We describe microwestern arrays, which enable quantitative, sensitive and high-throughput assessment of protein abundance and modifications after electrophoretic separation of microarrayed cell lysates. This method allowed us to measure 91 phosphosites on 67 proteins at six time points after stimulation with five epidermal growth factor (EGF) concentrations in A431 human carcinoma cells. We inferred the connectivities among 15 phosphorylation sites in 10 receptor tyrosine kinases (RTKs) and two sites from Src kinase using Bayesian network modeling and two mutual information-based methods; the three inference methods yielded substantial agreement on the network topology. These results imply multiple distinct RTK coactivation mechanisms and support the notion that small amounts of experimental data collected from phenotypically diverse network states may enable network inference.

Systems-level understanding of protein functions in biological processes remains a challenge. The western blot is a powerful protein analysis method because the electrophoretic separation step allows for reduction in sample complexity, and the antibody detection step then results in signal amplitude proportional to the abundance of the immobilized antigen at a physical location on the detection membrane that can be related to molecular size standards. Because western blots require a relatively large amount of sample and a great deal of human labor, they have been of limited utility in large-scale protein studies. Reverse-phase lysate arrays (RPAs), performed by arraying lysates directly on nitrocellulose-coated slides and probing them with antibodies, are useful for quantifying large numbers of proteins from limited amounts of material such as in biomarker discovery. In contrast to western blots, however, RPAs lack confirmatory data for signal veracity; in a side-by-side comparison of measurements from RPAs and western blots, only 4 of 34 phospho-specific antibodies examined had generated equivalent information. The authors of the study had concluded that antibody cross-reactivity contributed substantial noise to RPAs, confounding true protein measurements. Many antibodies have been validated for use with the Luminex xMAP bead-sorting system, but this approach requires ~1,000-fold more cell material per protein analysis than RPAs, and the cost of detection reagents per protein is ~30-fold greater. Flow cytometry permits a (relatively small) cohort of proteins to be examined simultaneously in individual cells; this multiplexing feature has been exploited with Bayesian network modeling to predict new signaling network causalities.

In contrast to antibody-based methods, mass spectrometry can be used to identify new proteins. Using mass spectrometry, thousands of peptides have been assessed in lung cancers to identify commonly activated receptor tyrosine kinases and downstream signaling pathways. Relative abundances can be examined quantitatively using isotopic labels across time points, cell types or perturbations as in examination of phosphorylation dynamics of HeLa and mammary epithelial cells after epidermal growth factor (EGF) or heregulin treatment. However, the large sample amount required by mass spectrometry can limit the number of conditions that can be analyzed; ~10^8 cells are typically required for a mass spectrometry experiment versus ~10^5 cells for an immunoblot or ~10^3 cells for RPAs.

Here we describe microwestern arrays (MWA), which combine the scalability of RPAs and retain vital attributes of western blots for highly multiplexed proteomic measurements: reduction of sample complexity and signals that can be related to protein size standards. In combination with suitable pan- and modification-specific antibodies, dynamics of protein abundance and modification may be simultaneously monitored across many samples. We demonstrate that MWA in combination with computational modeling techniques can yield useful systems-level biological insights for EGF receptor (EGFR) signaling dynamics.

RESULTS Fabrication of MWAs
Our strategy (Fig. 1) allows us to compare protein abundances and differences in post-translational modifications for cells stimulated under different conditions. To interface the microscopic western blots with microtiter-based liquid handling methods, we printed cell lysates via a noncontact microarrayer on gels in 96 identical blocks with dimensions of a 96-well plate. Using these dimensions, 6 different lysates may be examined with 96 different antibodies.
or 24 different lysates may be examined with 24 different antibodies. To increase the migration rate of large proteins and slow the rate of smaller ones, we used an acetate running buffer, obviating the need for a stacking gel. For each spot, 6 nl of sample was arrayed over the same gel position ten times, allowing for greater spotting density and signal than microdepositing the entire 60 nl in a single dispense. We arrayed one spot of size standard and six spots of experimental sample at 1 mm pitch at the top edge of each block. After printing, we subjected the samples to semidry electrophoresis and transfer of a single band of the LI-COR ladder across the area that was quantified. Orange circles depicted to the left of gels directed to indicated proteins and detected with goat anti-rabbit Alexa Fluor 680-labeled secondary antibody. Arrows to the left of gels point to the spot that was quantified. Orange circles depicted to the left of gels indicate positions of protein molecular weight standards. Numbers to the left of arrows indicate known sizes of the proteins in the Odyssey protein standard adjacent to the quantified spots.

Validation of MWA method
We compared the resolution and linearity in signal of MWAs with macroscopic gels using the Odyssey labeled protein molecular weight standard (LI-COR) (Fig. 2a). For proteins of 150, 50 and 25 kDa, the intensity of each ladder spot was proportional to the fold dilution over two orders of magnitude for both methods (Fig. 2b, c). The coefficient of variation from arraying, rehydration and transfer of a single band of the LI-COR ladder across the area of the membrane was <9%.

We then tested the linearity of signal response in quantifying proteins from A431 human carcinoma cell lysates using a two-stage fluorescence immunodetection system (Fig. 2d, e). We used five phospho- and two pan-specific antibodies to analyze 15–175 kDa proteins in EGF-stimulated A431 cells lysates. All MWAs showed a linear relationship between relative antigen concentration and signal intensity over their detectable range (100- to 1,000-fold). Assuming an expression level of 1.2 × 10^6 receptors per A431 cell, EGFR was detectable down to one cell equivalent (~2 attomoles; ~340 femtograms). We assumed linearity for all further analyses.

