Supporting Online Material for

A Systems Model of Signaling Identifies a Molecular Basis Set for Cytokine-Induced Apoptosis

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(available at www.sciencemag.org/cgi/content/full/310/5754/1646/DC1

Database S1 as a zipped archive

MATERIALS AND METHODS

P-JNK immunoblotting

HT-29 cells (ATCC) were plated and sensitized as described (*S1*), then stimulated with combinations of 0, 1, 5, or 100 ng/ml TNF and EGF for 15 min and lysed by standard techniques (*S2*). 100 μg of each lysate was run on a 10% SDS-PAGE gel and transferred to PVDF (Millipore). The membranes were blocked with 5% nonfat skim milk in 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 0.1% Tween-20 and probed overnight with anti-phospho-SAPK/JNK (Cell Signaling) at 1:1000 dilution. The membranes were then probed with horseradish peroxidase conjugated anti-rabbit secondary antibody (Amersham) at 1:10,000 dilution and visualized by enhanced chemiluminescence (Amersham) on a Kodak Image Station (Perkin Elmer). Bands were selected and quantified according to the manufacturer's recommendations. To improve quantitation, each membrane was normalized to an internal positive control (100 ng/ml TNF, 15 min). The membranes were stripped and reprobed with anti-tubulin (Calbiochem) to confirm equal protein loading.

Large-scale apoptotic marker measurements

HT-29 cells were plated in 24-well plates and were sensitized and treated identically to the cells used in the corresponding signaling measurements on the same day (*S1*). After 12, 24, or 48 hours of cytokine treatment, the cells were trypsinized until all cells were detached from the plate. The growth medium was combined with the trypsinized cells to ensure capture of both floating and adherent cells in each well. The combined pool of cells from each well was split into thirds; one third was analyzed for phosphatidylserine exposure and membrane permeability, one third was fixed with methanol and analyzed for cleaved caspase-cytokeratin, and one third was fixed with methanol (MeOH) and analyzed for nuclear fragmentation. For the phosphatidylserine-membrane permeability analysis, the cells were washed once with Annexin Binding Buffer (ABB, 10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$) and stained with Alexa Fluor 488-conjugated Annexin-V (Molecular Probes) and 1 ug/ml propidium iodide (PI) for 10 minutes at room temperature. Excess ABB was added and the cells were analyzed by a Becton Dickinson FACScan or FACSCalibur flow cytometer. For the cleaved caspase-cytokeratin assay, MeOH-fixed cells were stored at -20 C for up to 1 week. After centrifugation to remove the MeOH, cells were washed in PBS + 0.1% Tween-20 (PBS-T), and were then stained with anti-cleaved caspase-3 (Cell Signaling, Becton Dickinson) and anti-cleaved cytokeratin (Roche) antibodies in PBS-T + 1% bovine serum albumin (PBS-TB) for 1 hour at room temperature. The cells were washed and stained with secondary antibodies, Alexa Fluor 488-conjugated donkey anti-mouse IgG and Alexa Fluor 647-conjugated donkey anti-rabbit IgG (Molecular Probes) in PBS-TB for 1 hour at room temperature. The cells were then washed and analyzed on a Becton Dickinson FACSCalibur flow

cytometer. For the nuclear fragmentation assay, MeOH cells were stored at -20 C for up to one week. After centrifugation to remove the MeOH, cells were washed once with PBS-T, and then incubated with 1 mg/ml RNAse A in PBS-T for 2.5 hours. PI was then added at a final concentration of 50 ug/ml, and the cells were analyzed on a FACSCalibur flow cytometer to quantify nuclear fragmentation. For all flow cytometry assays, data was analyzed using FlowJo software (TreeStar).

To fuse quantitative measurements on different days, 100 ng/ml TNF and mock treatments were always included as fixed positive and negative controls. Experimental samples were then scaled by the TNF (membrane permeability and cleaved caspase-cytokeratin) or mock (phosphatidylserine exposure and nuclear fragmentation) index for that day. By cross-validation we verified that the magnitude and dynamics of these normalized apoptotic indices were highly reproducible, with $R^2 = 0.79–0.98$ (*S3*). The entire dataset of 1440 flow cytometry runs is available in the Supporting Online Material.

