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## Kinome siRNA-phosphoproteomic screen identifies networks regulating AKT signaling

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### Abstract

To identify regulators of intracellular signaling we targeted 541 kinases and kinase-related molecules with siRNAs and determined their effects on signaling with a functional proteomics reverse phase protein array (RPPA) platform assessing 42 phospho and total proteins. The kinome wide screen demonstrated a strong inverse correlation between phosphorylation of AKT and MAPK with 115 genes that when targeted by siRNAs demonstrated opposite effects on MAPK and AKT phosphorylation. Network based analysis identified the MAPK subnetwork of genes along with p70S6K and FRAP1 as the most prominent targets that increased phosphorylation of AKT, a key regulator of cell survival. The regulatory loops induced by the MAPK pathway are dependent on TSC2 but demonstrate a lesser dependence on p70S6K than the previously identified FRAP1 feedback loop. The siRNA screen also revealed novel bi-directionality in the AKT and GSK3 interaction, whereby genetic ablation of GSK3 significantly blocks AKT phosphorylation, an unexpected observation as GSK3 has only been predicted to be downstream of AKT. This method uncovered novel modulators of AKT phosphorylation and facilitated the mapping of regulatory loops.

### Keywords

AKT; MAPK; proteomics; signaling networks; siRNA

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## Introduction

Cancer-causing mutations frequently alter homeostatic signaling leading to increased cell proliferation and survival (Hanahan and Weinberg, 2000; Hunter, 2000). Activating mutations target multiple components of the phosphatidylinositol 3-kinase (PI3K)/AKT (aka PKB) pathway in a greater number of tumors than any other signaling pathway, emphasizing the importance of the PI3K/AKT pathway in tumor initiation and progression (Brugge *et al.*, 2007). Based on the importance of the PI3K/AKT pathway, many small molecule inhibitors targeting this pathway are in preclinical and early clinical evaluation (Hennessy *et al.*, 2005).

The upstream regulators and downstream targets of AKT are diverse (Engelman *et al.*, 2006; Manning and Cantley, 2007). Phosphorylation and activation of AKT can be achieved through activation of receptor tyrosine kinases (RTKs), as well as G protein-coupled receptors (GPCRs) (Engelman *et al.*, 2006). Regulatory loops have been proposed to maintain pathway homeostasis. One well characterized negative feedback loop involves the tuberous sclerosis complex (TSC), the Rheb RAS superfamily member, the kinase mTOR, and the mTOR target p70 ribosomal S6 kinase (p70S6K) that inhibits insulin receptor substrate 1 (IRS1) and insulin-like growth factor receptor (IGFR) signaling (Avruch *et al.*, 2006; Manning *et al.*, 2005; O'Reilly *et al.*, 2006).

An improved understanding of the homeostatic mechanisms that control the PI3K/AKT pathway is required to efficiently implement targeted therapeutics and, in particular, to facilitate the design of rational therapeutic combinations that bypass the effects of homeostatic loops. To address this gap in knowledge, we used a systems approach that combined a screen of 540 siRNA pools targeting kinases and related molecules with a high-throughput functional proteomics reverse phase protein array (RPPA) platform (Amit *et al.*, 2007; Iadevaia *et al.*; Muller *et al.*, 2008; Tibes *et al.*, 2006) assessing abundance of 42 phosphorylated and parental proteins. This analysis revealed an inverse correlation between AKT and MAPK1,2 phosphorylation as well as new feedback loops within the AKT-MAPK network.

## Results

To identify regulators of the PI3K/AKT pathway, we used a systems approach combining a screen of 541 siRNA pools targeting kinases and related molecules (Table 1 and Supplementary Table 1) with the RPPA platform against phospho and total forms of 25 different proteins (Supplementary Table 2). The screen was performed in the MDA MB 468 cell line, which due to overexpression of the EGFR and mutational inactivation of PTEN, has high constitutive activation the PI3K/AKT pathway.

Protein abundance for 15 of the siRNA targets was available on the RPPA array to validate knock down (Table 2). Of the 15 targets assessed, all but 3 were significantly decreased by at least one isogenic siRNA (Supplementary Table 3) when the cells were exposed to specific siRNAs, but not by nontargeting siRNA or other siRNA pools assessed. We observed a 60-70% knock down efficiency based on the siRNA-RPPA pairs (10/15) as well

as revalidation studies (13/18), these numbers are in line with previous siRNA screen as reported by other investigators (Berns *et al.*, 2007; MacKeigan *et al.*, 2005). In the case of AKT and GSK3 for which multiple isoforms exist, at least one of the siRNAs knocked down the target. Because the antibodies assessed recognize all AKT and GSK3 isoforms, this likely represents targeting of the dominant isoforms in the MDA MB 468 breast cancer cell line model used in the screen. For example, MDA-MB-468 express higher levels of AKT1 and AKT3 than AKT2 and knock down of only these two isoforms decreased total AKT protein levels (Supplementary Table 3 and Supplementary Figure 1).

Because of the importance of the PI3K/AKT pathway in breast cancer (Brugge *et al.*, 2007; Neve *et al.*, 2006), we determined which siRNAs altered AKT phosphorylation (pAKT) at S<sup>473</sup> and T<sup>308</sup> in serum-starved MDA-MB-468 cells (EGFR overexpressed, PTEN mutant) using a z-score of +/- 1.4 for the average change in both AKT phosphosites for significance (Fig. 1A and Table 1). Thirty-four of the 541 targeted kinase and kinase-associated proteins decreased the abundance of pAKT (including AKT1 and AKT3 siRNAs, green in Supplementary Table 4), whereas 15 increased the abundance of pAKT (red in Supplementary Table 4). We identified five known upstream modifiers of AKT phosphorylation, including receptors, intracellular kinases, and PI3K isoforms, eight downstream effectors, as well as thirty-four genes not previously related to AKT function (Supplementary Table 4, AKT1 and AKT3 are not counted as up or downstream bringing the total to 49). The sequences of these siRNA are shown in Supplementary Table 5.