Comparison of macrowestern blots and MWAs
To compare performance of MWAs with macrowestern blots for monitoring phosphorylation dynamics, we selected a representative test set of 11 antibodies. Four had been previously shown to generate equivalent quantitative data by RPAs and western blots; another four had been shown to result in substantial compression of dynamic range by RPAs owing to antibody cross-reactivity.

Measurements we obtained by MWAs were similar to those obtained by macrowestern blots for all antibodies (Fig. 3) and did not display the dynamic range compression observed for

Figure 1 | Microwestern array (MWA) method. Schematic of the procedure.

Figure 2 | MWA validation of linear response. (a) Traditional 10% SDS-PAGE of 5 µl aliquots (left) and MWA of 60 nl (right) of twofold serial dilutions of the Odyssey protein ladder. (b, c) Median net signal intensities quantified for the indicated bands of the Odyssey protein ladder in the traditional western blot (b) and MWA (c) in a. (d) MWA analysis of twofold serial dilutions of lysates from A431 cells stimulated for 5 min with 200 ng ml⁻¹ EGF and probed with seven rabbit primary antibodies directed to indicated proteins and detected with goat anti-rabbit Alexa Fluor 680-labeled secondary antibody. Arrows to the left of gels point to the spot that was quantified. Orange circles depicted to the left of gels indicate positions of protein molecular weight standards. Numbers to the left of arrows indicate known sizes of the proteins in the Odyssey protein standard adjacent to the quantified spots. (e) Median net signal intensity of each band versus relative concentration from the gels in d.
RPAs. For many protein phosphosites, including EGFR, IRS1 and AKT, we observed bands at the predicted size as well as at additional sizes that could obscure quantitative measurements by RPAs. The precision in estimating sizes of proteins >100 kDa by MWAs was ±10 kDa, and for smaller proteins ±5 kDa. Although we could determine protein sizes with precision approaching that of a standard western blot, proteins were not completely resolved unless they differed by more than the following: 75 kDa for >200 kDa proteins; 50 kDa for 100–200 kDa proteins; 25 kDa for 50–100 kDa proteins; and 10 kDa for <50 kDa proteins, corresponding to a migration distance of about 1.5 mm, twice the diameter of spotted protein (Figs. 2d and 3). Resolution equal to a macrowestern blot could be obtained by electrophoresing the samples for ~1.5 times the distance (Fig. 2a).

Application of MWAs to analysis of EGFR signaling network
To examine EGF signaling dynamics using MWAs, we chose antibodies to a wide range of phosphosites to monitor many molecular biological processes (Supplementary Fig. 1 and Supplementary Table 1): early positive growth factor response regulators, negative signaling regulators, downstream proliferation indicators, nutrition-sensing indicators, adhesion and migration indicators, phospholipid and calcium-state indicators, stress indicators, and transcription and cell-cycle indicators.

To observe signaling dynamics at doses approximating physiological levels, we stimulated cells with 2, 50, 100 and 200 ng ml⁻¹ EGF. We performed a mock stimulation to distinguish EGF-mediated signaling events from nutrition-related events. We probed all wells with a combination of rabbit and mouse antibodies to observe temporal dynamics in phosphorylation and control for variation in loading (Fig. 4 and Supplementary Table 2). The coefficient of variation from arraying, rehydration, transfer, binding of primary antibody and secondary antibody was <17%.

We quantified 91 phosphosites from 67 proteins and 18 pan-specific protein abundances. We analyzed a total of 75 proteins in technical triplicate replicates resulting in ~9,800 signaling observations. Sufficient lysates remained for many subsequent analyses. We recorded integrated intensity, signal-to-background ratio and inferred sizes from spots detected with each antibody (Supplementary Table 1 and Supplementary Figs. 2, 3). Seventeen of 91 phosphosites that we quantified here had been previously quantified in one recent mass spectrometry report using pan-phospho enrichment and 22 phosphosites had been quantified in another study using phosphotyrosine-specific enrichment (Supplementary Table 3). Many ubiquitous EGFR signaling proteins that we quantified by MWAs, including Tyr845 phosphorylation on EGFR (p-EGFR(Tyr845)), p-SHP2(Tyr542), p-p70S6K(Ser380), p-Raf-(c)(Ser338), p-p90RSK (Ser380) and p-Stat3(Ser727), had not been quantified in either mass spectrometry study suggesting that mass spectrometry detects only a fraction of phosphorylation events elicited by EGF. Of the 91 phosphosites that we quantified here, only four had been quantitatively measured in an equivalent manner as western blots by others using the RPA method.
Comparison of signaling network at different EGF input levels

We next asked whether biological insights could be revealed using the MWA method. We organized five clusters of signaling profiles based on the time after stimulation at which maximal phosphorylation occurred (Fig. 5). Phosphosites within clusters were rank-ordered by fold-change. At the 2 ng ml\(^{-1}\) EGF input level, we observed several phosphosites from EGFR, ErbB2, PLCγ, Gab1, Mek, p90RSK, p70S6K and Crkl that were absent upon mock treatment (Fig. 5 and Supplementary Figs. 4, 5).

