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A TORC2-Akt feedforward topology underlies HER3 resiliency in HER2-amplified cancers

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Abstract

The requisite role of HER3 in HER2-amplified cancers is beyond what would be expected as a dimerization partner or effector substrate and it exhibits a substantial degree of resiliency that mitigates the effects of HER2-inhibitor therapies. To better understand the roots of this resiliency, we conducted an in-depth chemical-genetic interrogation of the signaling network downstream of HER3. A unique attribute of these tumors is the deregulation of TORC2. The upstream signals that ordinarily maintain TORC2 signaling are lost in these tumors, and instead TORC2 is driven by Akt. We find that in these cancers HER3 functions as a buffering arm of an Akt-TORC2 feed-forward loop that functions as a self-perpetuating module. This network topology alters the role of HER3 from a conditionally engaged ligand-driven upstream physiologic signaling input to an essential component of a concentric signaling throughput highly competent at preservation of homeostasis. The competence of this signaling topology is evident in its response to perturbation at any of its nodes. Thus a critical pathophysiological event in the evolution of HER2-amplified cancers is the loss of the input signals that normally drive TORC2 signaling, repositioning it under Akt dependency and fundamentally altering the role of HER3. This reprogramming of the downstream network topology is a key aspect in the pathogenesis of HER2-amplified cancers and constitutes a formidable barrier in the targeted therapy of these cancers.

Keywords

HER2; HER3; ErbB2; ErbB3; TORC2

Introduction

The paradigm that oncogene-driven cancers can be effectively treated using drugs that inhibit their driving oncogene is now well established. The track record of success includes the use of inhibitors of Bcr-abl, EGFR, BRAF, or Ros, which are highly effective in the treatment of cancers driven by these oncogenes (1-5). However exceptions to this paradigm

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are increasingly being identified, and often implicate complexities in the larger signaling network as the underlying cause of treatment failure. Signaling context has become particularly relevant to the oncogene HER2. A subset of human cancers are driven by the amplification and overexpression of the HER2 receptor tyrosine kinase. A wealth of experimental evidence has confirmed that the overactive functions of HER2 underly the pathogenesis and progression of these cancers. Mouse genetic models of this disease confirm that even in the advanced and metastatic stages, this disease continues to be entirely dependent on its driving oncogene and will regress in the absence of continued overactive oncogene signaling (6). As such, the development of pharmacologic inhibitors of HER2 function affords a highly promising approach for the treatment of these cancers and a wealth of pharmaceutical agents have been developed based on this treatment hypothesis. However, the efficacies of HER2-targeting therapies in the monotherapy of patients with advanced HER2-amplified cancers has been incremental at best, falling short of scientific expectations (7-12). There is measurable efficacy associated with the clinical use of HER2-targeting antibodies, but this appears to be mediated through immunologic mechanisms rather than inactivation of HER2 signaling (13-16).

Work of the past few years has begun to highlight some of the complexities associated with the HER2 oncogene, particularly its signaling partner HER3, that underly its resiliency to drug intervention (17). HER3 is an important dimerization partner for HER2, functioning both upstream of HER2 as its allosteric activator (18), and downstream of HER2 as an important catalytic substrate linking it with downstream PI3K/Akt pathway signaling (19). HER3 function is essential for the progression of HER2-driven cancers (20-22). Although HER3 is expressed at far lower levels than HER2, its expression and signaling functions are dynamic and highly regulated by a multitude of mechanisms, linked with the downstream PI3K/Akt pathway signaling (23). HER3 is constitutively phosphorylated in HER2 amplified cancer cells, and although the phosphorylation of HER3 is directly effected by the HER2 kinase, the steady state phosphorylation of HER3 is not proportional to HER2 kinase activity. This is most evident when the activity of HER2 is reduced by kinase inhibitors, yet the steady state phosphorylation of HER3 is maintained after a brief interruption (24). This tenacity in HER3 signaling undermines the efficacy of HER2 inhibitors in these cancers.

We have been attempting to better understand the nature of the downstream network topology linked with HER3, and protective of HER3, in HER2-amplified cancer cells. We have previously described that the upregulation of HER3 involves an Akt-driven negative feedback loop (17, 24). But further analysis of this downstream pathway has revealed it to be more complex than a simple feedback loop. In the current study, we describe a much more in-depth analysis of the network topology downstream of HER2-HER3 in this cancer subtype, with a particular focus on identifying the key nodal relationships that underlie its competence at adaptation. The underlying rational premise is that the downstream network in normal epithelial cells does not demand or require constitutive HER3 signaling to maintain homeostasis; rather HER3 merely mediates an afferent signaling input when stimulated by extracellular ligands to influence biological behavior. But in HER2-amplified tumor cells, HER3 signaling appears to be essential and protected, suggesting that there must be key differences in the topology linking HER3 with the downstream network between normal cells and HER2-driven cancer cells. The study indeed reveals key

differences, at the heart of which is an Akt-TORC2 feedforward loop which functions to perpetuate the oncogenic signaling program including the requirement for HER3 signaling.

Materials and Methods

Reagents

All cell lines otherwise indicated were from American Type Culture Collection, and obtained within the past 10 years. Authentication was done in 2012 using DNA Diagnostics Center (Fairfield, OH). Cells were cultured at 37C, 5% CO2 in DMEM/HamF12 media supplemented with 10% fetal bovine serum, penicillin, streptomycin, and L-glutamine. The human mammary epithelial cell lines (HMECs) and their derivation were described previously (25-28) and their sources are identified in supplementary information Table 1. Some HMECs including MCF10A cells were cultured in DMEM/HamF12 media supplemented with 5% Horse serum, bovine insulin, recombinant EGF, hydrocortisone. Some HMECs were cultured in Clonetics MEGM media containing EGF, hydrocortisone, insulin, and bovine pituitary extract. 4-Hydroxytamoxifen was purchased EMD-Calbiochem and reconstituted in ethanol. The AKT allosteric inhibitors AKTXII and AKTXIII and Neuregulin α and β and doxycycline were purchased from Sigma. Lapatinib was purchased as tablets and the active ingredient purified by organic extraction as previously described (24). GSK690693, CI1040 were purchased from SYN thesis med chem and Rapamycin from Cell Signaling. All the other drugs were provided by Kevan Shokat (UCSF). All pharmaceutical drugs were reconstituted in DMSO.

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), Cell Signaling (p-HER2, pAKTth308, pAKTser473, pGSKbser9, pS6K, pS6, p4EBP1Th36/47, p-4EBP1ser65, p-NDRG1, p-MAPK, Rictor, Raptor), Enzo Life Sciences (pPRAS40) and Bethyl laboratories (PHLPP1 and PHLPP2), and Millipore (DEPTOR). The custom made anti-pY1289-HER3 was previously described (17).

Constructs generated

The inducible AKT (pBABEpuro-myrAKTΔER) and control AKT (pBABEpuro-myr*AKTΔER) constructs were a kind gift of Martin McMahon and have been previously described (29, 30). The HER3-myc construct was in pcDNA4/TO (Invitrogen). For inducible knockdowns of HER3, Rictor, Raptor and Scramble-control knockdowns, the below described oligonucleotides were synthesized and annealed and then ligated into the pTER (HER3) and pSuperior (Rictor and Raptor) vectors. pTER vector was a kind gift from Hans Clever (Utrech, Netherlands). Standard protocols were used according to the manufacturers (OligoEngine). shRNA sequences used are described below:

HER3shRNA sequences

5′-
GATCCCAAGAGGATGTCAACGGTTATTCAAGAGATAACCGTTGACATCCTCTTT
TTTTA-3′

5′-
AGCTTAAAAAAAGAGGATGTCAACGGTTATCTCTTGAATAACCGTTGACATCCT
CTTGG-3′

Rictor shRNA sequences

5′-
GATCCCGCAGCCTTGAACTGTTTAATTCAAGAGATTAAACAGTTCAAGGCTGCT
TTTTGGAAA-3′

5′-
AGCTTTTCCAAAAAGCAGCCTTGAACTGTTTAATCTCTTGAATTAAACAGTTCAA
GGCTGCGG-3′

Raptor shRNA sequences

5′-
GATCCCAGGGCCCTGCTACTCGCTTTTCAAGAGAAAGCGAGTAGCAGGGCCCTT
TTTTGGAAA-3′

5′-
AGCTTTTCCAAAAAAGGGCCCTGCTACTCGCTTTCTCTTGAAAAGCGAGTAGCA
GGGCCCTGG-3′

Scramble shRNA sequences

5′-
GATCCCCCTAAGGTTAAGTCGCCCTTTCAAGAGAAGGGCGACTTAACCTTAGGT
TTTTGGAAA-3′

5′-
AGCTTTTCCAAAAACCTAAGGTTAAGTCGCCCTTCTCTTGAAAGGGCGACTTAA
CCTTAGGGG-3′

Viral infections and transfection

Viral preps were obtained by transfecting Pheonix cells with pBABEpuro-myrAKTΔER and pBABEpuro-myr*AKTΔER constructs and collecting the media 48-72hrs post transfection followed by filtration through 0.45uM filter. MCF10A and B1400i cells were infected on 2 consecutive days by incubating for 4 hours with viral supernatants supplemented with 5ug/ml Sequebrene (Sigma). MCF10A cells were selected with 1ug/ml puromycin (Gemini Bio Products). SKBr3/TR cells were generated by lentiviral infections using viral preps of pLenti6/TR produced in 293FT cells followed by clonal selection after Blastacidin (Invitrogen) treatment of infected cells. Inducible HER3sh, Rictorsh, and Raptorsh and HER3-myc expressing cells were generated by transfecting the constructs into SKBr3/TR

cells using Lipofectamine 2000 (Invitrogen) and standard protocols and then clonal selection (knockdown) or pools (HER3 myc) of G418 (Gibco) or Zeocin (Invitrogen) resistant cells.