The fifteen siRNAs that increased pAKT could target negative regulators acting upstream of AKT or potentially components of feedback loops. With PathwayOracle (Ruths *et al.*, 2008a; Ruths *et al.*, 2008b), we reconstructed a directed signaling network based on published literature (PubMed) to identify possible pathways accounting for the increase in pAKT levels. Of the 15 siRNAs that increased the abundance of pAKT, 3 of the siRNA targets were predicted to increase pAKT phosphorylation based on existing literature (Supplementary Table 4). These included siRNAs targeting *MAP2K4*, which encodes a mitogen-activated protein kinase kinase kinase (MAPKKK) upstream of JNK (Song and Lee, 2005), *RPS6KB1*, which encodes p70 ribosomal S6 kinase (p70-S6K) (O'Reilly *et al.*, 2006), and Raf1 which is upstream of MAPK (Buck *et al.*, 2008) which is consistent with the known actions of these proteins with respect to AKT.

To capture the information contained in the data, we implemented a network-based approach probing three protein-protein interaction databases [HPRD (Human Protein Reference Database) ([www.hprd.org](http://www.hprd.org)), BIND (Biomolecular Interaction Database) ([www.bind.ca](http://www.bind.ca)), and TRANSPATH ([www.biobase-international.com](http://www.biobase-international.com)) for connectivity of the siRNA targets and AKT. Using a z-score of +/- 1.4 (assigned based on the change in the shape of the curve) for effects on the average abundance of pAKT S473 (Figure 1B) and pAKT T308 (Figure 1C) phosphosites, the only interconnected genes that when targeted increased the abundance of pAKT are components of the MAPK network. In order to explore the entire data set and not assign any cut-off values we used a random walk network-based approach using NetWalk (Komurov *et al.*). Analysis of the entire data set revealed that the MAPK module was the largest sub-network of genes that increased AKT phosphorylation at T308 and S473 (Fig. 1D and 1E). NetWalk analysis also identified 3 members of the CaMK network as genes

that when targeted increased AKT phosphorylation at S473 (Fig 1E). The two phosphosites act coordinately on AKT activity. Therefore we averaged the data from the two phosphosites of AKT and statistically re-analyzed the data. Strikingly, 6 out of the 15 genes that, when targeted, increased pAKT on both phosphorylation sites and were members of the MAPK signaling network (Supplementary Table 1). Statistical analysis of averaging the two phospho sites revealed a significant enrichment of MAPK pathway genes ( $p < 0.0001$  chi-square test Figure 1F) as compared to all other genes in the siRNA screen. In order to determine if the effect of targeting the MAPK pathway on increasing pAKT was specific we assessed calcium-calmodulin kinase (CaM kinase) pathways ( $p = 0.2$ , chi-square test, Figure 1G) but failed to identify a significant enrichment, supporting the argument that the MAPK and AKT pathways are highly interrelated.

Analysis of the abundance of phosphorylated MAPK1,2 and phosphorylated AKT across all siRNA targets showed a clear negative correlation between AKT and MAPK1,2 phosphorylation (Fig 2A and Supplementary Table 6). Regression analysis of the tails of the AKT and MAPK response curves and their intersections revealed 115 siRNAs that showed a significant inverse correlation coefficient of  $-0.50$  or greater suggesting that the MAPK and AKT pathways are tightly counterbalanced with potentially extensive negative crosstalk. Multiple siRNAs targeting members of the MAPK pathways (MAPK1,2, p38, and JNK) increased AKT phosphorylation (Supplementary Table 1). To validate the siRNA screen, we independently decreased MAPK2 and MEK1 abundance using different siRNA sequences and repeated the experiment in a different breast tumor cell line MDA-MB-231, which contains mutation in genes encoding Ras and Raf thus resulting in constitutive activation of the MAPK1,2 pathway. Because MDA-MB-231 cells have wild type PTEN, lack EGFR amplification and potentially due to the activated MAPK pathway, have no measurable basal AKT phosphorylation. Therefore, we added epidermal growth factor (EGF) to stimulate AKT phosphorylation and then measured the effects of the siRNAs on AKT phosphorylation. When MAPK1 (p42 MAPK – lower band of p42/p44 doublet on the MAPK blot) or MEK1 was knocked down with an independent siRNA, there was an increase in AKT phosphorylation compared to that in cells transfected with control siRNA (Fig. 2B&C). We also validated the effect of siRNAs targeting the MAPK pathway on AKT phosphorylation by applying pharmacological inhibitors of the MAPK pathway. MEK was blocked with two different inhibitors, U2016 and PD98059, in MDA-MB-231 cells, resulting in an increase in AKT phosphorylation in the presence of EGF (Fig. 2D&E). We further confirmed that pharmacological inhibitors of MEK could also increase AKT phosphorylation in the MDA-MB-468 cells and our data shows that treating cells (MDA 468 and MDA 231) with MEK inhibitor increases AKT phosphorylation (Fig. 2F &G). To determine if there was a difference in the phosphorylation of specific AKT isoforms in response to MEK inhibitors we determined the expression of the three AKT isoforms. AKT1 and AKT3 (Supplementary Figure 1) are expressed in both cell lines and both isoforms showed increased levels of phosphorylation in the presence of MEK inhibitor (Fig 2H).