Conversely, many phosphosites related to phosphoinositide signaling displayed substantial fold change in mock stimulation but not EGF treatment, including sites from PDK1 and its downstream targets AKT, PKCy and PKCδ; downstream targets of AKT including mTOR and FOXO1; and mTor substrate p70S6K and its downstream target S6 ribosomal protein. We speculate that activation of PLCγ after EGF stimulation led to hydrolysis of phosphatidylinositol 4,5-bisphosphate, causing downregulation of PDK1 and AKT. Reduced AKT activity could produce the observed A431 cell-cycle inhibition through decreased phosphorylation (and therefore increased inhibitory activity) of cyclin-dependent kinase (CDK) inhibitors, including CDK61A(Thr145) and CDK1B1(Thr157). Consistent with this notion, insulin-like growth factor (IGF), which stimulates PI3K and AKT, is also a potent mitogen for A431 cells.

We then asked how the dynamic range and timing of the EGF signaling network were influenced by EGF input amount. The first ‘wave’ of phosphorylation peaking at 1 min after EGF input included 33 tyrosines from EGFR and other receptor tyrosine kinases (RTKs) and membrane-localized proteins (Fig. 5, Supplementary Figs. 3–5 and Supplementary Table 1). At 5 min after EGF input, we observed serine and threonine sites from downstream kinases and transcription factors including Raf, MEK, p70S6 kinase, mTor and ATF2. At 15 min after EGF input, we observed phosphosites from Erk, P38 MAPK and cell cycle–related kinases and substrates. Sites with phosphorylation peaking at 30 min included those of the Crkl adaptor protein and MAPKAPK2, a substrate of P38 MAPK. Proteins with sites peaking at 60 min included the PDK1 substrates AKT and PKCδ, and the AKT substrate 4EBP1, among others. The timing of most phosphorylation events was not affected by EGF concentrations.

Bayesian network modeling of receptor layer connectivity

To elucidate the directional influences among phosphosites, we applied Bayesian network modeling approaches to phosphosites from proteins representing cell membrane-level influences of the EGF signaling network. This permitted us to verify known influences and identify new directional relationships underlying receptor-level cross-talk. Bayesian networks are graphical representations of conditional independencies in a probability distribution over a set of variables and can potentially be inferred from experimental data such as those generated by MWAs. The network we analyzed comprised 17 phosphosites: two from the Src kinase and 15 from the ten RTKs for which we specifically observed fold-change measurements with all four EGF treatments and for which the basic local alignment search tool (BLAST) predicted little similarity with the 57 other human-genome-encoded RTKs and thus indicated a relatively low probability of antibody cross-reactivity (Fig. 6 and Supplementary Table 4). We considered each time point as an independent sample of the EGF-stimulated network state, giving 20 samples for each phosphosite (4 conditions across 5 nonzero time points of one biological replicate), and we normalized all data to the zero time point.

Given typically limited amounts of data, a variety of graph structures can be generated by Bayesian inference modeling that describe the data reasonably well, so a consensus model is often sought rather than aiming to find a unique best-scoring graph. Accordingly, we created a consensus model (Fig. 6) containing only edges with a score > 0.3, derived from exact Bayesian network
model averaging over all directed acyclic graph structures having at most three parents per node\textsuperscript{16,17}. By considering only those directed acyclic graph (DAG) structures in the equivalence class of the consensus model with a directed edge from p-SRC(Tyr416) to p-EGFR(Tyr845), we determined directionality of the remaining compelled edges\textsuperscript{18} (Supplementary Note 1). Signs of directional influences (positive versus negative) could also be discerned.

EGFR(Tyr845) is a known Src kinase substrate that is not phosphorylated by the EGFR kinase\textsuperscript{19}. We used this prior knowledge only to distinguish edge directionality in the equivalence class; we used no prior structural knowledge to derive the consensus model.

The three linked root nodes from which we derived most downstream influences in the graph structure included p-SRC(Tyr416), p-EGFR(Tyr845) and p-PDGFRB(Tyr1009). The model suggests that the EGFR and PDGFR\textsubscript{A,B} influence one another, with p-EGFR(Tyr1068), p-EGFR(Tyr1173), p-ERBB4(Tyr1284) and both p-FGFR1(Tyr653, Tyr654) activation loop isoforms, was distinct among EGFR sites in displaying maximal phosphorylation at 5 min and sustained phosphorylation amplitude for the duration of the time course. The edge directed from p-EGFR(Tyr1068) to p-FGFR1(Tyr653, Tyr654) (145 kDa) displayed a relatively high edge score (0.80; see Supplementary Fig. 7 for all consensus Bayesian network edge weights), similar to that between p-EGFR(Tyr1068) and p-EGFR(Tyr1173) (edge score of 0.89), suggesting that EGFR can mediate FGFR1 activation. We speculate that the 145 kDa and 100 kDa forms of FGFR1 represent hyper- and hypoglycosylated forms of the receptor, respectively. Hyperglycosylation of FGFR1 has been shown to inhibit its interaction with both FGF2 and heparin-derived oligosaccharides\textsuperscript{21}, which has been predicted to decrease its activity. Our model depicted only the 100 kDa form phosphosite to have downstream targets among the 17 phosphosites modeled. The only site negatively regulated in the model was p-PDGFRA(Tyr754), which recruits the SHP2 phosphatase\textsuperscript{22}.
resulting in dephosphorylation of RASGAP recruitment sites on PDGFRA and B and increased MAPK signaling. Therefore down-regulation of p-PDGFR(A/Tyr754) would be predicted to decrease MAPK signaling. Consistent with previous reports\(^2\), our model suggested that p-SRC(Tyr527), a known inhibitory site of Src kinase, is disconnected from the EGF network.