Cell death analysis

FACS analysis of nuclear degradation was performed as described (24). % Sub G1 cells are reported from three independent repeats of experiments. Error bars are calculated from S.E.M. Student's t-test were performed and p-values of <0.05 reported.

Results

The response to perturbation of homeostatic signaling studied in extreme depth in SkBr3 cells

We began this effort by interrogating, in extreme depth, the signaling network downstream of HER3 in the HER2-amplified SkBr3 breast cancer cells. Although studies of a single cell line can sometimes lead to findings that are not generalizable, the depth of analysis that is possible by focusing on an individual cell line can reveal signaling relationships not otherwise discoverable by more cursory evaluations in larger panels of cell lines. The general relevance of any specific functionally revealing findings can then be readily tested in many other cell models.

Steady state signaling was negatively or positively perturbed in SkBr3 cells by a variety of pharmacologic and genetic means described below and the signaling throughput was assayed at many points within the HER3-PI3K-Akt-mTOR signaling network. The full range of the signaling nodes being experimentally perturbed and the nodes being read out are schematically depicted in Figure 1. We began with a panel of kinase inhibitors selective for targets spanning upstream to downstream based on current understanding of the signaling throughput downstream of PI3K. Multiple PI3K inhibitors were used to dilute the effects attributable to off-targets, and to exploit their differences in selectivity for p110α vs p110β. Both active site and allosteric type inhibitors of Akt and mTor were used since these agents differ substantially in their modes of action and selectivities. For each drug, we first tested a range of concentrations and selected a low or lowest concentration that inactivates its immediate target within one hour of treatment (supplementary Figure 1). We specifically used lower but effective concentrations not just to minimize the confounding effects of off-targets, but more importantly, to enable us to detect compensatory restoration of signaling throughput where such mechanisms are in place. The use of high concentrations can overpower cellular adaptive mechanisms, and in some cases induce cell death, and prove uninformative as an experimental tool. The selected concentration of each drug was then applied and cell signaling was interrogated at a short time point (T=1 hour) to assay the immediate effects on signaling throughput, and again at delayed timepoints (T=48,72 hrs) to interrogate the compensatory responses enacted by adaptational mechanisms. Two delayed timepoints were assayed to query whether the signaling throughput had yet stabilized at 48 hours, confirming the establishment of a new steady state and a new point of homeostasis in the cellular adaptive machinery. The readouts of signaling activity were taken at various signaling nodes upstream, midstream, downstream, and cross-stream. p-S6K1, p-S6, and p-4EBP1 were used as readouts of TORC1 signaling activity, and p-NDRG1 was used as a

readout of TORC2 signaling activity. The fidelity of these substrates to the corresponding mTor complexes has been well described by others and these are widely used as reporters of the two mTor complexes (31-37). Other substrates of TORC2, sometimes used as reporters, failed to show specificity for this complex in our hands, as has been reported by others (38).

Since certain key signaling nodes deemed critically relevant (HER3, TORC1, TORC2) are not directly or individually druggable, we complemented these pharmacologic studies with genetic targeting of these nodes using doxycycline-inducible shRNA knockdowns of HER3, raptor (to inactivate TORC1), and rictor (to inactivate TORC2) in stably transfected SkBr3 cells. In addition to the negative perturbation of the network using the pharmacologic library of kinase inhibitors and selected shRNAs, we also positively perturbed the network using HER3 ligand stimulation of SkBr3 cells, doxycycline-inducible induction of HER3 overexpression in engineered SkBr3 cells, and tamoxifen-inducible induction of an engineered myr-AKT-ER fusion construct in SkBr3 cells. The doxycycline-induced models were thoroughly investigated to determine the timeline of gene induction or suppression following doxycycline treatment in order to query the signaling throughput at timepoints before and at early and late timepoints after the genetically induced events. Genetically induced events don't perturb cellular signaling immediately as do pharmacologically induced events, and these manipulations may not create dips or spikes in signaling dynamics seen with drug treatments. But the cellular compensatory mechanisms, albeit more gradual in onset, can yet be informative, especially when used to complement the pharmacologically derived data sets.

Much of the network downstream of HER3 is resilient to perturbation

The full data sets emerging from this extensive analysis are shown in supplementary Figures 2-4. Since our goal was to obtain a deeper understanding of the network topology that underlies the resiliency in HER3-PI3K-AKT-mTOR signaling in these cancers, we simplified this data set into three-dimensional schematic forms depicting how the network reacts to perturbation at each of its nodes. The schematic summaries are shown in Figure 2 and 3 (for pharmacologic inhibitors), Figure 4 (for shRNA inhibition) and Figure 5 (for stimulations). The basic schematic is constructed based on our current understanding of signaling throughput in this network, realizing that the data may lead us to either challenge the validity of certain components of this topology, or to identify additional arms or components of the topology in these cancer cells. The first thing that is immediately apparent is that the signaling throughput along each of the nodes in this network from upstream HER3 to downstream mTOR can be upregulated to compensate for inhibition by kinase inhibitors (supplementary Figure 5). The restoration of signaling is a property of the signaling network, and not an artifact resulting from the loss of drug activity since it is observed despite various schemas of repetitive drug replenishment (supplementary Figure 6).

TORC2 is driven by Akt in SkBr3 cells

When the perturbations of Figures 2-5 are examined in depth, a key finding that becomes apparent is that in these cancer cells TORC2 appears to signal downstream of Akt, a position not previously reported. The reduction in TORC2 signaling is evident rapidly following drug

inhibition of the upstream nodes HER2 (2A), or PI3Kα (2B), or Akt (2D, 2E), or following the shRNA knockdown of HER3 (4A). This is corroborated by the positive perturbation studies where an increase in TORC2 signaling is evident following neuregulin stimulation (5A), or following HER3 overexpression (5B), or following tamoxifen induced activation of an activatable myrAkt construct (5C). These negative and positive perturbations are all consistent with the finding that Akt activates TORC2 in these cells. This does not negate the well known reciprocal relationship that TORC2 directly activates Akt through phosphorylation of its hydrophobic motif (39)., best seen with the direct inhibition of TORC2 by an mTOR kinase inhibitor (3B) or disruption of the TORC2 complex by shRNA knockdown of RICTOR (4C). The fact that Akt activates TORC2 and that TORC2 activates Akt describes an Akt-TORC2 positive feedback loop in these cancer cells. Positive feedback loops are commonly deployed in biologic systems, underlying the property of bistability and can function to robustly maintain a state of high output signaling.

HER3 signaling is negatively regulated by TORC2

A second finding, not previously known, that emerges from the collective analysis of the signaling data in these cells is that HER3 signaling is negatively regulated by TORC2. This is most evident when TORC2 is directly inhibited by mTOR kinase inhibitors or shRNA knockdown of RICTOR, both of which relieve the constraint on HER3 signaling, leading to a substantial upregulation of HER3 signaling (3B and 4C). HER3 is an upstream activator of Akt (through PI3K), and thus the negative regulation of HER3 by TORC2 describes a pathway through which TORC2 negatively regulates Akt in these cells. This constitutes a buffering arm for the Akt-TORC2 positive feedback loop described above, and completes a concentric signaling topology highly competent at response to perturbation at each of its nodes (Figure 6). It remains possible that additional proteins can function as a TORC2-Akt buffering arm. We observed a regulation of PHLPP phosphatase expression by TORC2 in our studies, and these may also function to establish the set-point for the Akt-TORC2 bistable switch. The PHLPP proteins are known to be regulated by TORC1 (40, 41), although their regulation by TORC2 has not previously been described. Since we were unable to directly assay or experimentally manipulate their signaling throughput, their role remains unconfirmed at this time.

Akt reconciles different signaling inputs into its output

There is additional complexity inherent in the Akt protein that enables us to further query and establish the competence of this homeostatic network topology. Signaling from inputs emanating from HER3-PI3K leads to phosphorylation of Akt on T308 within its activation loop, whereas signaling from TORC2 results in phosphorylation of Akt on S473 within its hydrophobic motif (39, 42). This is reaffirmed here since inhibition of PI3K induces a more profound dephosphorylation of Akt T308 (Figure 2B, supplementary Figure 2; blots 4B,4C, 4D,4F compared with 5B,5C,5D,5F) whereas inhibition of mTor kinase produces a more profound inhibition of Akt S473 (Figure 3B, a more profound dephosupplementary Figure 2; blots 5N,5O compared with 4N,4O). However when one of the signaling inputs to Akt is pharmacologically inhibited, a compensatory increase in the other input is often seen in the new steady state, driving the restoration of Akt signaling output. This is particularly evident in the compensatory response to inhibition of mTor kinase which restores Akt signaling with

a far greater phosphorylation of T308 than S473 (supplementary Figure 2, blots 4N,4O), or the compensatory response to PI3K inhibition which typically restores Akt signaling through a greater phosphorylation of S473 than T308 (supplementary Figure 2, blots 5B,5D).

Redundancy in TORC1 negative feedback signaling

The role of Akt in the regulation of TORC1 appears to be as an upstream activator as is well described, and a bidirectional relationship of these two nodes similar to the Akt-TORC2 relationship does not appear to exist in these cells. There are however negative feedback loops associated with TORC1 activity. This is evident when TORC1 is directly inhibited by rapamycin (3A) or shRNA knockdown of RAPTOR (4B) which result in a compensatory upregulation of HER3 signaling at the new steady state timepoints. But this feedback mechanism is far weaker than then the Akt-TORC2 feed-forward mechanism. In fact, TORC1-S6 signaling is fully and durably inhibited by the lapatinib-induced inactivation of HER2-HER3 signaling, and the compensatory mechanisms in place function to ensure the restoration of HER3-Akt-TORC2 signaling throughput while there is no mechanism robust enough to restore TORC1-S6 signaling.