To better understand the functional relationship between the target of the siRNA and AKT phosphorylation we developed a directed network map in PathwayOracle (Ruths *et al.*, 2008a; Ruths *et al.*, 2008b) using published literature of pair wise-interactions from PubMed

searches and *Science Signaling* (<http://stke.sciencemag.org/cm/>). siRNAs that decreased the abundance of proteins which increased AKT phosphorylation are colored in shades of red, whereas siRNAs that decreased the abundance of proteins which decreased AKT phosphorylation are colored in shades of green. (Fig. 3A). The intensity of the color is determined by the corresponding z-scores, and proteins in blue were not assessed with siRNA. siRNA targeting p70S6K and mTOR, which had been previously shown as part of a homeostatic loop (O'Reilly *et al.*, 2006; Wan *et al.*, 2007), increased AKT phosphorylation, thus enhancing confidence in the ability of the siRNA screen to accurately identify feedback loops (Figure 3A and Supplementary Table 1). Previously reported pair-wise interactions show that MAPK1,2 can activate p90RSK, which in turn can phosphorylate TSC2 altering its activity (Ballif *et al.*, 2005; Roux *et al.*, 2004). Additional reports suggest that MAPK1,2 can directly phosphorylate TSC2 (Ma *et al.*, 2005). TSC2 in turn regulates the Rheb-mTOR-S6K feedback loop leading to changes in IRS1 activity, thus altering AKT phosphorylation (Avruch *et al.*, 2006; Kwiatkowski and Manning, 2005; Manning *et al.*, 2005). We reconstructed the network in PathwayOracle, modeled the inhibition of MAPK1,2, and measured the changes in AKT phosphorylation, as well as all members of the network. A signaling petri-net model (Ruths *et al.*, 2008a; Ruths *et al.*, 2008b) predicted that the negative regulatory Rheb-mTOR-S6K loop from MAPK would increase AKT phosphorylation (Fig. 3A) when MEK or MAPK1,2 are inhibited. The model further indicated that knockdown of TSC2 would decrease the effect of inhibition of MEK on AKT phosphorylation.

We experimentally tested the activation state (based on phosphorylation) of MAPK and AKT in response to depletion of TSC2 by siRNA or gene deletion. As predicted, knockdown of TSC2 with siRNA decreased AKT phosphorylation induced by MEK inhibitors (Fig. 3B). In an independent approach to determine whether TSC2 plays a role in connecting the MAPK and AKT pathways, we assessed TSC2-knockout mouse embryonic fibroblasts (MEFs). In the TSC2-knockout cells, there was only a 0.6 fold increase in AKT phosphorylation when MEK was inhibited compared to a 2.5 fold increase in TSC2 wild-type cells (Fig. 3 C). Thus, a TSC2 mediated feedback loop appears to play an important role in connecting MAPK signaling to AKT activity.

Previous studies show a p70S6k-IRS-1 mediated negative feedback loop from mTOR leading to increased AKT phosphorylation (O'Reilly *et al.*, 2006). Recent studies suggest that the MEK/MAPK mediated regulatory feedback loop to AKT may overlap with the mTOR loop and converge on IRS1 (Buck *et al.*, 2008). To examine this further, we determined the effect of concurrent MEK inhibition and mTOR inhibition on AKT phosphorylation and found that there was a slightly additive effect, and that the MEK inhibitor induced increase in AKT phosphorylation is greater than when mTOR is inhibited alone (Fig 3D) suggesting that the MAPK induced loop may be dominant over the mTOR loop. The data shows that rapamycin completely inhibits phospho-p70S6K while inducing the negative feedback loop as previously described (O'Reilly *et al.*, 2006). However, most strikingly MEK inhibition only partially decreases p70S6K phosphorylation yet causes a higher increase in AKT phosphorylation suggesting parallel and overlapping feedback loops to AKT (Fig 3D).

We next analyzed the genes that when targeted by siRNA decreased AKT phosphorylation. Network analysis of the targeted genes above the 1.4 z-score cutoff revealed known upstream regulators of AKT including EGFR (Harari and Yarden, 2000) and PKC (Li *et al.*, 1999) as decreasing pAKT S473 (Fig 4A). The only common interacting genes that when targeted decreased both pAKT S473 and T308 were AKT3 and GSK3 $\alpha$  (Figure 4A and 4B). We performed NetWalk analysis of the averaged data set and found that the only significant network is composed of receptors that are known to activate AKT and the GSK3 $\alpha$  –AKT1,3 subnetwork (Fig 4C). We further examined the relationship between AKT and GSK3 in the siRNA kinome screen and analysis of the abundance of phosphorylated AKT and phosphorylated GSK3 showed a strong positive correlation coefficient of +0.7 between the paired proteins which is expected, based on their known functional relationship. However, based on published literature, GSK3 $\alpha$  is proposed to be downstream of AKT (Manning and Cantley, 2007) and a role as an upstream regulator of pAKT was not expected. Nevertheless, we confirmed that knockdown of GSK3 $\alpha$  using independent siRNA preparations does indeed block phosphorylation of AKT induced by EGF (Fig. 4D). Experiments with GSK3  $\alpha$  or  $\beta$  knockout MEFs validated this connection between AKT and GSK3 $\alpha$  as EGF-induced AKT phosphorylation was markedly decreased in GSK3 $\alpha$ -knockout as compared to wild-type MEFs (Figure 4E). Previous reports show that GSK3 can regulate TSC2 activity (Inoki *et al.*, 2006), therefore we determined if TSC2 is involved in the GSK3 regulation of AKT phosphorylation. Silencing GSK3 in TSC2 null and wild type MEF's decreased phospho AKT (Fig 4F) suggesting that the connection between GSK3 and AKT is independent of TSC2.