To corroborate the Bayesian network results, we also inferred network connectivities using the ‘algorithm for the reconstruction of accurate cellular networks’ (ARACNe) and ‘context likelihood of relatedness’ (CLR) algorithm\(^2\),\(^4\),\(^5\). ARACNe and/or CLR also identified 22 of 24 edges in the Bayesian network, though as undirected edges because these latter methods are based on mutual information notions (Fig. 6b\,c and Supplementary Figs. 8,9). Experimental evidence suggests that consistency across network inference methods improves edge prediction accuracy\(^2\),\(^4\),\(^5\), and in our case here, data permutation studies showed that the topologies inferred by the Bayesian network, ARACNe and CLR were significant ($P < 0.01$) (Supplementary Note 1 and Supplementary Fig. 10). Because in the context of proteomic signaling networks it is problematic to make broad assumptions about edge directionality absent extensive prior knowledge (for example, concerning particular kinase-substrate relationships), we believe that predicting edge directionality using methods such as Bayesian network modeling offers an appealing advantage.

Figure 6 | Consensus model of EGF receptor level influences modeled by Bayesian network inference with comparison to ARACNe and CLR. (a) A consensus model of the EGF signaling network obtained by exact Bayesian model averaging following Bayesian network inference. Significant ($P < 0.001$) positive edges (green), significant ($P < 0.05$) negative edges (red, blunt edges), and interactions with a nonsignificant correlation coefficient (black) are shown. Edges for which the directionality could not be determined using equivalence class analysis are shown as undirected. (b) Heatmaps show the undirected adjacency matrices comparing the Bayesian network to the ARACNe and CLR networks. An edge between node $i$ and node $j$ is represented by matrix value ($i$, $j$). Because the undirected networks are compared, the adjacency matrix is symmetric across the diagonal, and thus only the lower triangular matrix of the adjacency matrix is shown. Edge weight thresholds were set to $> 0.3$ for the Bayesian network and ARACNe (using ARACNe data processing inequality parameter $\tau = 0.03$) and to $Z > 1.13$ for CLR. Eight of 11 edges present only in the Bayesian network and not in the ARACNe network would induce three-node triplets in the ARACNe network, which is precisely what ARACNe is designed to prune out. (c) Venn diagram comparing edges across the three networks. The ARACNe network forms a complete subnetwork of the CLR network and a near complete subnetwork of the Bayesian network, which forms a near complete subnetwork of the CLR network.
DISCUSSION

In contrast to RPAs, MWAs can reduce the complexity of lysates after arraying, minimizing effort in experimental scale-up. Most of the information of a traditional western blot can be obtained, using 200-fold less protein and antibody. MWAs should be useful for analysis of proteins from cell lines and tissues from which there are sufficient lysates to print hundreds of MWAs that could be distributed en masse in an analogous manner to spotted DNA microarrays for interrogation with the user’s choice of antibodies. The only devices required after printing are commercially available 96-well gaskets and an imager. The ability to obtain information regarding hundreds of proteins with the MWA method should allow advances in our understanding of cell context–specific networks underlying human disease when combined with appropriate computational modeling methods.

MWAs could also be very useful for large-scale, systematic validation of antibodies. Antibody collections could be systematically verified for selectivity by examining lysates from cells transfected with a CDNA or depleted for the cognate protein by RNAi. The amount of antibody obtained from a single rabbit immunization (~5 mg) would be sufficient for over 100,000 MWAs, thus minimizing lot-to-lot variability of polyclonal antibodies. MWAs could be useful for current efforts to build a human protein atlas; samples from tissues used for in situ analyses could be examined with MWAs to verify that signals observed with each antibody resulted from proteins of the predicted molecular weight(s).

The ability to gather dynamic information regarding hundreds of proteins under many conditions poses new challenges for computational modeling. The Bayesian network described here represents direct and/or indirect effects of a given node on other nodes as indicated by high–probability connecting arcs, which are hypothesized to represent relationships of influence among the phosphoproteins in the network. Using prior knowledge to restrict edge directionality across a Bayesian network equivalence class, one can bolster the case for assigning directionality to these edges. To further support a case for interpreting network connections as causal, one could explicitly model the temporal data and/or use interventional data, which will be the subject of future inquiry.

The timing and amplitude of phosphorylation dynamics observed here coupled with the connectivities modeled in the Bayesian network suggest several candidate sources of RTK co-activation, each of which may be important in specific cancer contexts: (i) direct dimerization and/or phosphorylation by EGFR or other downstream tyrosine kinases as suggested by the rapid phosphorylation kinetics of Src, ErbB2 and ErbB4, coupled with their close proximity at the top of the network; (ii) activation of proteases that activate precursor growth factors or latent RTKs as might be predicted from the delayed phosphorylation amplitudes of FGFR1 (100 kDa) and MET activation loop sites coupled with their distance from EGFR in the network; and (iii) inactivation of tyrosine phosphatases through oxidation by reactive oxygen species. Phosphorylation of Tyr542 of Shp2 phosphatase displayed the highest fold change of any site in our analysis; this site has been suggested to relieve inhibition of phosphatase activity. The sustained phosphorylation of this and other tyrosine sites at EGF concentrations ≥50 ng ml⁻¹ suggests that it (and other cysteine-based tyrosine phosphatases) may be inactivated at such concentrations, thus unmasking many tyrosine kinase activities. Each of these mechanisms may have distinct roles in the context of cancers that have become resistant to single kinase inhibitors; systems-level analysis of other tyrosine kinase-driven cancers may be helpful in revealing appropriate therapeutic targets.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemethods/.