The Akt-TORC2 feed-forward loop does not exist in normal mammary epithelial cells

To determine whether the Akt-TORC2 feed-forward network topology is a pre-existing and conserved part of homeostatic signaling or whether it is acquired in the process of tumorigenesis, we conducted a similar, albeit more limited, chemical and genetic interrogation of this signaling pathway in non-malignant MCF10A human mammary epithelial cells (HMECs)(supplementary Figure 7). While the general signaling circuitry in MCF10A cells has some similarities to the circuitry in SkBr3 cells, the described central Akt-TORC2 feed-forward loop is lacking in MCF10A cells. This is principally due to the absence of a forward relationship between Akt signaling and TORC2 signaling in MCF10A cells. In SkBr3 cells, the activity of Akt drives the activity of TORC2. This becomes evident when the activity of Akt is directly inhibited (Figure 2d,2E, supplementary Figure 2 blots 12H,12J,12L) or directly stimulated (Figure 5C, supplementary Figure 4 blot 14E). However there is no such relationship in MCF10A cells and in these HMECs TORC2 signaling is not reduced when Akt is directly inhibited (supplementary Figure 7, blot 6G,6I,6J,6K). The inputs that drive TORC2 activity in MCF10A cells remain unknown and not identified here, but it's not Akt.

The Akt-TORC2 feed-forward loop is widely seen in HER2-amplified cancer cells

To further investigate the relationship between Akt and TORC2 in HMECs cells, we engineered MCF10A and B1400is16m HMECs to express a tamoxifen-inducible Akt construct. The superactivation of Akt in these cells does not further increase TORC2 signaling as seen by p-NDRG1 (Figure 7). Therefore, in contrast to SkBr3 cancer cells, the activity of TORC2 in MCF10A HMECs is uncoupled from Akt and is neither dependent on or modulated by Akt.

The above data derived through an in-depth analysis of the signaling throughput downstream of HER3 conducted in SkBr3 cells revealed the fact that Akt drives TORC2 in these cells, constituting one arm of a positive feedback loop that forms a robust bistable

switch highly competent at maintaining signaling throughput. To determine whether this Akt-TORC2 relationship is unique to SkBr3 cells or whether it is generalizable to HER2-amplified cancers or to other types of cancer, we further studied this relationship in a panel of untransformed HMECs and cancer cell lines. Indeed the finding is generalizable, as TORC2 function is not interrupted by Akt inhibition in HMECs, whereas it is interrupted in the HER2-amplified cancer cells (Figure 8 and supplemental Figure 8). There are variable degrees of restoration of TORC2 signaling in HER2-amplified cancer cells, however the Akt-dependency is clearly evident in them through an initial rapid decline in TORC2 signaling following inhibition of Akt activity. However there is no such dependency in the HMECs. Therefore TORC2 activity is dependent on Akt in HER2-amplified cancer cells, and this appears to be unique to these cancer cells since it is not seen in HMECs. We also expanded the analysis to a panel of cancer cells lines that are not driven by HER2 amplification and find that the coupling of TORC2 to Akt is not seen in most cancer cells but appears to be unique to HER2-amplified cancers (Figure 8). There do exist however some cancer cells, such as MDA-468 and PC3 that show a delayed downregulation of TORC2 in response to Akt inhibition, suggesting a relationship between Akt and TORC2 but not a more direct regulation of TORC2 by Akt. The Akt-TORC2 coupling observed in HER2-amplified cancers is not due to non-specific effects of the Akt inhibitors since it is seen with four different Akt inhibitors (Figure 2D, 2E, supplementary Figures 2 blots 12H, 12J, 12L, supplementary Figure 9) and also seen with the direct activation of Akt (Figure 5C, supplementary Figure 4 blot 14E). The signaling inputs that drive the apparent constitutive activity of TORC2 in HMECs and most cancer cells remain to be identified, but this upstream input to TORC2 appears to be lost in HER2-amplified cancer cells, instead rendering TORC2 dependent on Akt.

These data place TORC2 in a critical position in the biology of HER2-amplified cancer cells, particularly in their highly competent ability to restore HER3 signaling in the face of HER2-targeting tyrosine kinase inhibitor therapy. This key role of TORC2 in HER2-amplified cancer cells was confirmed with the inducible knockdown of RICTOR. In the absence of TORC2 function, lapatinib-treated SkBr3 cells fail to restore HER3 signaling, and fail to restore either the PI3K-driven or TORC2-driven inputs to Akt (Figure 9A). Similar results are seen in BT474 cancer cells subjected to shRNA-induced inactivation of TORC2 function (supplemental Figure 10). The critical role of TORC2 in the preservation of cellular homeostasis in HER2-amplified cancer cells is also apparent in the cell fate that ensues following the selective inactivation of TORC2. When TORC2 is selectively inactivated through shRNA targeting in SkBr3 cells, apoptotic cell death ensues, whereas this is not seen with the selective inactivation of TORC1 (Figure 9B).

Discussion

Oncogenes are often accompanied by an altered network topology

Oncogenes are the major biological driving force underlying the growth and progression of some cancers, making them ideal targets for therapeutic drugs and this treatment paradigm has now been validated in several types of cancers. An important lesson learned from the first generation of attempts to inhibit kinase oncogenes has been a better appreciation for the

broader signaling network within which they function and the fact that the pathological basis for the disease is not solely embodied in the overactivity of the oncogene itself, but also encompasses a reprogramming of the signaling network within which the oncogene functions. The functional significance of the altered signaling network may not be easily appreciated on snapshot analysis of signaling in such cancer cells, but can become readily apparent by the adaptive nature of the cellular response over time following inhibition of the driving oncoprotein.

In this study, we undertook to develop a deeper understanding of the signaling network linked with HER3 in HER2-amplified cancers cells. In these cancer cells, although HER2 is the genetically altered and overexpressed oncogene, the presence of HER3 is known to be essential (20-22), and its resiliency at its orthogonal position lies at the heart of resistance to HER2-targeting drugs (17, 24, 43). We began this analysis by first exploring, in extreme depth, how the network responds to perturbation using a library of pharmacologic inhibitors and genetic manipulations. These observations did not fully reconcile with the known topology along the HER3-PI3K-Akt-mTOR signaling network leading us to suspect unanticipated links between Akt and TORC2 and between TORC2 and HER3. The validity of these signaling links was confirmed in more direct experiments and their general relevance to the context of HER2-amplified cancer also confirmed in panels of cancer cell lines and controls.

Altered downstream network topology in HER2-amplified cancer cells

We find a fundamental reprogramming of the downstream network topology in HER2-amplified cancers that alters the role of HER3 from a mere upstream growth factor receptor input to an essential node in a highly competent homeostatic signaling network driven by a Akt-TORC2 feed-forward engine, schematically depicted in Figure 6. At the heart of this reprogramming is the deregulation of TORC2 signaling in these cancer cells. The upstream signals that drive TORC2 signaling in most cell types are not yet known. Growth factors are thought to activate TORC2 although the signaling mechanisms for such a pathway are not yet defined. In this study, we find that in HER2-amplified cancer cells, TORC2 is activated by Akt, a relationship not seen in most cell types, or most cancer cells, and not previously reported in any other contexts. How Akt activates TORC2 is not clear and most likely involves intermediaries not yet known. All previous evidence reveals TORC2 to function upstream of Akt and to activate Akt by phosphorylation of its hydrophobic motif (39) and this function remains intact in these cells as seen by the rapid dephosphorylation of Akt S473 following treatment with mTOR inhibitors (Figure 3B, suppl Figure 2, blots 4N, 4O) or shRNA knockdown of RICTOR (Figure 4C, suppl Figure 3 blot 6C). However the position of TORC2 both upstream and downstream of Akt in HER2-amplified cancers defines an Akt-TORC2 feed-forward loop not previously encountered in other cellular contexts. In untransformed cells and in most other types of cancers, TORC2 signaling is not dependent on Akt, and is driven by currently unknown signaling inputs. Why these same inputs are unable to sustainTORC2 signaling in HER2-amplified cancer cells remains unknown at this time, but one can speculate that the loss of such a signaling input, through additional genetic or epigenetic events, may be an essential step in the evolution of HER2-driven tumorigenesis, and constitutes the key step in tumorigenesis wherein HER2-HER3

evolves from an overactive but non-essential input to a signaling node essential for the preservation of homeostasis.

Positive feedback loops function as bistable switches

Signal transduction motifs are ubiquitously used in biological systems to shape the behavior of cells in space and time (44). Both positive and negative feedback loops are widely seen in biological systems, each with its own advantages and attributes. A specific attribute of positive feedback loops is that they convert graded inputs into switch-like irreversible outputs creating so-called bistable switches that establish a self-perpetuating state (45-47). Positive feedback between Cdc2-cyclin B and its activator Cdc25 trigger the sustained high output activity required to propel the progression and completion of the mitotic phase of the cell cycle (48). Similarly, positive feedback within the MAPK cascade accounts for the irreversible commitment of xenopus oocytes to biological maturation in response to progesterone (49). In the process of adipogenesis, glucocorticoid and cAMP initiate cascades of positive feedback loops that lock cells in the differentiated state committed to the accumulation of lipid (50). As such, positive feedback loops function to establish a form of biologic memory or commitment that sustains a regulatory program in the face of wavering inputs. The malignant state is a committed cellular state similar to many of the physiologic states described above, and as such, it is easily conceivable that cancer cells would rely on this robust network motif to secure and perpetuate their signaling programs. In HER2-amplified cancer cells, the Akt-TORC2 positive feedback loop commits this signaling pathway to a set level of constitutive activity likely required for the needs of the tumorigenic state. The robustness provided by this signaling motif underlies the resiliency encountered upon attempts to interfere with signaling throughput by pharmacologic targeting at any point within the network (Supplementary Figure 5). The observed Akt-TORC2 positive feedback loop is a specific attribute of HER2-amplified cancers that appears to be acquired during the process of tumorigenesis, as it is not apparent in non-transformed cells or most other types of cancer cells. The Akt activation of TORC2 most likely involves intermediaries not currently defined. Such an indirect positive feedback loop is common in biological systems and is thought to protect the system from noise (51).