## Discussion

The kinase and kinase-associated siRNA screen coupled to a high-throughput functional proteomics platform revealed differences in connectivity of the network of kinases that alter AKT phosphorylation at Thr 473 and Ser 308 (Fig. 1B, C, 4A, B). Identification of the previously known regulatory loops from mTOR and MAPK show that unbiased systematic interrogation of the network is highly valuable in uncovering regulators of network function. Analysis of the two loops suggest regulation of AKT via pathways with differential dependence on p70S6K activity. The screen also identified an anti-correlation between phosphorylation of MAPK1,2 and AKT suggesting that the activities of MAPK and AKT and their corresponding pathways are counterbalanced maintaining homeostasis in the cell. Indeed, this counterbalance may contribute to the coordinate regulation of the AKT and MAPK pathways in cancer. For example, RAS mutations that activate both the MAPK pathway and PI3K/AKT pathway are usually seen in the absence of other mutations in the pathway; whereas mutations in dedicated pathway members, such as RAF and PIK3CA or PTEN are commonly coordinated. This also suggests that targeting the PI3K, MAPK, or mTOR pathways could be counterbalanced by activation of the other pathway and that coordinate inhibition of several pathways will be necessary for optimal therapeutic potential to be manifest. We identified a new functional relationship between AKT and GSK3 $\alpha$  and can now position GSK3 $\alpha$  both downstream and upstream of AKT, such that knockdown of GSK3 $\alpha$  decreases AKT phosphorylation induced by EGF. A recent study used a similar siRNA knockdown approach with an assessment of only three proteins by RPPA: pERK,



pSTAT3 and actin and identified a number of unexpected links between the EGFR and pERK (Komurov *et al.*, 2010). Thus a systems approach combining knockdown of targets by siRNA with high-throughput quantitative analysis of functional proteomics can increase our understanding of the signaling networks and particularly cross talk present in cells and may greatly aid in the development and implementation of targeted therapeutics.

## Materials and Methods

### siRNA library screen by RPPAs

siRNA library targeting 541 kinases and kinase-related genes was designed by Dharmacon Research, Inc., using their proprietary algorithm whereby each mRNA is targeted by a pool of siRNAs consisting of a combination of four siRNA duplexes directed at different regions of the gene. This siRNA library has been previously characterized extensively by us (Lin *et al.*, 2007; Morgan-Lappe *et al.*, 2006; Morgan-Lappe *et al.*, 2007; Sarthy *et al.*, 2007; Tahir *et al.*, 2007; Zheng *et al.*, 2008). The breast cancer cell line MDA-MB-468 (mutant PTEN, RB1, TP53 and SMAD4 and over expressing EGFR) was seeded in 96-well plates at the density of 10,000 cells per well. Cells were transfected with 100 nM of the pool of siRNA consisting of 25nM of each siRNA targeting each gene in each well by Mirus TransIT TKO reagent. Triplicate plates were performed with each plate containing the same set of controls including mock transfection, siRNA targeting PLK (polo like kinase) as a positive control, scrambled siRNA as a negative control, siTOX as a marker for siRNA delivery, and staurosporin as a broad kinase inhibitor. Forty-eight hours after siRNA transfection, cells were serum starved overnight. Toxicity and cell death after siRNA transfection were measured using the Toxilight assay (Cambrex Corporation) according to the manufacturer's instructions, and siRNAs with marked toxicity were removed from the analysis. Cells were either unstimulated or stimulated with EGF (20 ng/ml) for 10 minutes prior to washing in PBS, then were lysed in 1% Triton X-100, 50 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 100 mM NaF, 10 mM Na pyrophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10% glycerol, containing freshly added protease and phosphatase inhibitors. Cellular proteins were denatured by 1% SDS (with β-mercaptoethanol) and diluted in six 2-fold serial dilutions in dilution buffer (lysis buffer containing 1% SDS). Serial diluted lysates were arrayed on nitrocellulose-coated FAST slides (Whatman, Inc) by G3 Arrayer (Genomic Solutions). Total 960 array spots were arranged on each slide including 96 spots corresponding to positive and negative controls prepared from mixed cell lysates or dilution buffer, respectively. Each slide was probed with a validated primary antibody plus a biotin-conjugated secondary antibody. The signal was amplified using a DakoCytomation-catalyzed system (Dako) and visualized by DAB colorimetric reaction. The slides were scanned, analyzed, and quantified using a customized-software Microvigene (VigeneTech Inc.) to generate spot intensity. Each dilution curve was fitted with the logistic model ("Supercurve Fitting" developed by the Department of Bioinformatics and Computational Biology in MD Anderson Cancer Center, "<http://bioinformatics.mdanderson.org/OOMPA>"). The program fits a single curve using all the samples (that are in the dilution series) on a slide with the signal intensity as the response variable and the dilution steps as the independent variable. The protein concentrations of each set of slides were then normalized by median polish, which was corrected across samples by the linear expression values using

the median expression levels from all antibody experiments to calculate a loading correction factor for each sample.

### Detection of AKT phosphorylation in MEF cell lines by RPPA

MEF cell lines differentially expressing wild-type GSK3  $\alpha$  and  $\beta$  isoforms, GSK3 $\beta^{-/-}$ , or GSK3 $\alpha^{-/-}$  were kindly provided by Dr. Jim Woodgett (University of Toronto). Cells were seeded in 12-well plates at 100,000 cells per well. Cells were serum starved overnight prior to stimulation with IGF at 75 ng/ml for 10, 30, 60, 120, and 240 minutes. Unstimulated cells were used as a control and designated as “0” minute. Triplicates were performed for each treatment. Cells were lysed and the lysates were denatured by 1% SDS as described above. Cellular proteins were serially diluted for five 2-fold dilutions and arrayed on nitrocellulose-coated Fast Slide by Aushon 2470 Arrayer (Aushon Biosystems). The slide was probed by anti-phospho-AKT (S473) and the data was analyzed as described above. The intensity of AKT phosphorylation was presented in Bar-Graph as the average plus standard deviation from the triplicates of each treatment.