Note: Supplementary information is available on the Nature Methods website.

ACKNOWLEDGMENTS

A2780 cells were kindly provided by S. Liao (The University of Chicago). We thank C.Y. Chuang for technical assistance with MWAs, M.R. Rosner, K.P. White and W.L. McKeenan for helpful discussions, C. May for the graphic design of Figure 1, and J. Barkinge for operating support with microarraying. This work was supported, in part, by awards from The University of Chicago Cancer Research Center, The American Cancer Society, The University of Chicago Breast Cancer Specialized Program of Research Excellence, The Cancer Research Foundation and The Illinois Department of Health (86280156 to R.B.J.), the US National Institutes of General Medical Sciences P50-GM068762 Cell Decision Processes Center grant and US National Cancer Institute CA96504 to D.A.L.; M.F.C. was supported by a National Institutes of Health Systems Biology of Oxygen Predoctoral Training grant; J.P.W. was supported by a National Science Foundation Graduate fellowship; and C.-P.C. was supported by a National Institutes of Health Cancer Biology Postdoctoral Training grant.

AUTHOR CONTRIBUTIONS

C.-P.C., M.F.C. and R.B.J. designed the experiments. C.-P.C. and M.F.C. performed the cell culture and growth factor stimulations. M.F.C. and R.B.J. designed the MWA method, M.F.C. carried out microwestern experiments and organized the data into heat maps. J.P.W. and D.A.L. performed Bayesian network, CLR and ARACNe analysis of the data. M.F.C., J.P.W., D.A.L. and R.B.J. wrote the manuscript. All authors read and revised the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

Published online at http://www.nature.com/naturemethods/. Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/.

1. Burnette, W. “Western blotting”: electrophoretic transfer of proteins from sodium dodecyl sulfate polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radiodinated protein A. Anal. Biochem. 112, 195–203 (1981).
2. Paweletz, C.P., Liotta, L.A. & Petricoin, E.F. New technologies for biomarker analysis of prostate cancer progression: Laser capture microdissection and tissue proteomics. Urology 57, 160–163 (2001).
3. Paweletz, C.P. et al. Reverse phase protein microarrays which capture disease progression show activation of pro-survival pathways at the cancer invasion front. Oncogene 20, 1981–1989 (2001).
4. Sevecka, M. & MacBeath, G. State-based discovery: a multidimensional screen for small-molecule modulators of EGF signaling. Nat. Methods 3, 825–831 (2006).
5. Sachs, K., Perez, O., Pe’er, D., Lauffenburger, D.A. & Nolan, G.P. Causal protein-signaling networks derived from multiparameter single-cell data. Science 308, 523–529 (2005).
6. Rikova, K. et al. Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. Cell 131, 1190–1203 (2007).
7. Olsen, J.V. et al. Global, in vivo and site-specific phosphorylation dynamics in signaling networks. Cell 127, 635–648 (2006).
8. Wolf-Yadlin, A. et al. Effects of HER2 overexpression on cell signaling networks governing proliferation and migration. Mol. Syst. Biol. 2, 54 (2006).
9. Tibes, R. et al. Reverse phase protein array: validation of a novel proteomic technology and utility for analysis of primary leukemia specimens and hematopoietic stem cells. Mol. Cancer Ther. 5, 2512–2521 (2006).
10. Jones, R.B., Gordus, A., Krall, J.A. & MacBeath, G. A quantitative protein interaction network for the ErbB receptors using protein microarrays. Nature 439, 168–174 (2006).
11. Sunada, H., Magun, B.E., Mendelsohn, J. & MacLeod, C.L. Monoclonal antibody against epidermal growth factor receptor is internalized without stimulating receptor phosphorylation. Proc. Natl. Acad. Sci. USA 83, 3825–3829 (1986).
12. Gill, G.N. & Lazar, C.S. Increased phosphotyrosine content and inhibition of proliferation in EGF-treated A431 cells. *Nature* **293**, 305–307 (1981).

13. Wolf-Yadlin, A., Hautaniemi, S., Lauffenburger, D.A. & White, F.M. Multiple reaction monitoring for robust quantitative proteomic analysis of cellular signaling networks. *Proc. Natl. Acad. Sci. USA* **104**, 5860–5865 (2007).

14. Chang, F. *et al.* Involvement of PI3K//Akt pathway in cell cycle progression, apoptosis and neoplastic transformation: a target for cancer chemotherapy. *Leukemia* **17**, 590–603 (2003).

15. Pe’er, D. Bayesian network analysis of signaling networks: a primer. *Sci. STKE* **2005**, 14 (2005).

16. Koivisto, M. & Sood, K. Exact Bayesian structure discovery in Bayesian networks. *J. Mach. Learn. Res.* **5**, 549–573 (2004).

17. Eaton, D. & Murphy, K. Exact Bayesian structure learning from uncertain interventions. in *Proc. 12th Conf. on AI and Stats*, 107–114 (2007).

18. Chickering, D.M. Learning equivalence classes of bayesian-network structures. *J. Mach. Learn. Res.* **2**, 445–498 (2002).

19. Downward, J., Parker, P. & Waterfield, M.D. Autophosphorylation sites on the epidermal growth factor receptor. *Nature* **311**, 483–485 (1984).