This however is not a simple positive feedback loop as the activation of Akt by TORC2 is not a simple link. TORC2 is well known to activate Akt directly through phosphorylation of its hydrophobic motif (39), but in these cancer cells this is also tempered through the indirect regulation of HER3 (Figure 6). The negative regulation of HER3 signaling by TORC2 has not been described before but is clearly evident when TORC2 is inhibited following treatment with mTOR kinase inhibitors (Figure 3B; supplementary Figure 2 blots 3N, 3O) or shRNA knockdown of RICTOR (Figure 4C, supplementary Figure 3 blot 4C). Given the well-described position of HER3 upstream of PI3K and Akt (19), this completes the second arm of a TORC2-Akt feedforward loop. As such, TORC2 activates Akt through an incoherent feedforward loop (52, 53) in HER2-amplified cancers and thus the overall topology is best described as a nested or coupled feedforward positive feedback loop. Feedforward loops are widely seen in biologic systems and commonly seen nested within feedback loops, such as during the elaborate and committed programs involved in development (54). In particular, feedforward motifs are known for their stability and rapid

signaling response times (53, 55). The coupling of network motifs is also frequently seen in biological systems, and in particular, the coherent coupling of motifs, such as seen here with the Akt-TORC2 positive feedback loop and the TORC2-HER3-Akt feedforward loop, substantially enhances the robustness of the overall network (56). This also functionally reassigns HER3 from its natural role as a conditionally engaged upstream signaling input to an essential node in the network topology that is committed to the maintenance of homeostasis. The robust nature of this coupled feedforward positive feedback network ensures stability in the oncogenic program in the face of perturbations encountered due to environmental conditions including interference from drugs that target any of its components.

Since overactive HER2-HER3 signaling is fixed by the genomic level amplification of HER2 in these cancers, it seems unlikely that a downstream positive feedback loop would be required to perpetuate the oncogenic signaling coming from upstream HER2. But although the expression and activity of HER2 is fixed, the expression and signaling activity of HER3 is highly dynamic and regulated through a number of mechanisms (23, 57, 58). Therefore, even though HER2 output is fixed at the genomic level in these cancer cells, HER3 output is not, and the Akt-TORC2 positive feedback loop can function to maintain adequate HER3 signaling in the face of numerous regulatory influences upon it. It is also possible that the Akt-TORC2 positive feedback loop functions to counteract pre-existing negative feedback signaling typically seen in growth factor receptor signaling networks which in some circumstances protects against tumorigenesis induced by the constitutive activation of the growth factor receptor signaling cascade, such as the feedback mechanisms that underlie senescence induced by mutational activation of Ras or Raf (59).

The non-redundant functions of TORC1

Much of the functions of mTor signaling in cancers has been attributed to the functions of TORC1 and indeed, these functions, including anabolic processes such as protein and lipid synthesis, the regulation of cellular metabolism, autophagy, and control of the cell cycle are all important for maintaining the malignant state. The analysis here does not undermine the importance of TORC1 functions in HER2-driven tumors; rather our analysis is focused on the network topology that constitutes the heart of the signaling machinery driving homeostasis and underlying the resiliency seen in the face of drug interference. Indeed the selective loss of TORC2 function in SkBr3 cells disrupts cellular homeostasis in such a fundamental way that apoptotic cell death ensues, whereas the selective loss of TORC1 function merely resets the state of homeostasis into a new non-proliferative state.

Complexities in targeting Akt

The analysis of pharmacologic inhibitors of Akt reveals differences between allosteric site and active site inhibitors of Akt. Specifically, inhibiting Akt with an active site inhibitor induces the paradoxical hyperphosphorylation of Akt on its regulatory T308 and S473 sites (Figures 2E, supplementary Figure 2 blot 4K-L, 5K-L and supplementary Figure 7, blot 3J-K, 4J-K). This has previously been reported and thought to be due to conformational changes induced by inhibitor binding that expose the PH domain, promote the membrane localization of Akt and its consequent phosphorylation by upstream kinases at the membrane

(60, 61). These paradoxical effects are not seen with the allosteric class of Akt inhibitors. Therefore although the increased phosphorylation of Akt T308 and S473 seen with active site Akt inhibitors is depicted as increased PI3K-Akt and increased TORC2-Akt signaling in Figure 2E, this should not be attributed to the network response to inhibition since it is a direct consequence of drug-induced conformational and localizational changes. Both inhibitor classes inhibit the kinase activity of Akt as seen through its substrate PRAS40. There are subtle differences in the assay results from the two classes of Akt inhibitors which may in part suggest that the two classes of inhibitors may have different biological effects, although some of this may also be due to different potencies or different selectivities associated with these molecules.

Implications for the treatment of HER2-amplified cancers

This work has significant implications for the treatment of HER2-amplified cancers. It has been recognized that PI3K/Akt signaling is critically important in the biology of these cancers, leading to suggestions for combining novel PI3K, Akt, or mTOR inhibitors to current HER2 inhibitors. An inherent problem with these combination approaches is the limited therapeutic index associated with targeting these downstream pathways due to their critical functions in physiologic cell signaling in many normal tissues. In addition, targeting the PI3K/Akt pathway in these cancers induces a compensatory activation of MAPK pathway that further mitigates the efficacy of such combination therapy approaches. The network topology described in our work identifies TORC2 as a highly attractive novel target for combination therapy with HER2 inhibitors. Although selective inhibitors of TORC2 are not currently available, their narrower selectivity would be expected to translate into a wider therapeutic index than mTor, PI3K, or Akt kinase inhibitors, and would not induce a compensatory activation of MAPK pathway signaling. These attributes make a highly compelling case for the development of TORC2-selective allosteric inhibitors for combination with HER2 or HER3 inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Janne PA, Johnson BE. Effect of Epidermal Growth Factor Receptor Tyrosine Kinase Domain Mutations on the Outcome of Patients with Non-Small Cell Lung Cancer Treated with Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors. Clin Cancer Res. 2006; 12:4416s–20s. [PubMed: 16857820]

2. Camidge DR, Bang YJ, Kwak EL, Iafrate AJ, Varella-Garcia M, Fox SB, et al. Activity and safety of crizotinib in patients with ALK-positive non-small-cell lung cancer: updated results from a phase 1 study. Lancet Oncol. 2012; 13:1011–9. [PubMed: 22954507]

3. Shaw AT, Camidge DR, E JA, Solomon BJ, Kwak EL, Clark JW, et al. Clinical activity of crizotinib in advanced non-small cell lung cancer (NSCLC) harboring ROS1 gene rearrangement. J Clin Oncol. 2012; 30 suppl; abstr 7508.

4. Flaherty KT, Puzanov I, Kim KB, Ribas A, McArthur GA, Sosman JA, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. N Engl J Med. 2010; 363:809–19. [PubMed: 20818844]

5. Kantarjian H, Sawyers C, Hochhaus A, Guilhot F, Schiffer C, Gambacorti-Passerini C, et al. Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. N Engl J Med. 2002; 346:645–52. [PubMed: 11870241]

6. Moody SE, Sarkisian CJ, Hahn KT, Gunther EJ, Pickup S, Dugan KD, et al. Conditional activation of Neu in the mammary epithelium of transgenic mice results in reversible pulmonary metastasis. Cancer Cell. 2002; 2:451–61. [PubMed: 12498714]

7. Burstein HJ, Storniolo AM, Franco S, Forster J, Stein S, Rubin S, et al. A phase II study of lapatinib monotherapy in chemotherapy-refractory HER2-positive and HER2-negative advanced or metastatic breast cancer. Ann Oncol. 2008; 19:1068–74. [PubMed: 18283035]

8. Toi M, Iwata H, Fujiwara Y, Ito Y, Nakamura S, Tokuda Y, et al. Lapatinib monotherapy in patients with relapsed, advanced, or metastatic breast cancer: efficacy, safety, and biomarker results from Japanese patients phase II studies. Br J Cancer. 2009; 101:1676–82. [PubMed: 19844234]

9. Blackwell KL, Burstein HJ, Storniolo AM, Rugo H, Sledge G, Koehler M, et al. Randomized study of Lapatinib alone or in combination with trastuzumab in women with ErbB2-positive, trastuzumab-refractory metastatic breast cancer. J Clin Oncol. 2010; 28:1124–30. [PubMed: 20124187]

10. Burstein HJ, Sun Y, Dirix LY, Jiang Z, Paridaens R, Tan AR, et al. Neratinib, an irreversible ErbB receptor tyrosine kinase inhibitor, in patients with advanced ErbB2-positive breast cancer. J Clin Oncol. 2010; 28:1301–7. [PubMed: 20142587]

11. Baselga J, Tripathy D, Mendelsohn J, Baughman S, Benz CC, Dantis L, et al. Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. J Clin Oncol. 1996; 14:737–44. [PubMed: 8622019]

12. Cortés J, Baselga J, Petrella T, Gelmon K, Fumoleau P, Verma S, et al. Pertuzumab monotherapy following trastuzumab-based treatment: Activity and tolerability in patients with advanced HER2-positive breast cancer. J Clin Oncol. 2009; 27