Signaling metric extraction

Signaling network measurements

Details of the signaling network measurements are described elsewhere (*S1, S3*). Briefly, 19 quantitative signaling network measurements—IKK activity, JNK1 activity, MK2 activity, P-EGFR (Y1068), total EGFR, P/total EGFR, P-MEK (S217/221), ERK activity, P-IRS1 (S636), P-IRS1 (Y896), P-Akt (S473) by Western blot, total Akt, Akt activity, P-Akt (S473) by antibody microarray, total Akt, P/total Akt, P-FKHR (S256), procaspase-8, cleaved caspase-8, and procaspase-3—were compiled from triplicate biological samples treated with 0, 0.2, 5, 100 ng/ml TNF and 0, 1, 100 ng/ml EGF or 0, 1, 5, 500 ng/ml insulin for 5, 15, 30, 60, 90 min or 2, 4, 8, 12, 16, 20, 24 hr. With each treatment, sextuplicate 0-min samples were also prepared to measure the level of baseline signaling. Exact treatment combinations are shown in Fig. 1F and described in the legend. The total number of individual protein measurements in the initial compendium was 7980: 19 molecular signals x 3 replicate measures x 10 treatment combinations x the equivalent of 14 time points (i.e., zero in sextuplicate) = 7980 individual signals.

For autocrine perturbations, cells were pretreated with 10 μg/ml C225 for one hr or cotreated with 10 μg/ml IL-1ra, then stimulated with 5 or 100 ng/ml TNF (*S1*). These data provided an additional 1596 protein measurements (19 molecular signals x 3 replicate measures x 2 treatment combinations x the equivalent of 14 time points) to test model predictions.

Metric extraction

For each signaling time course, the following signaling metrics were derived:

a. Thirteen time-point metrics, defined as the mean signal at each point in the time course.

b. Thirteen instantaneous-derivative metrics, defined as the forward slope between the current time point and the subsequent time point. For the final time point (24 hr), the instantaneous derivative was set to zero.
c. Four summary metrics, which included the area under the curve for the entire time course, the maximum signal, the mean signal, and the steady-state signal (defined as the mean of the final four time points).
d. A variable number of peak metrics. For each signal a tolerance value (defined as a percentage of the dynamic range of the signal) was set for the algorithm to classify an upward fluctuation as a peak. Tolerances were set at: 50% for Akt activity, IKK activity, and all antibody microarray measurements; 5% for JNK1 activity and MK2 activity; and 20% for all other signaling network measurements. For each peak identified, three metrics were extracted: area under the curve, activation slope (defined as the best linear fit between the preceding local minimum and the peak maximum), and decay rate (defined as the best exponential fit between the peak maximum and the proceeding local minimum).

These signaling metrics are summarized in table S2. Metric extraction algorithms were coded in MATLAB and are available upon request.

PLS model construction and validation

Prior to all analyses, the signaling and apoptosis matrices were variance scaled to nondimensionalize the different measurements. The PLS model was constructed in the SIMCA-P 10.0 (Umetrics) software suite according to the following iterative formulas:

$$E_1 = X - t_1 p_1^T;\ E_2 = E_1 - t_2 p_2^T,\ t_2 = E_1 w_1;\ E_i = E_{i-1} - t_i p_i^T,\ t_i = E_{i-1} w_i$$
$$F_1 = Y - b_1 t_1 q_1^T;\ F_2 = F_1 - b_2 t_2 q_2^T;\ F_i = F_{i-1} - b_i t_i q_i^T$$

where E_i represents the residual of the i^{th} principal component, with score vector t_i, weight vector w_i, and loading vector p_i, and ^{T} represents transpose. F_i represents the residuals of the i^{th} dependent principal component, with score vector t_i and loading vector q_i, and b_i represents the coefficient characterizing the inner relation between the independent and dependent principal components. Model predictions were made by leave-one-out crossvalidation for the TNF-EGF-insulin treatments and by unbiased prediction for the autocrine perturbations (*S3*). Model uncertainties were calculated by jack-knifing (*S4*). Signaling axes and treatment mappings (Fig. 4, E–G) were plotted using w_1, w_2 and t_1, t_2 respectively after a 60^o subspace rotation (*S5*). Centered and scaled coefficients were used as the regression weights.