20. Saito, Y., Haendeler, J., Hojo, Y., Yamamoto, K. & Berk, B.C. Receptor heterodimerization: essential mechanism for platelet-derived growth factor-induced epidermal growth factor receptor transactivation. *Mol. Cell. Biol.* **21**, 6387–6394 (2001).

21. Duchesne, L., Tissot, B., Rudd, T.R., Dell, A. & Fernig, D.G. N-glycosylation of fibroblast growth factor receptor 1 regulates ligand and heparan sulfate co-receptor binding. *J. Biol. Chem.* **281**, 27178–27189 (2006).

22. Ekman, S., Kallin, A., Engström, U., Heldin, C. & Rönnstrand, L. SHP-2 is involved in heterodimer specific loss of phosphorylation of Tyr771 in the PDGF beta-receptor. *Oncogene* **21**, 1870–1875 (2002).

23. Gould, K.L. & Hunter, T. Platelet-derived growth factor induces multisite phosphorylation of pp60c-src and increases its protein-tyrosine kinase activity. *Mol. Cell. Biol.* **8**, 3345–3356 (1988).

24. Margolin, A.A. *et al.* ARACNE: an algorithm for the reconstruction of gene regulatory networks in a mammalian cellular context. *BMC Bioinformatics* **7** (Suppl. 1), S7 (2006).

25. Faith, J.J. *et al.* Large-scale mapping and validation of Escherichia coli transcriptional regulation from a compendium of expression profiles. *PLoS Biol.* **5**, e8 (2007).

26. Taylor, R.C., Acquaah-Mensah, G., Singhal, M., Malhotra, D. & Biswal, S. Network inference algorithms elucidate Nrf2 regulation of mouse lung oxidative stress. *PLoS Comput. Biol.* **4**, e1000166 (2008).

27. Cantone, I. *et al.* A yeast synthetic network for in vivo assessment of reverse-engineering and modeling approaches. *Cell* **137**, 172–181 (2009).

28. Husmeier, D. Sensitivity and specificity of inferring genetic regulatory interactions from microarray experiments with dynamic Bayesian networks. *Bioinformatics* **19**, 2271–2282 (2003).

29. Lou, Y.W. *et al.* Redox regulation of the protein tyrosine phosphatase PTP1B in cancer cells. *FEBS J.* **275**, 69–88 (2008).

30. Lu, W., Shen, K. & Cole, P.A. Chemical dissection of the effects of tyrosine phosphorylation of SHP-2. *Biochemistry* **42**, 5461–5468 (2003).
ONLINE METHODS

Cell stimulation and lysis. A431 cells were passaged three times after thawing from frozen stocks. For the final passage, plates at 80% confluence were split 1:10 into fresh plates (6 plates per EGF concentration tested). We incubated 0.7 ml of trypsin-EDTA with cells for 7 min at 37 °C. Newly plated cells were grown in 8% FBS with 30 µg ml^{-1} penicillin and 50 µg ml^{-1} streptomycin, and the medium was replaced with serum-free medium when they reached 50% confluence. Cells were maintained in serum-free DMEM with penicillin and streptomycin for 48 h. A stock solution of 200 µg ml^{-1} EGF was applied at the appropriate dilution in serum-free medium after washing twice with PBS. At each time point the cells were again washed twice with PBS (pH 7.4) (see Supplementary Note 2 for all buffer compositions). We added 1.0 ml of lysis buffer and scraped the cells into a 2 ml microcentrifuge tube. After lysis, the samples were boiled for 10 min and then frozen at −80 °C. On the next day, the cells were sonicated at power 5 on a model W140 Sonifier cell disruptor (Heat Systems, Ultrasonic, Inc) for 10 s, alternating one second on, one second off (total 20 s time). The samples were boiled for 2 min, and 500 µl of the sample were immediately placed in a microconcentration device with a 10 kDa molecular weight cutoff. The tubes were spun at 12,000g for ~10 min until the volume was reduced fivefold. Each sample was then topped off to 100 µl with lysis buffer so that each sample was of equal volume. Samples were divided into 20 µl aliquots and frozen at −80 °C to avoid repeated freeze-thaw cycles before printing. Samples were boiled for 2 µl immediately before printing.

Gel fabrication. Glass casting plates (one measuring 14 × 27 cm, the other measuring 14 × 28 cm) were sprayed with BlueSlick (Serva) and wiped thoroughly with a paper towel to provide an even layer of solution over the entire side of the glass plate. Rubber spacers were placed on three sides of the inner coated sides of the glass plate (two long sides and one short side) making sure there were no gaps between the spacers on the corner (as this would be a potential source of acrylamide leakage). One rectangle of Netflix (Serva) was placed on the glass plate on top of the spacers. The second glass plate was placed on top with the coated surface facing down, leaving a small lip (about 1 cm) to inject the unpolymerized gel. Twelve clamps were placed around the three gasketed edges of the sandwich.

Gel reagents (Supplementary Note 2) were gently mixed to avoid bubble formation in a 50 ml conical tube. One corner of the sandwiched plates was placed on an object about three inches high so that gel could run at a slant in two dimensions. A 30 ml syringe with a 19 gauge needle was used to inject the gel mixtures so that it ran down the length of one long side, then along the length of the bottom short side and then up the second length to avoid any trapped air. After the sample was poured, the sandwich was laid horizontally during polymerization to avoid leakage. After one hour, the clamps were removed and the top plate was pried off with a plastic wedge. The gel was removed from the bottom glass plate by lifting manually from the plate and placed between two plastic Gelfix (Serva) sheets.