13. Romond EH, Perez EA, Bryant J, Suman VJ, Geyer CE Jr, Davidson NE, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. N Engl J Med. 2005; 353:1673–84. [PubMed: 16236738]

14. Perez EA, Thompson EA, Ballman KV, Anderson SK, Asmann YW, Kalari KR, et al. Genomic Analysis Reveals That Immune Function Genes Are Strongly Linked to Clinical Outcome in the North Central Cancer Treatment Group N9831 Adjuvant Trastuzumab Trial. J Clin Oncol. 2015; 33:701–8. [PubMed: 25605861]

15. Bellati F, Napoletano C, Ruscito I, Liberati M, Panici PB, Nuti M. Cellular adaptive immune system plays a crucial role in trastuzumab clinical efficacy. J Clin Oncol. 2010; 28:e369–70. ; author reply e71. [PubMed: 20479394]

16. Gianni L. The “other” signaling of trastuzumab: antibodies are immunocompetent drugs. J Clin Oncol. 2008; 26:1778–80. [PubMed: 18347004]

17. Sergina NV, Rausch M, Wang D, Blair J, Hann B, Shokat KM, et al. Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3. Nature. 2007; 445:437–41. [PubMed: 17206155]

18. Jura N, Shan Y, Cao X, Shaw DE, Kuriyan J. Structural analysis of the catalytically inactive kinase domain of the human HER3 receptor. Proc Nat Acad Sci USA. 2009; 106:21608–13. [PubMed: 20007378]

19. Soltoff SP, Carraway KL 3rd, Prigent SA, Gullick WG, Cantley LC. ErbB3 is involved in activation of phosphatidylinositol 3-kinase by epidermal growth factor. Mol Cell Biol. 1994; 14:3550–8. [PubMed: 7515147]

20. Holbro T, Beerli RR, Maurer F, Koziczak M, Barbas CF 3rd, Hynes NE. The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. Proc Natl Acad Sci U S A. 2003; 100:8933–8. [PubMed: 12853564]
21. Lee-Hoeflich ST, Crocker L, Yao E, Pham T, Munroe X, Hoeflich KP, et al. A central role for HER3 in HER2-amplified breast cancer: implications for targeted therapy. Cancer Res. 2008; 68:5878–87. [PubMed: 18632642]
22. Vaught DB, Stanford JC, Young C, Hicks DJ, Wheeler F, Rinehart C, et al. HER3 is required for HER2-induced preneoplastic changes to the breast epithelium and tumor formation. Cancer Res. 2012; 72:2672–82. [PubMed: 22461506]
23. Amin DN, Sergina N, Lim L, Goga A, Moasser MM. HER3 signalling is regulated through a multitude of redundant mechanisms in HER2-driven tumour cells. The Biochemical journal. 2012; 447:417–25. [PubMed: 22853430]
24. Amin DN, Sergina N, Ahuja D, McMahon M, Blair JA, Wang D, et al. Resiliency and vulnerability in the HER2-HER3 tumorigenic driver. Sci Transl Med. 2010; 2:16ra7.
25. Bazarov AV, Hines WC, Mukhopadhyay R, Beliveau A, Melodyev S, Zaslavsky Y, et al. Telomerase activation by c-Myc in human mammary epithelial cells requires additional genomic changes. Cell Cycle. 2009; 8:3373–8. [PubMed: 19806010]
26. Bazarov AV, Lee WJ, Bazarov I, Bosire M, Hines WC, Stankovich B, et al. The specific role of pRb in p16 (INK4A) -mediated arrest of normal and malignant human breast cells. Cell Cycle. 2012; 11:1008–13. [PubMed: 22333593]
27. Bazarov AV, Van Sluis M, Hines WC, Bassett E, Beliveau A, Campeau E, et al. p16(INK4a) -mediated suppression of telomerase in normal and malignant human breast cells. Aging cell. 2010; 9:736–46. [PubMed: 20569236]
28. Stampfer MR, Garbe J, Levine G, Lichtsteiner S, Vasserot AP, Yaswen P. Expression of the telomerase catalytic subunit, hTERT, induces resistance to transforming growth factor beta growth inhibition in p16INK4A(-) human mammary epithelial cells. Proc Natl Acad Sci U S A. 2001; 98:4498–503. [PubMed: 11287649]
29. Kohn AD, Barthel A, Kovacina KS, Boge A, Wallach B, Summers SA, et al. Construction and Characterization of a Conditionally Active Version of the Serine/Threonine Kinase Akt. Journal of Biological Chemistry. 1998; 273:11937–43. [PubMed: 9565622]
30. Mirza AM, Kohn AD, Roth RA, McMahon M. Oncogenic transformation of cells by a conditionally active form of the protein kinase Akt/PKB. Cell Growth Differ. 2000; 11:279–92. [PubMed: 10910095]
31. Dibble CC, Asara JM, Manning BD. Characterization of Rictor phosphorylation sites reveals direct regulation of mTOR complex 2 by S6K1. Mol Cell Biol. 2009; 29:5657–70. [PubMed: 19720745]
32. Garcia-Martinez JM, Alessi DR. mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). The Biochemical journal. 2008; 416:375–85. [PubMed: 18925875]
33. James MF, Stivison E, Beauchamp R, Han S, Li H, Wallace MR, et al. Regulation of mTOR complex 2 signaling in neurofibromatosis 2-deficient target cell types. Mol Cancer Res. 2012; 10:649–59. [PubMed: 22426462]
34. Laplante M, Sabatini DM. mTOR signaling at a glance. Journal of cell science. 2009; 122:3589–94. [PubMed: 19812304]
35. Pearce LR, Sommer EM, Sakamoto K, Wullschleger S, Alessi DR. Protor-1 is required for efficient mTORC2-mediated activation of SGK1 in the kidney. The Biochemical journal. 2011; 436:169–79. [PubMed: 21413931]
36. Willems L, Chapuis N, Puissant A, Maciel TT, Green AS, Jacque N, et al. The dual mTORC1 and mTORC2 inhibitor AZD8055 has anti-tumor activity in acute myeloid leukemia. Leukemia. 2012; 26:1195–202. [PubMed: 22143671]
37. Zhang C, Wendel AA, Keogh MR, Harris TE, Chen J, Coleman RA. Glycerolipid signals alter mTOR complex 2 (mTORC2) to diminish insulin signaling. Proc Natl Acad Sci U S A. 2012; 109:1667–72. [PubMed: 22307628]

38. Manning BD. Comment on “A dynamic network model of mTOR signaling reveals TSC-independent mTORC2 regulation”: building a model of the mTOR signaling network with a potentially faulty tool. Sci Signal. 2012; 5:lc3. author reply lc4. [PubMed: 22786721]
39. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science. 2005; 307:1098–101. [PubMed: 15718470]
40. Liu J, Stevens PD, Gao T. mTOR-dependent regulation of PHLPP expression controls the rapamycin sensitivity in cancer cells. J Biol Chem. 2011; 286:6510–20. [PubMed: 21177869]
41. Warfel NA, Newton AC. Pleckstrin homology domain leucine-rich repeat protein phosphatase (PHLPP): a new player in cell signaling. J Biol Chem. 2012; 287:3610–6. [PubMed: 22144674]
42. Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, et al. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Curr Biol. 1997; 7:261–9. [PubMed: 9094314]
43. Garrett JT, Olivares MG, Rinehart C, Granja-Ingram ND, Sanchez V, Chakrabarty A, et al. Transcriptional and posttranslational up-regulation of HER3 (ErbB3) compensates for inhibition of the HER2 tyrosine kinase. Proc Natl Acad Sci U S A. 2011; 108:5021–6. [PubMed: 21385943]
44. Brandman O, Meyer T. Feedback loops shape cellular signals in space and time. Science. 2008; 322:390–5. [PubMed: 18927383]
45. Ferrell JE Jr. Self-perpetuating states in signal transduction: positive feedback, double-negative feedback and bistability. Current opinion in cell biology. 2002; 14:140–8. [PubMed: 11891111]
46. Mitrophanov AY, Groisman EA. Positive feedback in cellular control systems. BioEssays: news and reviews in molecular, cellular and developmental biology. 2008; 30:542–55.
47. Xiong W, Ferrell JE. A positive-feedback-based bistable /‘memory module/’ that governs a cell fate decision. Nature. 2003; 426:460–5. [PubMed: 14647386]
48. Pomerening JR, Sontag ED, Ferrell JE Jr. Building a cell cycle oscillator: hysteresis and bistability in the activation of Cdc2. Nat Cell Biol. 2003; 5:346–51. [PubMed: 12629549]
49. Ferrell JE Jr, Machleder EM. The biochemical basis of an all-or-none cell fate switch in Xenopus oocytes. Science. 1998; 280:895–8. [PubMed: 9572732]
50. Park BO, Ahrends R, Teruel MN. Consecutive positive feedback loops create a bistable switch that controls preadipocyte-to-adipocyte conversion. Cell reports. 2012; 2:976–90. [PubMed: 23063366]
51. Brandman O, Ferrell JE Jr, Li R, Meyer T. Interlinked fast and slow positive feedback loops drive reliable cell decisions. Science. 2005; 310:496–8. [PubMed: 16239477]
52. Alon U. Network motifs: theory and experimental approaches. Nat Rev Genet. 2007; 8:450–61. [PubMed: 17510665]
53. Mangan S, Alon U. Structure and function of the feed-forward loop network motif. Proceedings of the National Academy of Sciences. 2003; 100:11980–5.
54. Kim MS, Kim JR, Kim D, Lander AD, Cho KH. Spatiotemporal network motif reveals the biological traits of developmental gene regulatory networks in Drosophila melanogaster. BMC Systems Biology. 2012; 6:31. [PubMed: 22548745]
55. Doncic A, Skotheim JM. Feedforward regulation ensures stability and rapid reversibility of a cellular state. Mol Cell. 2013; 50:856–68. [PubMed: 23685071]
56. Kwon YK, Cho KH. Coherent coupling of feedback loops: a design principle of cell signaling networks. Bioinformatics. 2008; 24:1926–32. [PubMed: 18596076]
57. Morrison MM, Hutchinson K, Williams MM, Stanford JC, Balko JM, Young C, et al. ErbB3 downregulation enhances luminal breast tumor response to antiestrogens. The Journal of Clinical Investigation. 2013; 123:4329–43. [PubMed: 23999432]
58. Mujoo K, Choi BK, Huang Z, Zhang N, An Z. Regulation of ERBB3/HER3 signaling in cancer. Oncotarget. 2014; 5:10222–36. [PubMed: 25400118]
59. Courtois-Cox S, Genther Williams SM, Reczek EE, Johnson BW, McGillicuddy LT, Johannessen CM, et al. A negative feedback signaling network underlies oncogene-induced senescence. Cancer Cell. 2006; 10:459–72. [PubMed: 17157787]