PLS model perturbations

Reduced metric submodels

For the reduced metric submodels, the information content of each signaling metric was assessed by its variable importance in the projection (VIP):

$$VIP_k = \sqrt{\frac{k\sum_{a=1}^{A} w_{ak} SS_a}{\sum_{a=1}^{A} SS_a}}$$

where k is the number of signaling metrics, w_{ak} is the weight of the k^{th} metric for principal component a, A is the total number of principal components, and SS_a is the sum of squares explained by principal component a (*S6*). For the reduced signal submodels, the mean VIP score for each signal calculated across all metrics was used as the central estimate of the pure-signal information content.

REFERENCES

S1. K. A. Janes *et al*., in preparation.
S2. K. A. Janes *et al*., *Mol Cell Proteomics* **2**, 463-73 (2003).
S3. S. Gaudet *et al*., *Mol Cell Proteomics* **4**, 1569-1590 (2005).
S4. B. Efron, R. J. Tibshirani, *An Introduction to the Bootstrap* (Chapman and Hall, London, 1993).
S5. O. Alter, P. O. Brown, D. Botstein, *Proc Natl Acad Sci U S A* **97**, 10101-6 (2000).
S6. S. Wold, *Chemometrics and Intelligent Laboratory Systems* **23**, 149-161 (1994).
S7. Materials and methods are available as supporting material on Science online.
S8. K. A. Janes, J. G. Albeck, S. Gaudet, unpublished observations.
S9. K. A. Janes *et al*., *J Comput Biol* **11**, 544-61 (2004).

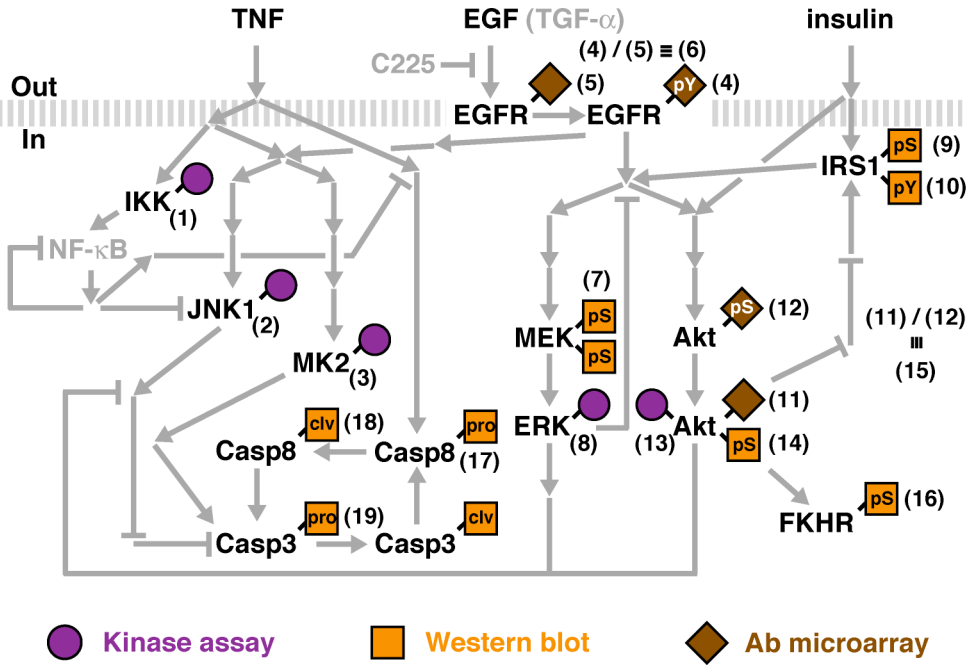

fig. S1. Schematic of the shared TNF-EGF-insulin signaling network. Nineteen molecular signals (numbers) from protein nodes highlighted in black were measured by high-throughput kinase activity assays, quantitative Western blotting, or antibody microarrays as described (*S1, S7*).

