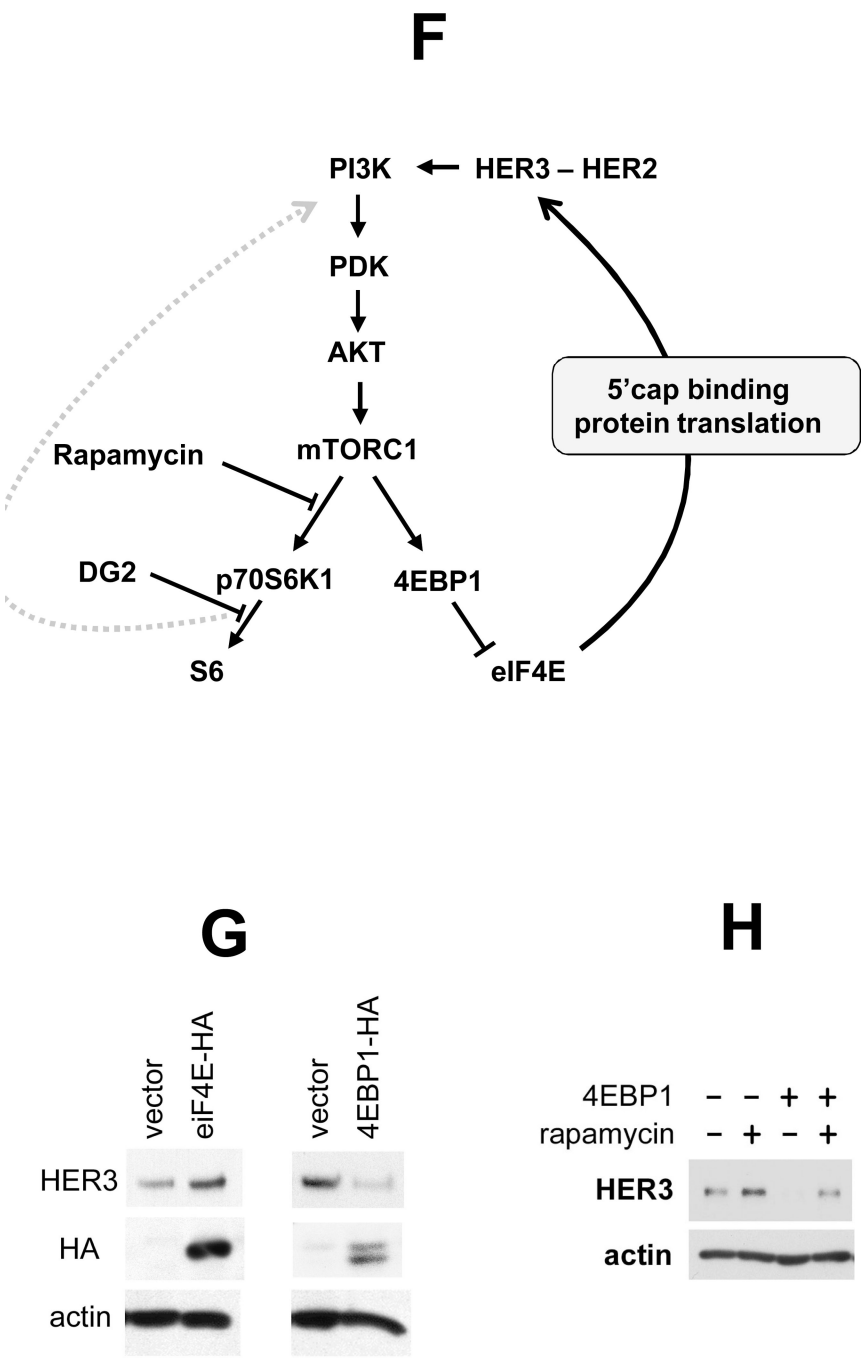


Figure 4. Induction of HER3 protein by translational upregulation

A) SkBr3 cells were treated with the Torc1 inhibitor rapamycin (25nM) for 0, 1, 48 and 72 hours and the lysates analyzed for total levels of HER3, phosphorylation of HER3, phosphorylation of S6 or phosphorylation of 4EBP1 at the Th37/46 site. B) Quantitative PCR was performed to measure HER3 transcript levels after treatment with rapamycin (25nM) for 0, 1, or 48 hours. Shown is the fold change in HER3 transcripts compared to time zero. C) SkBr3 cells were treated with the S6K inhibitor DG2 for 0, 1, 48 and 72 hours and the lysates analyzed as in part A. D) Western blot analysis of SKBr3 cell lysates after treatment for the indicated time points with 25nM rapamycin in the presence or absence of cycloheximide (100ug/ml). E) Diagram of the secondary structure of the 5'UTR regions of

HER3 and actin assembled by Genebee. F) Schematic representation of the effects of rapamycin and DG2 on Torc1 functions and how this affects HER3 signaling. G) Western blotting for HER3 protein expression after transfection of SkBr3 cells with HA tagged eiF4E or 4EBP1 or vector controls. Blotting for the HA tag was performed to confirm expression of the transfected genes. H) SkBr3 cells treated with rapamycin after transfection with 4EBP1-expressing vector.