60. Han EK, Leverson JD, McGonigal T, Shah OJ, Woods KW, Hunter T, et al. Akt inhibitor A-443654 induces rapid Akt Ser-473 phosphorylation independent of mTORC1 inhibition. Oncogene. 2007; 26:5655–61. [PubMed: 17334390]

61. Okuzumi T, Fiedler D, Zhang C, Gray DC, Aizenstein B, Hoffman R, et al. Inhibitor hijacking of Akt activation. Nat Chem Biol. 2009; 5:484–93. [PubMed: 19465931]

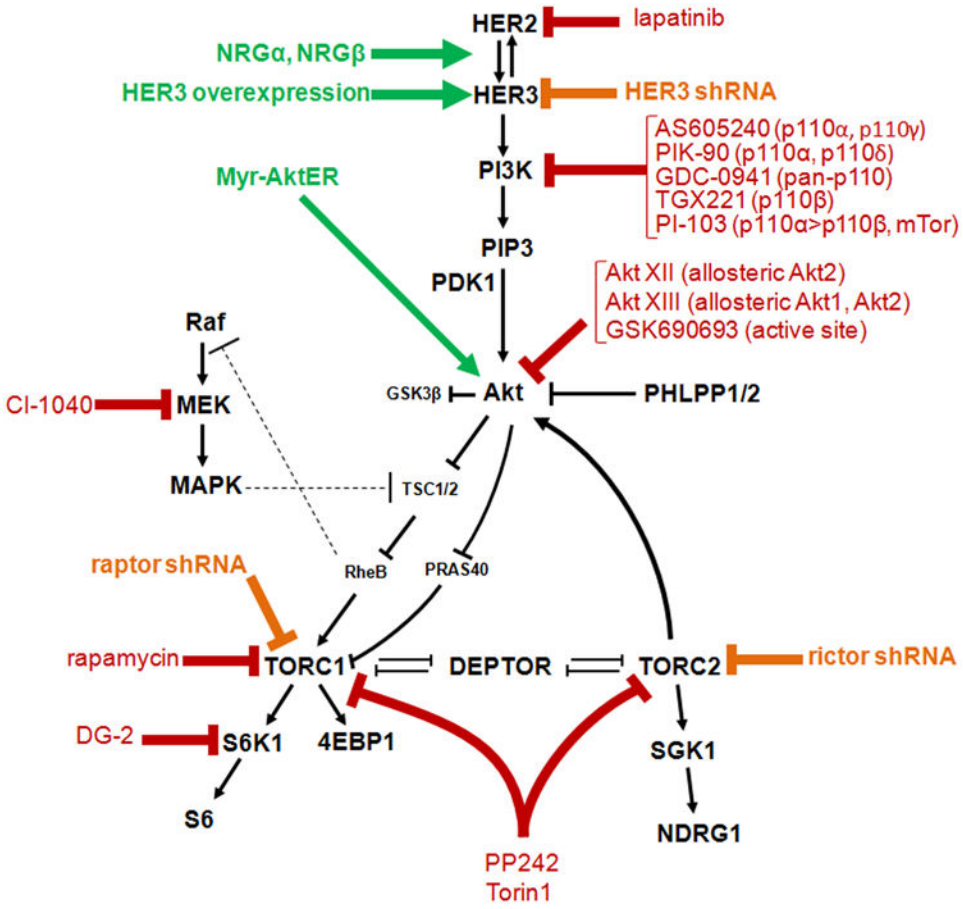

Figure 1.

A simplified version of the signal transduction pathway linking HER3 with the PI3K/Akt pathway is drawn in black based on currently known relationships. The pathway was perturbed negatively using the targeted drugs shown in red or using shRNA knockdown of targets shown in orange. The pathway was perturbed positively as shown in green, using ligand stimulation, HER3 overexpression, or a tamoxifen-inducible Akt construct.

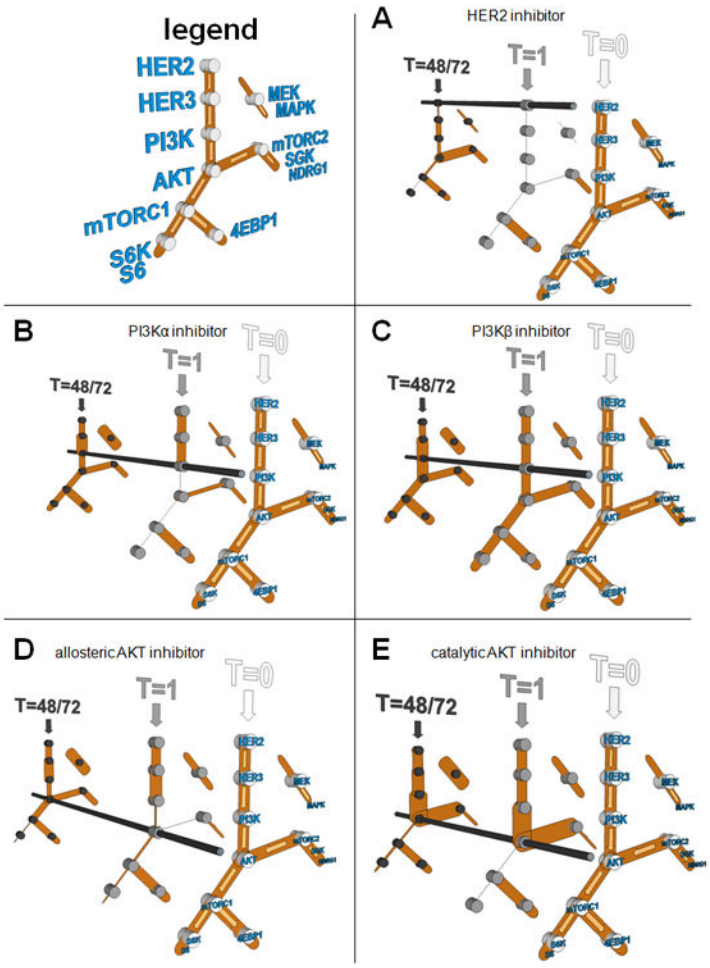

Figure 2.

Schematic depiction of signaling throughput along the HER3-PI3K-Akt-mTOR pathway in SkBr3 cells and its response to negative perturbation at the indicated nodes. The data is plotted in 3D format encompassing 3 layers reflecting 1) the state of the signaling throughput at baseline, 2) at initial inhibition at T=1 hour, and 3) at a late timepoint after establishment of a new steady state. The signaling throughput from node to node is reflected in the orange bands connecting the nodes. The front schematic reflects baseline signaling state and is identical in all the Figures. The width of the band in the treated timepoints reflects the relative strength of the signaling activity compared to baseline. A band thicker then baseline indicates an upregulation and the absence of a band indicates complete inhibition. The intervention in each Figure is identified in the caption and also schematically depicted by a black pipe drawn through the affected node. The western blots for these data sets are shown in supplementary Figure 2 and the concentrations of each drug is also specified there. The drug treatments experiments are assayed at T=0,1,48,72 hours. Drug-containing media is refreshed every 24 hours. A) The response to treatment with a HER2 inhibitor. B) The response to treatment with PI3Kα inhibitors. C) The response to treatment with a PI3Kβ-selective inhibitor. D) The response to treatment with an allosteric Akt inhibitor. E) The response to treatment with an active site Akt inhibitor.