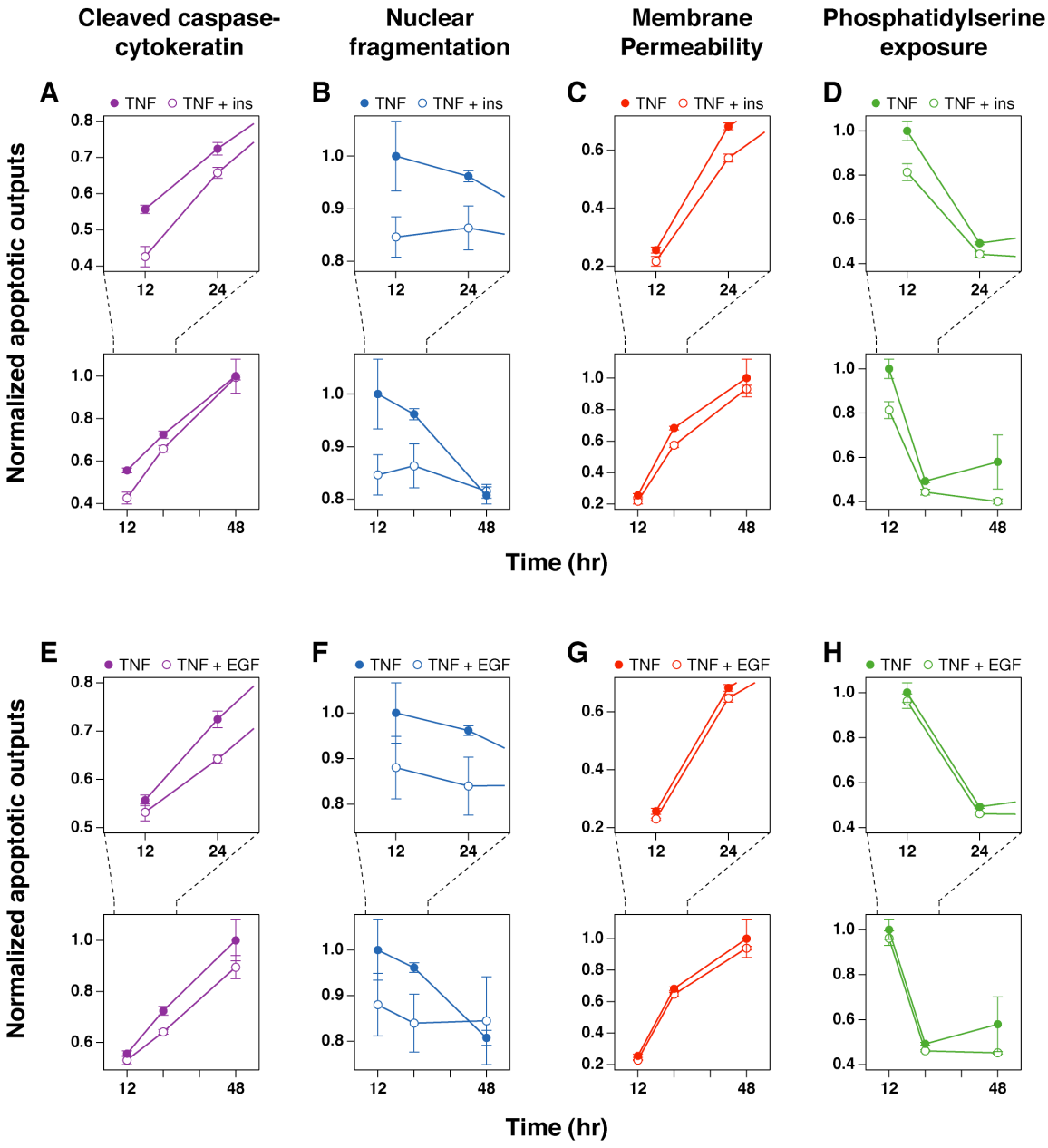

fig. S2. Insulin and EGF antagonize TNF-induced apoptosis. **(A–D)** Insulin significantly reduces all four apoptotic outputs at 12 and 24 hr and reduces membrane permeability and phosphatidylserine exposure from 12 to 48 hr ($p < 0.05$, two-way analysis of variance). **(E–H)** EGF significantly reduces caspase substrate cleavage from 12 to 48 hr and membrane permeability at 12 and 24 hr ($p < 0.05$). Nuclear fragmentation was also reduced but did not reach statistical significance (p = 0.07). Data are replotted from Fig. 1, M–O.