### Antibody validation for reverse phase protein arrays

Antibodies with a single or dominant band on Western blotting are further assessed by direct comparison to RPPA using cell lines with differential protein expression or modulated with ligands or inhibitors or siRNA for phosphorylated or structural proteins, respectively. Only antibodies with a Pearson correlation coefficient between RPPA and Western blotting of greater than 0.7 were used in RPPA study. Further, these antibodies are assessed for specificity and quantification using phosphopeptides and nonphosphorylated peptides arrayed on nitrocellulose-coated slides (Figure S3). Where is this figure. No response to the comment.

### Cell culture and stimulation

Human MDA-MB-231 breast cancer cells were maintained in RPMI supplemented with 10% FBS. Cells were serum starved for 16 hours and then subjected to treatments using U0126 (MEK inhibitor) (10  $\mu$ M) (Promega, Madison, WI), EGF (20 ng/mL) (Cell Signaling Technology, Beverly, MA), or both. MDA-MB-231 cells were transfected with 20 nM smartpool siRNA for 72 hours and stimulated with EGF (20 ng/ml) for 10 minutes. Cells were lysed and probed for phosphorylated AKT. For cells exposed to both reagents, the cells were pretreated with U0126 for 1 hour followed by EGF stimulation for 10 minutes. Controls were incubated for corresponding times with DMSO. For some experiments, cells were treated with siRNA for 72 hours prior to treatment to knockdown TSC2 or MEK (Dharmacon, Lafayette, CO).

### SDS-PAGE and immunoblotting

Cells were lysed by incubation on ice for 15 minutes in a sample lysis buffer (50 mM Hepes, 150 mM NaCl, 1 mM EGTA, 10 mM Sodium pyrophosphate, pH 7.4, 100 nM NaF, 1.5 mM MgCl<sub>2</sub>, 10% glycerol, 1% Triton X-100 plus protease inhibitors; aprotinin, bestatin, leupeptin, E-64, and pepstatin A). Cell lysates were centrifuged at 15,000 g for 20 minutes at 4°C. The supernatant was frozen and stored at -20°C. Protein concentrations were



determined using a protein-assay system (BCA, Bio-Rad, Hercules, CA), with BSA as a standard. For immunoblotting, proteins (25 µg) were separated by SDS-PAGE and transferred to Hybond-C membrane (GE Healthcare, Piscataway, NJ). Blots were blocked for 60 minutes and incubated with primary antibodies overnight, followed by goat antibody against mouse IgG-HRP (1:30,000; Cell Signaling Technology, Boston, MA) or goat antibody against rabbit IgG-HRP (1:10,000; Cell Signaling Technology, Boston, MA) for 1 hour. Secondary antibodies were detected by enhanced chemiluminescence (ECL) reagent (GE Healthcare, Piscataway, NJ). All experiments were repeated a minimum of three independent times. The following antibodies were used for immunoblotting: antibody against phosphorylated p44 and p42 MAPKs, antibody against phosphorylated GSK3 $\alpha/\beta$  (residues Ser<sup>21</sup> and Ser<sup>9</sup>); antibody against phosphorylated AKT (residue Ser<sup>473</sup>); antibody against phosphorylated TSC2 (residue Thr<sup>1462</sup>); antibody against phosphorylated mTOR (residue Ser<sup>2448</sup>); antibody against phosphorylated P70S6K (residue Thr<sup>389</sup>) (Cell Signaling Technology, Boston, MA); and antibody against  $\beta$ -actin (Sigma-Aldrich, St. Louis, MO).

### PathwayOracle network building

PathwayOracle (Ruths *et al.*, 2008a; Ruths *et al.*, 2008b), was used to overlay the measured AKT phosphorylation on the network connectivity map derived from Science Signaling database of connections (<http://stke.sciencemag.org/cm/>) as well as from the network by Ma'ayan et al (Ma'ayan *et al.*, 2005).

### NetWalk analysis

We performed NetWalk analysis (Komurov *et al.*) to identify networks of genes that when targeted by siRNA increased or decreased AKT phosphorylation in a co-ordinated manner. The algorithm uses data biased random walks on graphs to identify interactions that are more significant than others as determined by both the data values as well connectivity. The algorithm has recently been published and is explained in detail in our recent paper (Komurov *et al.*, 2010).

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgements

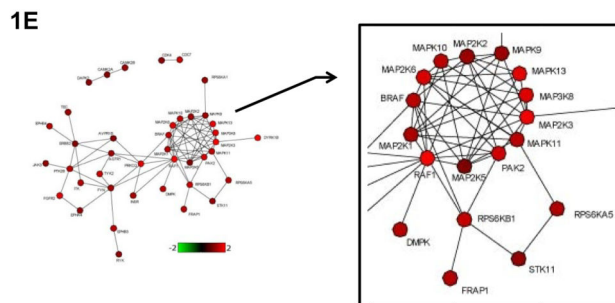
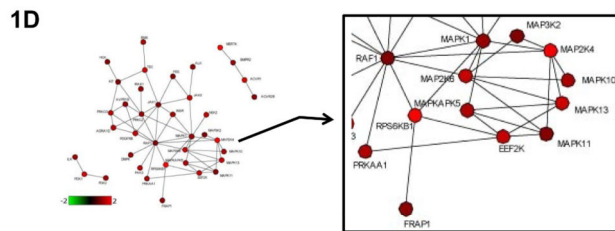
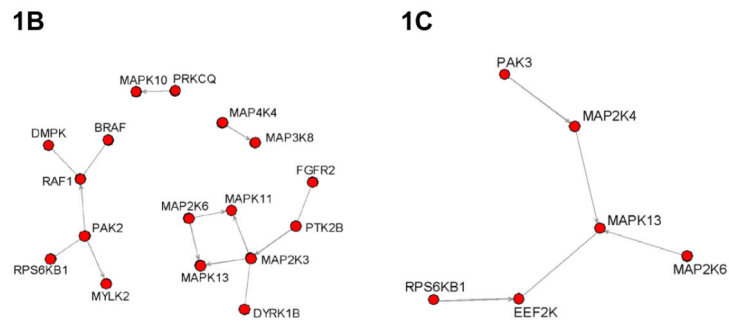
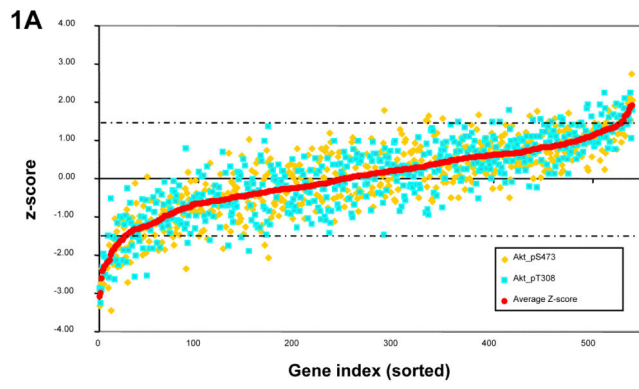
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**1F**