A standard paper cutter was used to remove the Netflix border and then used to divide the gel into two parts (with each half measuring at least 11.5 cm wide in each dimension). The gels were saved in vacuum-sealed bags and refrigerated at 4 °C before use. The performance was not found to differ substantially after storage for several weeks.

Microarraying. The lysates were spotted using a noncontact microarrayer (GeSiM Nanoplotter 2.1E) with active humidification. The printing performance of each tip was validated with a stroboscope before beginning each microarray print. If printing was inconsistent, 200 µl of 50% methanol–50% HCl was loaded into the tip and dispensed three times using manual mode. The tips were washed for 60 s using the wash/dry cycle and rechecked with the stroboscope. The Gelfix sheet was removed from the gel and the gels prehydrated in hydration buffer (100 ml) containing 1 ml of 1 M sodium bisulfite and 1 ml of 1 M DTT for 5 min. Excess fluid was blotted off with a piece of cardboard.

To keep the gel and samples hydrated, the relative humidity was maintained at 50−80%. Z-height measurements were taken before the print of each gel. MWAs were printed onto two gels per array run. Tip dispense height was held at 1.5 mm above the gel surface while printing.

Samples were placed with the ladder in well A1 of a 384 well plate and samples consecutively in A2–A7. Software NP2.15.46 was used. The TransferTipMultiSim04H9 (GeSiM) was run using the transfer text and the workplate definition file provided in Supplementary Note 3.

Odyssey protein ladder (LI-COR) was printed in lane 1 at a 1:2 dilution in lysis buffer. This ladder was printed at high concentration so that it may be visible on the nitrocellulose membrane after transfer to facilitate alignment of the gasket.

After print completion, the gel was maintained for an additional 10 min in the humidified environment to insure that all droplets were absorbed beneath the gel surface. The gel was subsequently rehydrated for 5 min in the rehydration buffer described above with gentle agitation. After rehydration, the gel was placed onto the GenePhor (GE Healthcare) for horizontal semidry electrophoresis.

Horizontal semidry electrophoresis. Samples were separated by size using a horizontal electrophoresis device prechilled to 10 °C. Three drops of kerosene were added on to the surface of the GenePhor and spread with a Kimwipe to provide a homogenous layer on the surface. A clean piece of Gelfix measuring 14 × 14 cm was added to the surface of the GenePhor. The hydrated gel was placed on top of the plastic sheet covering the area of both electrode rectangles. Three filter wicks (Serva) were cut in half and each half stacked three deep. Each stack was placed in electrode buffer (Supplementary Note 2) for several seconds and air was manually pressed out of them while the stacks were submerged. The first (bottom) lid of the GenePhor was placed over the gel making sure that there was no fluid making contact with the lid from the gel. The second stacks of hydrated wicks were placed in the center of each electrode slit on the gel. The second and third (upper) lids of the GenePhor were then closed. The power supply was set at 350 V, 30 W and unlimited amps. The lowest-molecular-weight ladder bands migrated about 9 mm (the length of one well of a 96-well plate) in 12 min.

Transfer. After electrophoresis, the gel was placed protein side down onto nitrocellulose (Bio-Rad) premoistened in transfer buffer (Supplementary Note 2). Filter paper was placed on either side of the nitrocellulose and gel, and was clamped in a transfer device with a 10 kDa molecular weight cutoff. The tubes were ultrasonically homogenized (Ultrasonic, Inc) for 10 s, alternating one second on, one second off (total 20 s time). The samples were boiled for 2 min, and 500 µl of the sample was printed in lane 1 at a 1:2 dilution in lysis buffer. This ladder was printed at high concentration so that it may be visible on the nitrocellulose membrane after transfer to facilitate alignment of the gasket.

After print completion, the gel was maintained for an additional 10 min in the humidified environment to insure that all droplets were absorbed beneath the gel surface. The gel was subsequently rehydrated for 5 min in the rehydration buffer described above with gentle agitation. After rehydration, the gel was placed onto the GenePhor (GE Healthcare) for horizontal semidry electrophoresis.
Blotting. Nitrocellulose was removed from the transfer apparatus and washed for 5 min in TBS (without Tween 20) to remove methanol. The gels were blocked for 1 h in Odyssey blocking buffer (LI-COR). The blot was aligned on the gasket by placing the visible ladder on the vertical lines and centering the ladder between the horizontal lines. The gasket was clamped into the 96-well isolation device, and primary antibodies were pipetted into the appropriate wells, making sure that the membrane remained wet during the process. The primary antibody was diluted in pure Odyssey blocking buffer (without Tween 20) overnight. We added 150 µl of diluted antibody per well. After incubation, the wells were washed four times with 200 µl of TBST while clamped in the gasket-device. Goat anti-rabbit Alexa Fluor 680-conjugated secondary (Invitrogen), goat anti-rabbit and goat anti-mouse IR800-conjugated secondary antibodies (1:5,000) (LI-COR) were diluted in 20% Odyssey blocking buffer, 80% TBS (without Tween 20). We added 150 µl of the diluted secondary antibody to the appropriate well. After incubation for an hour, the blot was washed three times with 200 µl TBST while clamped in the gasket-device (Arrayit). The blot was then removed from the gasket, placed in a box top and washed for an additional 5 min in TBST. For the fifth wash, TBS without Tween 20 was used, washing for 5 min. The membrane was completely dried using pressurized air and scanned using the LI-COR Odyssey imager at 24 µm resolution and high quality (using laser intensity 1.0 on the 700 nm channel, and using laser intensity 2.0 on the 800 channel) settings.