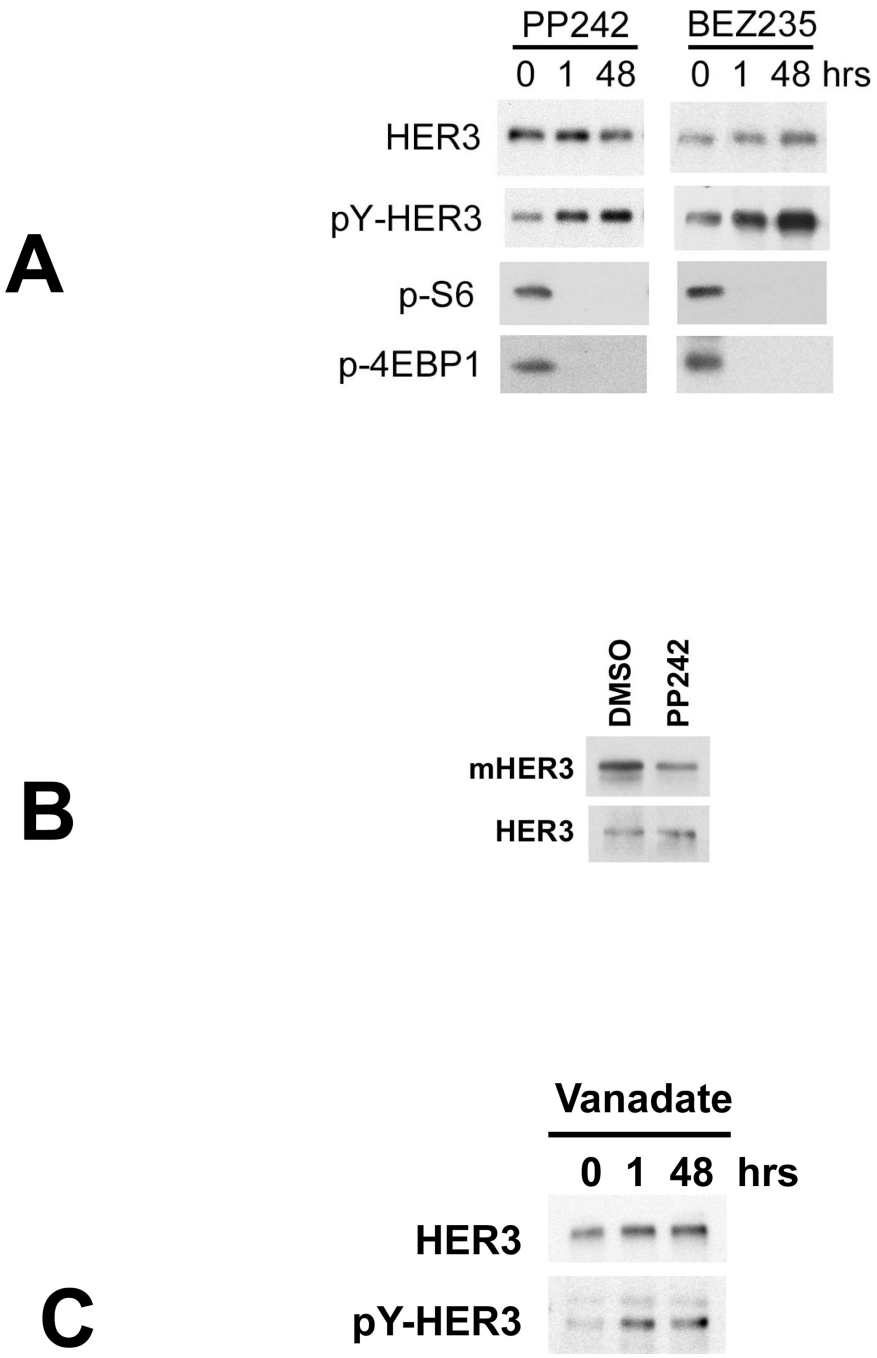


Figure 5. Induction of HER3 signaling by enhanced phosphorylation

A) SkBr3 cells treated with the mTor kinase inhibitor PP242 (1uM) or BEZ235 (250nM) for the indicated time points, and the expression of total and phosphorylated HER3 was assayed by immunoblotting. B) The cell surface HER3 expression was determined by biotinylation of the surface proteome using cell-impermeable reagents and subsequent streptavidin pulldowns followed by HER3 immunoblotting. Results are shown for control or PP242 (1uM) treatment for 48 hours. C) SkBr3 cells were treated with 1mM sodium vanadate for 0, 1, or 4 hours. Cell lysates were analyzed by western blotting as indicated.