$p < 0.00001$	MAPK Pathway	Non-MAPK Pathway	total
Positively Significant	6	9	15
Non-significant & Negatively Significant	36	490	526
total	42	499	541

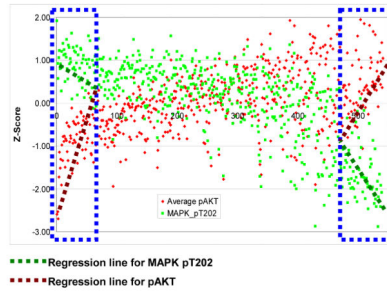
**1G**

$p = 0.2$	CaM-kinase	Non-CaM Kinase Pathway	total
Positively Significant	1	14	15
Non-significant & Negatively Significant	9	517	526
total	10	531	541

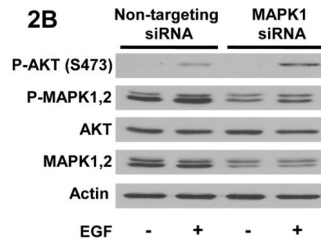
**Figure 1.**

(A) Scatter plot of changes in phospho Ser473 (yellow), Thr308 (blue), and average of the two phosphor sites (red) of AKT in response to siRNA. The siRNA are plotted individually on the x-axis and the z-score of Akt phosphorylation is plotted on the y-axis. (B) Network of genes that when targeted by siRNA increased phospho Ser473 AKT above 1.7 z-score. (C) Network of genes that when targeted by siRNA increased phospho Thr308 AKT above 1.7 z-score. (D) The most high scoring networks from the NetWalk analysis of the genes that when targeted by siRNA increased Ser473 AKT phosphorylation. (E) The most high scoring networks from the NetWalk analysis of the genes that when targeted by siRNA increased Thr308 AKT phosphorylation (F) Enrichment of MAPK genes in the total set of siRNA targets. Statistical analysis of all MAPK family genes targeted in the siRNA screen and their effect on the abundance of phosphorylated AKT shows a significant role of MAPK pathway in regulating AKT. (G) Targeting casein kinase A1 (CSNK1A1 in 2A) produced the fourth highest increase in AKT phosphorylation, however statistical analysis of the casein kinase pathway in the screen showed no significant increase in AKT phosphorylation.

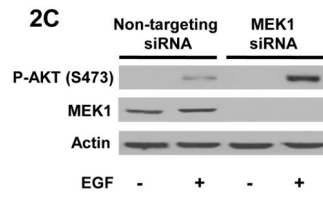
2A



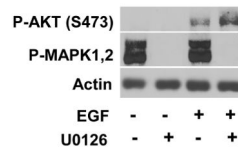
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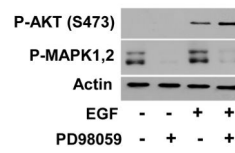
2C