Analysis. Scanned images were saved for analysis as 16-bit tiff files. Genepix 8.0 ( Molecular Devices) was used to record the mean by drawing an equally sized circle around the appropriately sized band for each sample. Appropriate size was defined as within 10 kDa of the size as defined by the antibody product sheet as measured in comparison to the LI-COR ladder bands. All bands within this region that were visible were recorded. Bands outside this region were noted but the intensities were not recorded or analyzed. The background fluorescence was recorded by placing an equal sized circle in the blank space to the left of the first sample (not covering sample or ladder space) and the minimum value of this circle was recorded. Net intensity was calculated by subtracting each sample intensity from the background. To normalize sample concentration, the net intensities were divided by a simple mean of the net intensities for GAPDH, tubulin and actin calculated separately for each array print. Fold change was calculated as the ratio of each normalized net intensity to the net intensity at the 0 min time point, minus one. Graphs, heatmaps and clustergrams were generated using Matlab 2007b (The Mathworks).

Signaling network inference modeling. Bayesian networks were modeled using a dynamic programming algorithm that computes the exact marginal posterior probability of edges in the Bayesian network derived from the dataset16. The algorithm was implemented using a modified version of the open-source Bayesian Network Structure Learning toolbox in Matlab17. Node conditional probability distributions were represented by multinomials using a uniform Dirichlet prior with equivalent sample size of one and a prior over graph structures was calculated by accounting for the number of ways to choose parents sets in a graph, as previously described16,31. Networks were scored using the Bayesian Dirichlet likelihood equivalent uniform (BDeu) score32. The BDeu score accounts for both model fit and complexity and thus avoids overfitting the data. Although this dynamic programming algorithm introduces a non-uniform prior over graph structures, it has been shown to perform better at structure learning tasks16,17 than local search methods that use a uniform prior over graph structures, such as Markov chain Monte Carlo searches over directed acyclic graphs33, as well as Markov chain Monte Carlo over node orderings, which uses a non-uniform prior31.

All nodes were discretized using three-level k-means clustering to indicate low, medium and high phosphoprotein levels (Supplementary Table 5). Clustering was done using the squared Euclidean distance metric and repeated 50 times for each node to find the optimal clustering assignments. It is believed that by using k-means clustering, we are better representing the physiological diversity in signaling states of the phosphoproteins in the network, compared to more arbitrary discretization schemes, like interval and quantile discretization, that do not try to explicitly capture clusters in the data.

CLR was implemented using Matlab code provided by the original authors, with Z scores (edge weights) calculated as previously described25. ARACNe was implemented using the minet package in R (ref. 34). To minimize the sources of variation between algorithms, the same discretized data that were used to learn the Bayesian network model were also used to learn the CLR and ARACNe models. The mutual information matrix for ARACNe was calculated using a simple histogram method in the minet package, and for CLR was calculated directly from the discretized data. The edge score thresholds for CLR and ARACNe were varied in an effort to maximize the similarity between the Bayesian network and ARACNe (or CLR), both given the >0.3 edge weight threshold for the Bayesian network (Fig. 6) and when this constraint on the Bayesian network edge weight threshold was removed, though in both cases staying within edge weight thresholds that gave significant network results (Supplementary Note 1 and Supplementary Figs. 8–10).

The sign of the influences between nodes in the Bayesian network was estimated using pairwise correlation coefficients. Seventeen of 24 pairwise interactions had a highly significant (P < 0.001) positive correlation coefficient. Two of 24 had a significant (P < 0.05) negative correlation coefficient. The remaining 5 of 24 pairwise interactions had a nonsignificant (P > 0.05) correlation coefficient but were edges in two- or three-parent interactions, suggesting a simple pairwise correlation coefficient was not sufficient to capture the parent-child behavior. Notably, both negative interactions were directed at p-PDGFRA(Tyr754). Five of the six two-parent interactions (including all four with p-EGFR(Tyr845) and p-PDGFRB(Tyr1009) as the parent set) were consistent with ‘and gate’ behavior. The parent-child raw data from all one-, two- and three-parent interactions are plotted versus one another in Supplementary Figure 11.

Considering up to three parents per node in the Bayesian network captured almost all higher-order interactions in the
dataset (Supplementary Note 1 and Supplementary Fig. 12). Although additional higher-order interactions may be present but there are simply not enough data for the Bayesian network to infer them, it may also be that such higher-order interactions are indeed not present, regardless of how much data are available describing the network. ARACNe and CLR, which only consider undirected pairwise interactions, thus represent useful, but likely not complete, approximations of interactions in this dataset.

31. Friedman, N. & Koller, D. Being Bayesian about Bayesian network structure: a Bayesian approach to structure discovery in Bayesian networks. *Mach. Learn.* 50, 95–125 (2003).
32. Heckerman, D., Geiger, D. & Chickering, D.M. Learning Bayesian networks: The combination of knowledge and statistical data. *Mach. Learn.* 20, 197–243 (1995).
33. Madigan, D. & York, J. Bayesian graphical models for discrete data. *Int. Stat. Rev.* 63, 215–232 (1995).
34. Meyer, P., Lafitte, F. & Bontempi, G. minet: a R/Bioconductor package for inferring large transcriptional networks using mutual information. *BMC Bioinformatics* 9, 461 (2008).