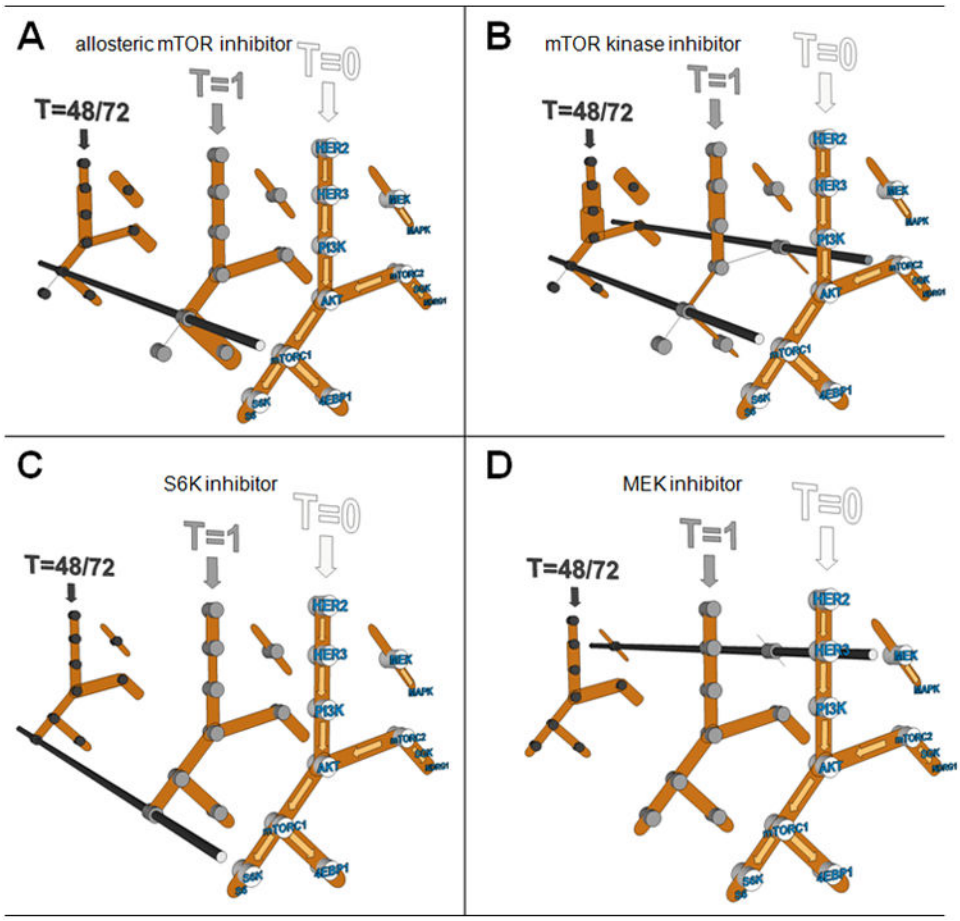

Figure 3.

Schematic depiction of signaling throughput along the HER3-PI3K-Akt-mTOR pathway in SkBr3 cells and its response to negative perturbation at the indicated nodes, as previously described in Figure 2 legend. A) The response to treatment with rapamycin. B) The response to treatment with an mTor kinase inhibitor. C) The response to treatment with and S6K inhibitor. D) The response to treatment with a MEK inhibitor.

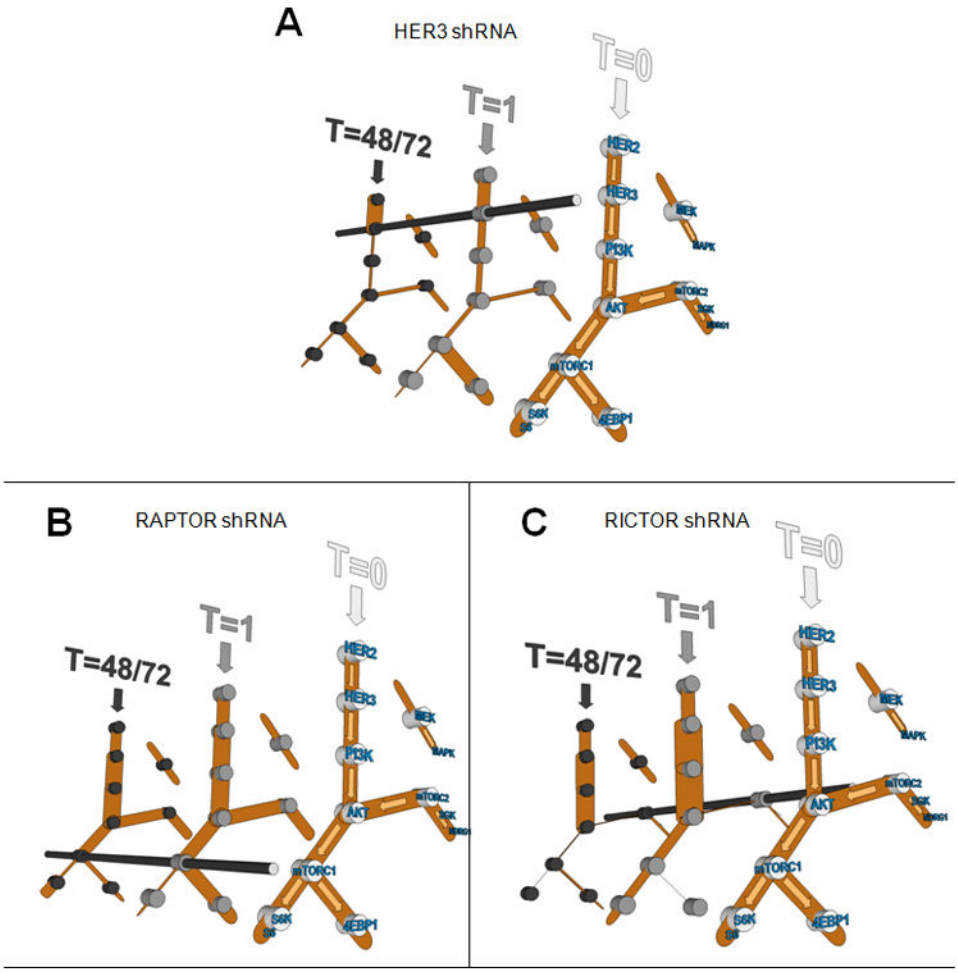

Figure 4.

Schematic depiction of signaling throughput along the HER3-PI3K-Akt-mTOR pathway in SkBr3 cells and its response to negative perturbation by doxycycline-induced shRNA knockdown of the indicated nodes. A black pipe is drawn through the node that is being inhibited by shRNA knockdown. The first timepoint after doxycycline treatment for each cell type is chosen to coincide with the earliest timepoint of knockdown or overexpression for that target. This was initially determined in pilot experiments designed to identify the kinetics of the doxycycline-induced perturbation. A) The response to shRNA suppression of HER3 expression and signaling. B) The response to shRNA suppression of Raptor expression and TORC1 signaling. C) The response to shRNA suppression of Rictor expression and TORC2 signaling.

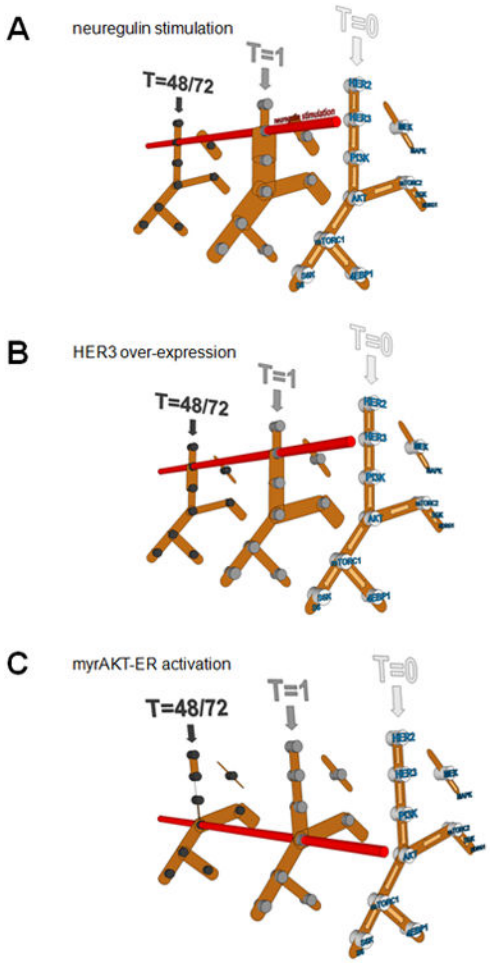

Figure 5.

Schematic depiction of signaling throughput along the HER3-PI3K-Akt-mTOR pathway in SkBr3 cells and its response to positive at the indicated nodes. A red pipe is drawn through the node that is directly stimulated. The schematic description is as described in Figure 2 legend. A) The response to upregualtion of HER3 signaling by neuregulin stimulation. B) The response to upregulation of HER3 signaling by doxycycline-induced overexpression of HER3 expression. C) The response to upregulation of Akt signaling by tamoxifen-induced activation of a stably transfected myrAKT-ER construct.

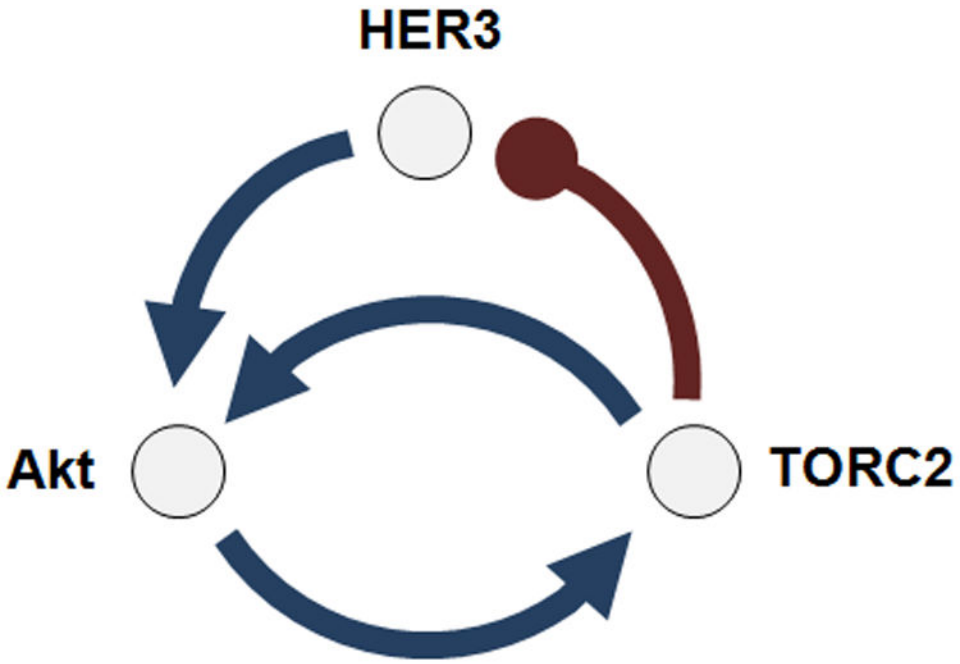

Figure 6.