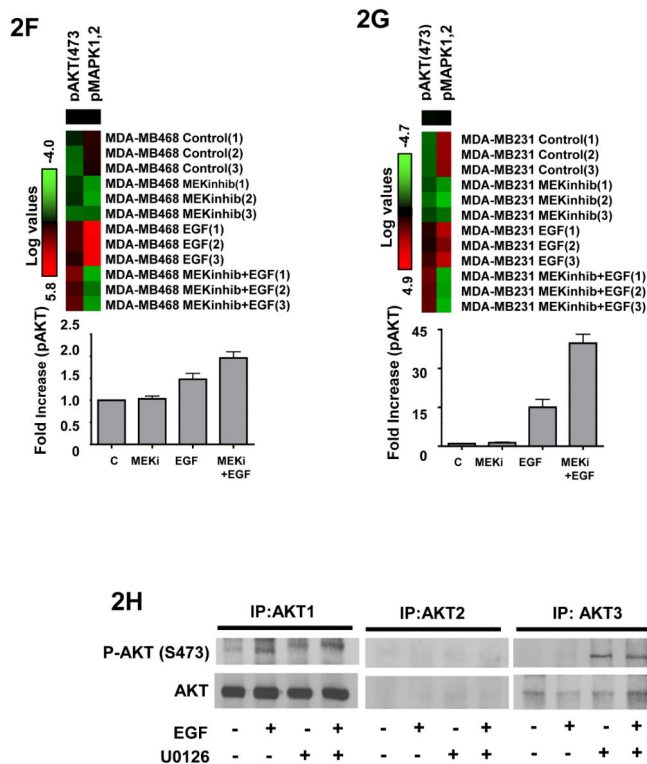
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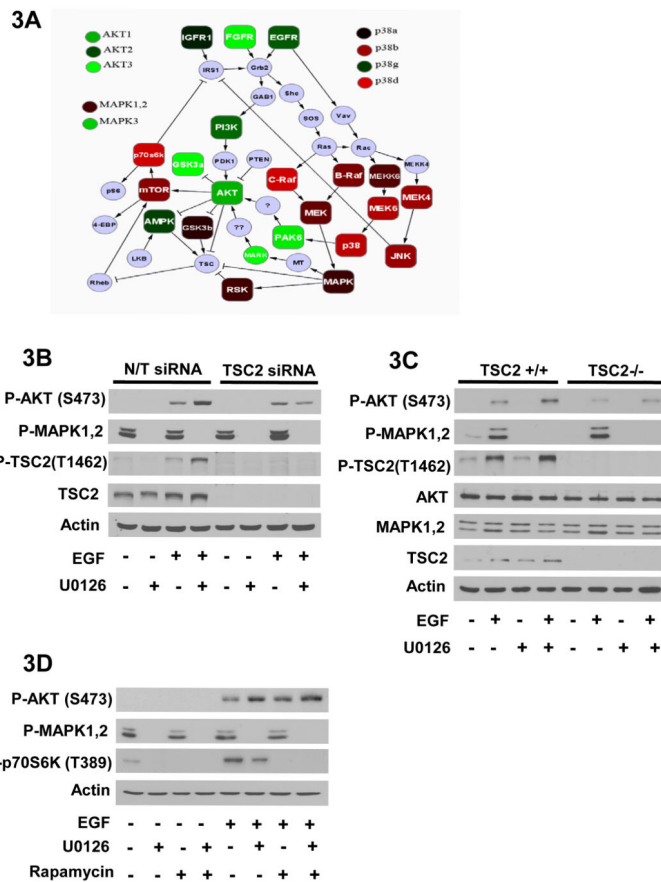
2E





**Figure 2.**

(A) AKT and MAPK phosphorylation are inversely correlated. Regression analysis of AKT and MAPK phosphorylation in response to the same siRNA reveals that 115 siRNA have significant inverse correlation ( $r = -0.5$ ) in their effect on the phosphorylation of AKT and MAPK (see table S5 for list of siRNA targets and their alteration of MAPK1,2 phosphorylation). (B) siRNA targeting of MAPK1 in an independent cell line (MDA-MD-231) shows increase in phosphorylation of Thr473 of AKT when MAPK1 is knocked down. (C) siRNA targeting of MEK1, which is upstream of MAPK, in MDA-MB-231 cells also increases AKT phosphorylation when MEK1 is knocked out. (D & E) Inhibition of MEK with different MEK inhibitors increases AKT phosphorylation. MDA-MB-231 cells were serum starved and incubated with MEK inhibitor U0126 (D) or PD98459 (10  $\mu$ M) (E) for 1 hour prior to stimulation with EGF (20 ng/ml) for 10 minutes. Cell lysates were probed for phosphorylated AKT. (F and G) MDA-MB-468 (F) and MDA-MB-231 (G) cells were serum starved and incubated with MEK inhibitor for 1 hour prior to stimulation with EGF (20 ng/ml) for 10 minutes, cell lysates were probed on the RPPA (heat map of data) and graphs show changes in pAKT in response to conditions as indicated. (H) Immunoprecipitation of individual AKT isoforms shows that AKT1 and AKT3 both show increased phosphorylation in response to EGF and MEK inhibition.



**Figure 3.** (A) Network model of putative regulatory loops involving AKT. Directed network of the siRNA targets, their interactions, and their effect on AKT phosphorylation was developed in PathwayOracle. Nodes in red are those that when targeted increased AKT phosphorylation, nodes in green are those that when targeted produced a decrease in AKT phosphorylation, and nodes in blue were not targeted in the siRNA screen. Shades of red and green indicate z-scores of AKT phosphorylation (See table S3 for corresponding values). (B) TSC2 knockdown in MDA MB-231 cells blocks the increase in AKT phosphorylation induced by inhibition of the MAPK pathway. MDA-MB-231 cells were transfected with smartpool TSC2 siRNA for 72 hours, followed by MEK inhibitor U0126 for 1 hour and stimulated with EGF for 10 minutes as indicated. Cell lysates were probed for phosphoproteins as indicated. (C) TSC2<sup>-/-</sup> mouse embryonic fibroblasts have a blunted increase in AKT phosphorylation when the MAPK pathway is inhibited. MEFs from TSC2 wildtype (TSC2<sup>+/+</sup>) and TSC2 knockout (TSC2<sup>-/-</sup>) animals were serum starved, incubated with MEK inhibitor U0126 for 1 hour, and stimulated with EGF for 10 minutes. Cell lysates were probed for phosphorylated AKT and other kinases as indicated. (D) MDA-MB-231 cells were treated with either MEK inhibitor (U0126), mTOR inhibitor (rapamycin) or both as indicated. The cells were stimulated with EGF and lysates probed for phospho AKT, MAPK1,2 and p70S6K. MEK inhibition only partially decreases p70S6K (lane 6) as