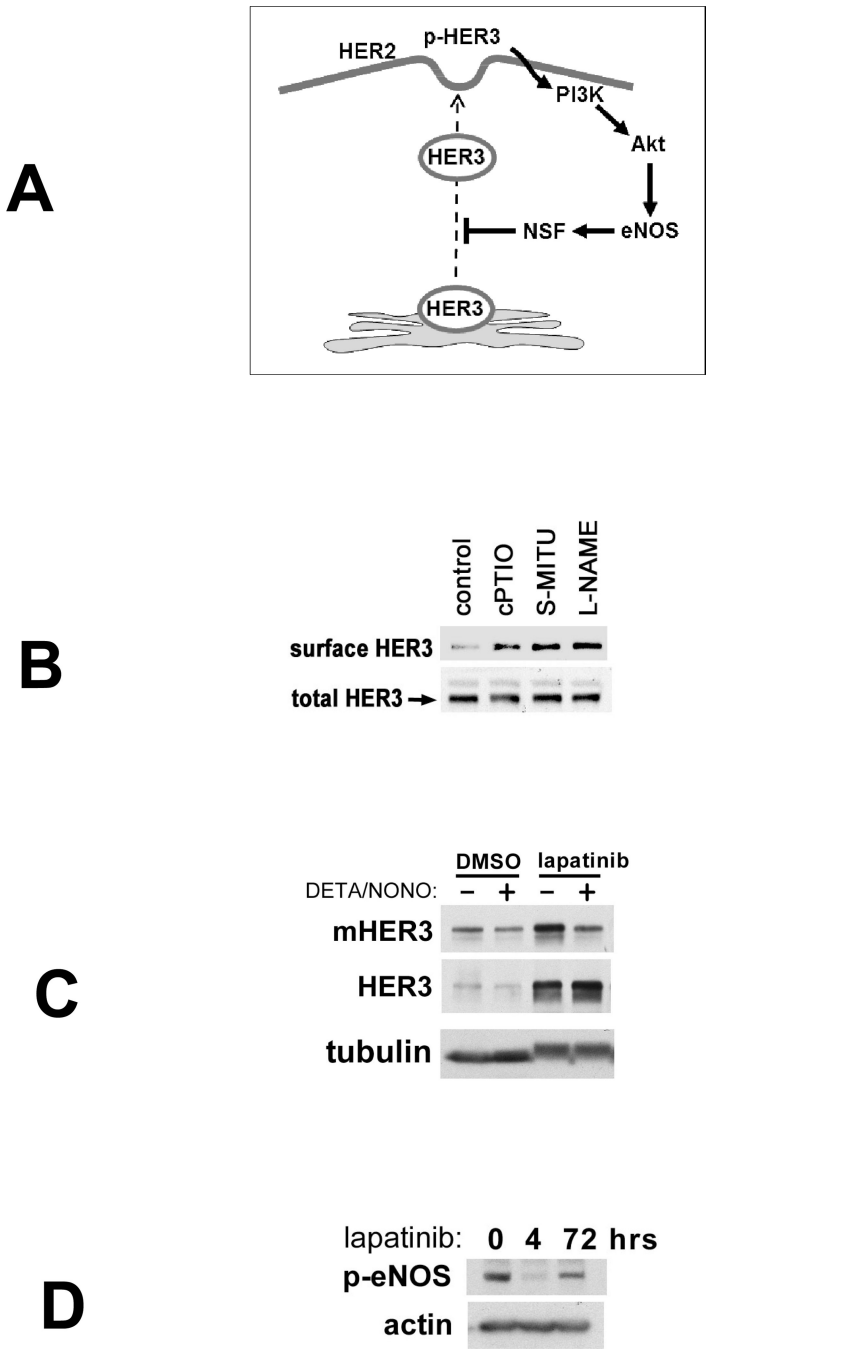


Figure 6. Induction of HER3 membrane trafficking

A) Schematic depicting how Akt can regulate protein exocytosis through nitric oxide signaling. B) SkBr3 cells were treated with the NO scavenger cPTIO (10uM), or the NOS inhibitors S-MITU (0.5mM) or L-NAME (5mM) for 24 hours and surface membrane HER3 expression was assayed by streptavidin pulldowns of the biotinylated surface proteome as shown. Total cellular HER3 levels are shown below for comparison. C) SkBr3 cells were treated with 200nM lapatinib for 48 hours or the NO donor DETA-NONO for 8 hours. The surface HER3 and total cellular HER3 expression were assayed as before. D) The phosphorylation of eNOS was assayed by immunoblotting following lapatinib treatment (200nM).