Schematic overview summarizing the core infallible network topology in HER2-amplified cancer cells. TORC2 activates Akt through a feedforward loop, one arm which directly activates Akt, and the second arm mediated through the regulation of HER3. This is nested within a larger positive feedback loop motif. Akt and TORC2 activate each other completing a positive feedback loop that functions as a bistable switch, a network motif, seen in biologic systems, that generates a self-perpetuating state.

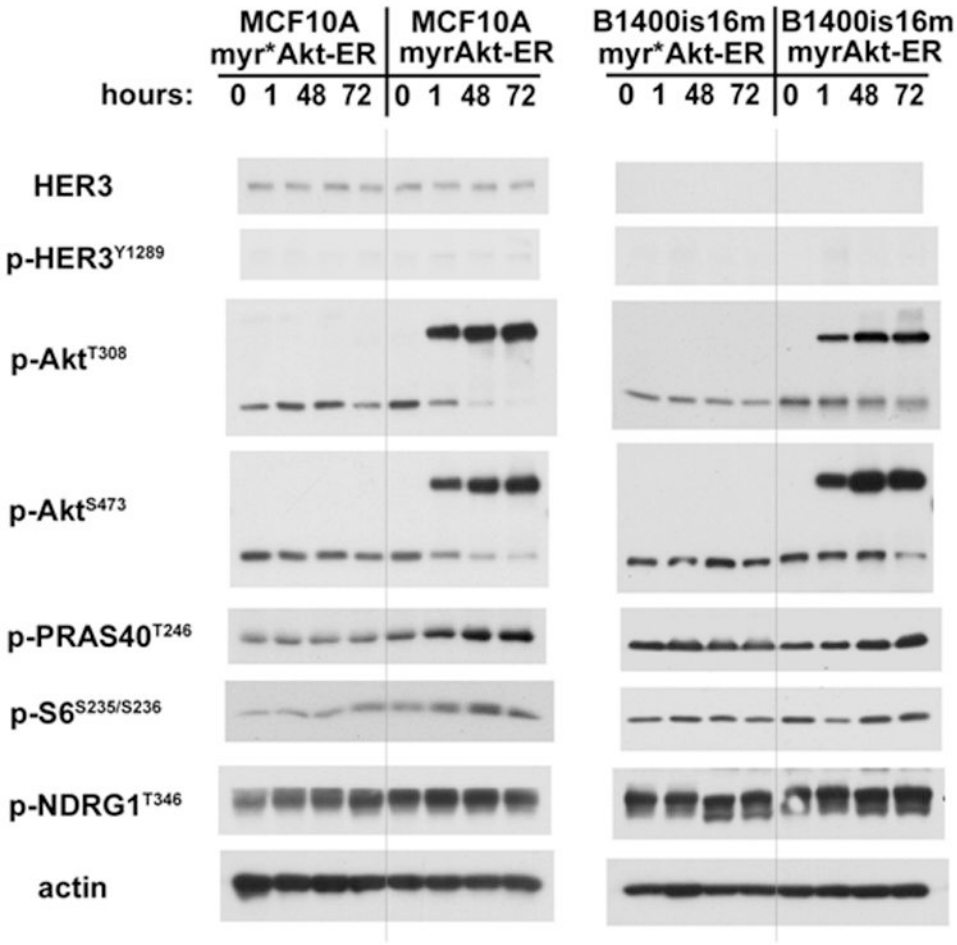

Figure 7.

MCF10A and B1400is16m human mammary epithelial cells were engineered to stably express a tamoxifen-inducible myristoylated Akt-ER fusion construct (myrAktΔER). The myr*AktΔER construct contains an inactivating point mutation in the myrisoylation domain and functions as a negative control. Cells were treated with tamoxifen to activate Akt directly and assayed at the indicated timepoints as shown.

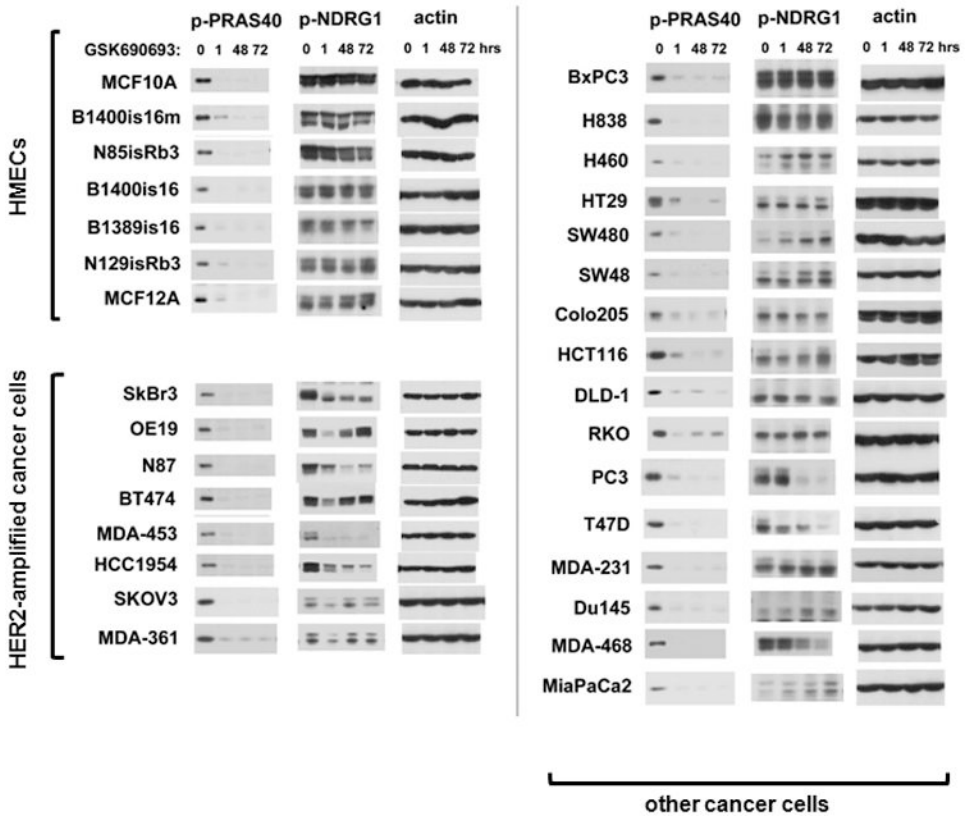

Figure 8.

A panel of human mammary epithelial cells, HER2-amplified cancer cells, and cancer cells of other types were treated with the Akt inhibitor GSK690693 at concentrations from 0.5-2uM (as found to be required to sufficiently inhibit Akt activity in each cell type) and assayed. The p-PRAS40 immunoblots serve to confirm the inactivation of Akt in all the cell types. The p-NDRG1 blots serve to show whether TORC2 activity is sensitive to the loss of Akt activity.

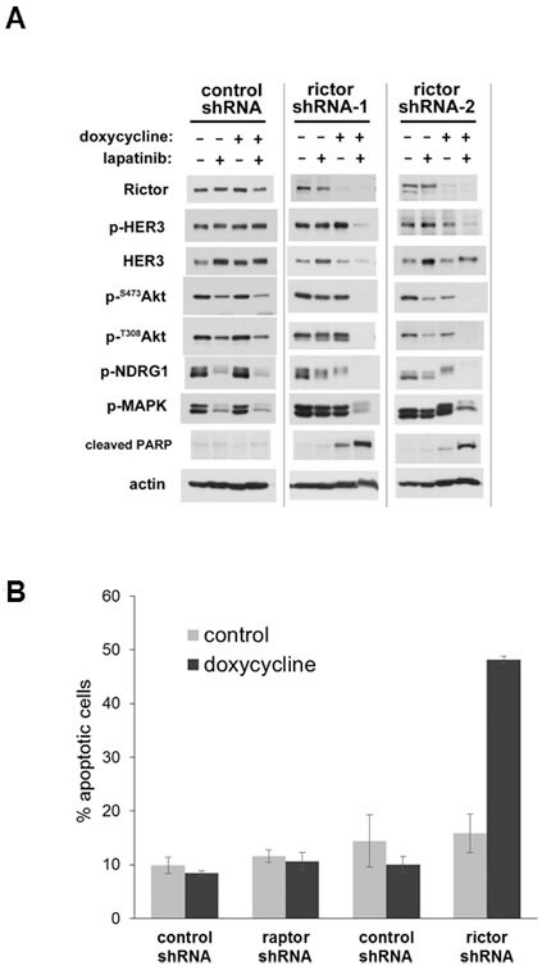

Figure 9.

A) SkBr3 cells engineered to express dox-inducible control or rictor shRNAs were treated with doxycycline or control for 8 days, and in the indicated arms this included the addition of 200nM lapatinib treatment for the last 48 hours. The 48 hour timepoint following lapatinib treatment is chosen because by this time the compensatory effects on HER3 have occurred. This enables direct determination of how Rictor suppression affects the compensatory upregulation of HER3 expression and the restoration of HER3 signaling. B) SkBr3 cells engineered to express dox-inducible shRNA constructs were treated with doxycycline or control and the fraction of apoptotic cells determined by flow cytometry. The duration of doxycycline treatment was 6 days for the raptor shRNA cells and their control shRNA, and 9 days for the rictor shRNA cells and their control shRNA. These timepoints are based on the timeline of raptor and rictor protein knockdowns established previously.