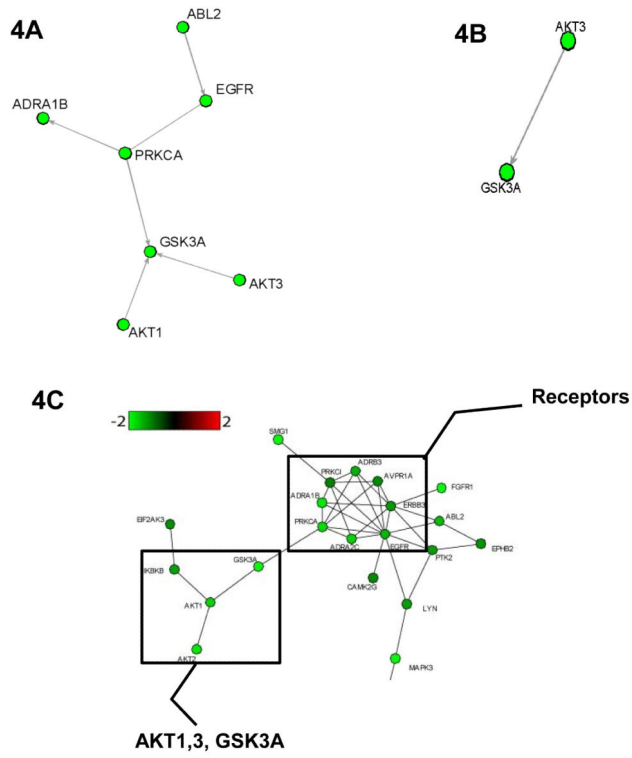
compared to rapamycin (lane 7) but the increase in pAKT is greater in lane 6 compared to lane 7.

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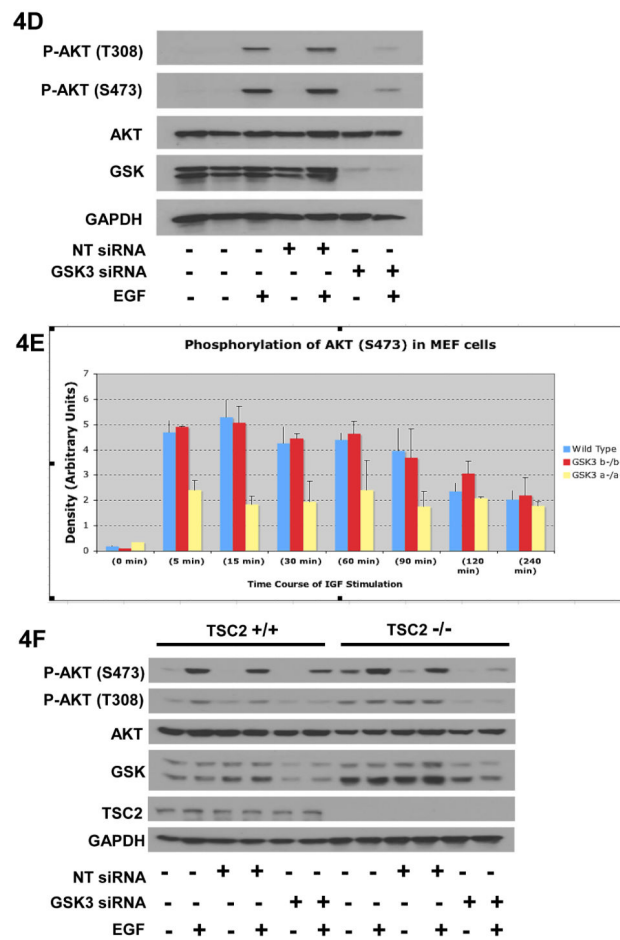


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**Figure 4.**

(A) Network of genes that when targeted by siRNA decreased phospho Ser473 AKT below  $-1.7$  z-score (B) Only 2 known interacting genes were found of the genes that when targeted by siRNA decreased phospho Thr308 AKT below  $-1.7$  z-score (C) The most low scoring network from the NetWalk analysis of the genes that when targeted by siRNA decreased Ser473 and Thr308 AKT phosphorylation. (D) GSK3 knockdown in MDA-MB-231 cells inhibits AKT phosphorylation. GSK3 was knocked down in MDA-MB-231 cells using smartpool siRNA. Cells were stimulated with EGF for 10 minutes and the lysates probed for phosphorylated AKT as indicated. (E) GSK3 $\alpha$   $-/-$  mouse embryonic fibroblasts show a blunted AKT response. MEFs from GSK3 $\alpha$ -deficient (GSK3 $\alpha$ -/ $\alpha$ -), GSK3 $\beta$ -deficient (GSK3 $\beta$ -/ $\beta$ -), or wild-type animals were serum starved and stimulated with IGF for different lengths of time. Cell lysates were probed for phosphorylated AKT showing the average and standard deviation of 3 independent experiments. (F) GSK3 $\alpha/\beta$  was silenced in wild type and TSC2 knockout MEF cells. The cells were stimulated with EGF for 10 minutes and the lysates probed for phosphorylated AKT, total AKT, TSC2, and GSK3 as indicated.

**Table 1**

Number of siRNA targets in primary screen	541
Number of pooled siRNA targeting each gene	4
Number of conditions per siRNA	2
Number of repeats per siRNA	3
Number of antibodies in RPPA	42
Number of dilutions per sample on RPPA	5
Total number of assayed points in siRNA-RPPA screen	~682,500
Number of gene targets re-tested	18
Number of cell lines re-tested	5
Number of different conditions per cell line tested	12
Number of phospho and total proteins assayed in each cell line	6-10
Total number of data points assayed to validate primary screen	~2,600

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**Table 2**

Number of siRNA in screen	541
Number of siRNA targets assayed for protein expression by RPPA in screen	15
Number (percent) of successful knockdowns in screen (measured by RPPA)	10/15 (67%)
Number of genes targeted that decreased pAKT in screen (z-score < -1.4)	34
Number of gene targeted that increased pAKT in screen (z-score >1.4)	15
Number of gene targets re-validated using different siRNA sequences	18
Number (percent) of genes that re-confirmed knockdown with different siRNA	13/18 (72%)
Number (percent) of genes targeted that reconfirmed change in pAKT with different siRNA	10/13 (77%)
Number (percent) of genes targeted that showed no change in pAKT with different siRNA	2/13 (15%)
Number (percent) of genes targeted that showed opposite change in pAKT with different siRNA	1/13 (8%)

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