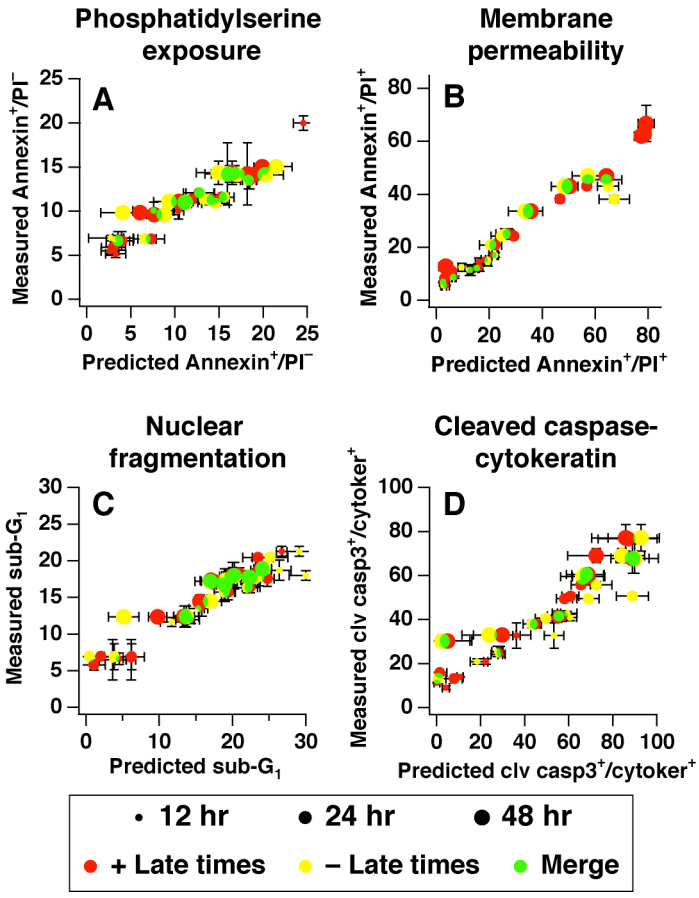

fig. S3. The partial least squares model does not require late time points to make predictions of apoptosis. Correlation plots between measured apoptotic indices and cross-validated predictions of **(A)** phosphatidylserine exposure, **(B)** membrane permeability, **(C)** nuclear fragmentation, and **(D)** cleaved caspase-cytokeratin. The full PLS model (red) was compared with a truncated PLS model (yellow) that omitted signaling measurements after four hours. The merged overlay is shown in green, and marker size corresponds to the response time point. Data are presented as the mean ± S.E.M., and model uncertainties were estimated by jack-knifing (*S9*).

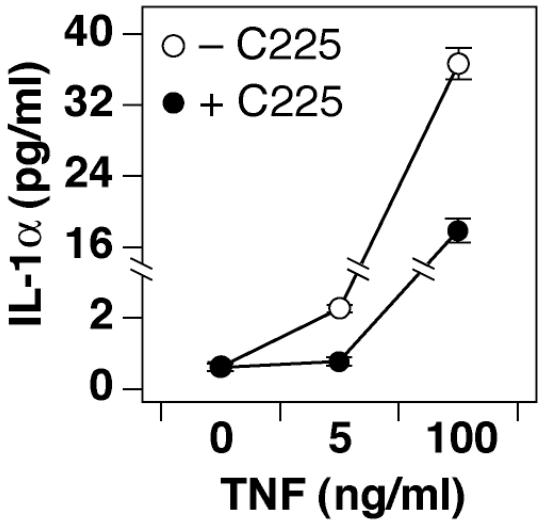

fig. S4. TNF-induced autocrine TGF-α causes the release of autocrine IL-1α. Autocrine TGF-α was blocked with 10 μg/ml C225 antibody and HT-29 cells then stimulated with 0, 5, or 100 ng/ml TNF for 24 hr and analyzed for IL-1α release (*S1*).

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table S1. Signaling metrics extracted from dynamic network measurements

Metric class*	Metrics extracted
Time point	0 min
	5 min
	15 min
	30 min
	60 min
	90 min
	2 hr
	4 hr
	8 hr
	12 hr
	16 hr
	20 hr
	24 hr
Instantaneous derivative	0 min
	5 min
	15 min
	30 min
	60 min
	90 min
	2 hr
	4 hr
	8 hr
	12 hr
	16 hr
	20 hr
	24 hr
Summary metrics	Area under the curve
	Maximum signal
	Mean signal
	Steady-state signal
Peak metrics	Area under the curve
	Activation slope
	Decay rate

*See (*S7*) for a complete description and definition of the signaling metrics.

table S2. Top 20 most informative signaling metrics of the PLS model

VIP*	Protein†	Signal†	Metric‡
1.57	IRS-1	P-Ser636	Decay rate, peak #2
1.43	Akt	P-Ser473	Activation slope, peak #3
1.39	IRS-1	P-Ser636	Activation slope, peak #2
1.35	JNK1	Activity	Area under the curve, peak #2
1.35	IKK	Activity	Area under the curve, peak #2
1.30	MK2	Activity	4 hr time point
1.30	MK2	Activity	8 hr time point
1.29	JNK1	Activity	8 hr time point
1.29	IKK	Activity	Steady-state
1.29	JNK1	Activity	Decay rate, peak #2
1.29	MK2	Activity	Mean
1.28	MK2	Activity	30 min time point
1.28	MK2	Activity	Area under the curve
1.28	JNK1	Activity	Mean
1.28	MK2	Activity	Maximum
1.28	IKK	Activity	20 hr time point
1.28	IKK	Activity	Area under the curve
1.27	IKK	Activity	12 hr time point
1.27	IKK	Activity	16 hr time point
1.27	MK2	Activity	90 min time point§

*Variable importance in the projection (*7*).
†See Table 1 and (*S1*) for a complete description of the network measurements.
‡See table S1 and (*S7*) for a complete description of the signaling metrics.
§The top caspase metric, mean cleaved caspase-8, was only 34th overall (*S8*).

table S3. Top 20 loadings in the first principal component of the PLS model

w_1c_1*	Protein†	Signal†	Metric‡
0.097	Caspase-8	Cleavage	2 hr derivative
0.095	EGFR	P-Tyr1068/total	16 hr derivative
0.095	MK2	Activity	5 min derivative
0.093	JNK1	Activity	5 min derivative
0.092	MK2	Activity	15 min time point
0.092	Akt	P-Ser473	Activation slope, peak #3
0.092	JNK1	Activity	Activation slope, peak #1
0.091	MK2	Activity	Area under the curve, peak #1
0.091	JNK1	Activity	15 min time point
0.090	JNK1	Activity	Maximum
0.090	Caspase-8	Cleavage	4 hr derivative
0.089	JNK1	Activity	30 min time point
0.089	MK2	Activity	90 min time point
0.088	MK2	Activity	60 min time point
0.088	Caspase-8	Cleavage	12 hr time point
0.087	MK2	Activity	30 min time point
0.087	Caspase-8	Cleavage	16 hr time point
0.087	Caspase-8	Cleavage	8 hr derivative
0.086	Akt	Total	20 hr derivative
0.086	MK2	Activity	Maximum
0.085	Caspase-8	Cleavage	Steady-state

*Loading weights of the first principal component (*S7*).
†See Table 1 and (*S1*) for a complete description of the network measurements.
‡See table S1 and (*S7*) for a complete description of the signaling metrics.

table S4. Top 20 loadings in the second principal component of the PLS model

w_2c_2*	Protein†	Signal†	Metric‡
-0.0695	IKK	Activity	Activation slope, peak #2
-0.0691	EGFR	Total	60 min derivative
-0.0622	Akt	P-Ser473	24 hr time point
-0.0615	MK2	Activity	Decay rate, peak #2
-0.0613	Akt	P-Ser473	8 hr derivative
-0.0608	FKHR	P-Ser256	16 hr time point
-0.0605	Akt	P-Ser473	16 hr time point
-0.0605	EGFR	Total	Area under the curve, peak #3
-0.0603	Akt	P-Ser473	Steady-state
-0.0600	Procaspase-3	Zymogen	12 hr time point
-0.0599	Procaspase-3	Zymogen	Steady-state
-0.0599	Procaspase-3	Zymogen	20 hr time point
-0.0596	Procaspase-3	Zymogen	24 hr time point
-0.0595	Procaspase-3	Zymogen	16 hr time point
-0.0593	Caspase-8	Cleavage	90 min time point
-0.0590	FKHR	P-Ser256	12 hr time point
-0.0587	Akt	P-Ser473	20 hr time point
-0.0586	FKHR	P-Ser256	Steady-state
-0.0583	Akt	P-Ser473	12 hr time point
-0.0581	IRS1	P-Tyr896	24 hr time point§

*Loading weights of the second principal component (*S7*).
†See Table 1 and (*S1*) for a complete description of the network measurements.
‡See table S1 and (*S7*) for a complete description of the signaling metrics.
§Five P-IRS1 (Ser636) metrics were identified within the top 30 loadings of the second principal component (*S8*).