Supporting Information for

Quantification of ERK kinase activity in biological samples using differential sensing

Diana Zamora-Olivares,[1,5,#] Tamer S. Kaoud,[2,3,#] Lingyu Zeng,[4] Jacey R. Pridgen,[2] Deborah L. Zhuang, [5] Yakndara E. Ekpo, [5] Jessica R. Nye, [5] Mitchell Telles,[5] Eric V. Anslyn*[1] and Kevin N. Dalby*[2, 6]

[1]Department of Chemistry, The University of Texas at Austin, Austin, TX 78712, USA;

[2]Division of Chemical Biology & Medicinal Chemistry, College of Pharmacy, The University of Texas at Austin, Austin, TX 78712, USA;

[3]Medicinal Chemistry Department, Faculty of Pharmacy, Minia University, Minia 61519, Egypt.

[4]College of Chemistry and Molecular Science, Wuhan University, Wuhan, Hubei, China;

[5]Texas Institute for Discovery Education in Science and Freshman Research Initiative, The University of Texas at Austin, Austin, Texas, USA.

[6]Department of Oncology, Dell Medical School, The University of Texas at Austin, Austin, TX 78712, USA.

*To whom correspondence should be addressed: Kevin N Dalby, email: dalby@austin.utexas.edu or Eric V. Anslyn, email: anslyn@austin.utexas.edu.

Author contributions: [#] D.Z.-O and T.S.K. contributed equally to this study.

Content

A. SUPPORTING METHODS

B. SUPPORTING SCHEMES

C. SUPPORTING FIGURES S1 – S13

D. SUPPORTING CHARACTERIZATION OF SYNTHESIZED PEPTIDES

E. SUPPORTING TABLES S1 – S32

SVM analysis Tables S8-S32

a) Input and output data sets of the quantitative analysis of ERK1 in MDA-MB-231 cell lysates. Tables S8-S13.
b) Input and output data sets of the quantitative analysis of ERK1 in A549 cell lysates. Tables S14-S18.
c) Input and output data sets of the quantitative analysis of ERK1 in treated tumor samples. Tables S20-S23.
d) Input and output data sets of the quantitative analysis of ERK1 in treated and control tumor samples. Tables S24-S26.
e) Input and output data sets of the quantitative analysis of ERK1 in treated cell and tumor samples using general model. Tables S27-S32.

A. SUPPORTING METHODS

Peptide synthesis, purification and characterization - Peptides were synthesized using standard Fmoc SPPS (Solid phase peptide synthesis (SPPS) based on Fmoc chemistry) with a peptide synthesizer, CEM Liberty Blue Automated Microwave. Peptides Sub-D and MEF2A were synthesized using P3 bioSystems rink AM resin, Sub-F peptide was synthesized using P3 bioSystems wang resin. NFAT4 peptide on-resin was purchased from BioSynthesis. Coupling reactions used 0.2 M of Fmoc-L-amino acid/ 0.5 M DIC /1.0 M Oxyma in DMF relative to 1 eq. of pre-loaded resin. In the final step, the *N*-terminus was acetylated using 1.2 M *N*-methylmorpholine/acetic anhydride in DCM. The resin-bound peptides were dried for at least 4 h under vacuum prior to the alkylation reaction. The resin-bound peptide (1 eq.) was swelled in dry DMF for 30 min. The Mmt protecting group was cleaved by exposing the resin to a solution of 1% TFA and 5% TIS in DCM for 3-5 min. This previous step was repeated until the yellowish solution changed to a transparent color. The resin was thoroughly washed with DCM and DMF. A solution of SOX-Br (2 eq.) and tetramethylguanidine (5 eq.) in dried DMF (6 mL) was stirred at room temperature. The solution was then added to the resin-bound peptide. The reaction mixture was allowed to shake for 24 h under argon. The reagents were filtered and the resin was thoroughly washed with DMF, DCM and MeOH. The resin was then dried under vacuum for 4 h. A cleaving solution of 10 mL of TFA/H_2O/TIS (95/2.5/2.5 % v/v) was added to the resin, and mixed for 4 h. The crude SOX-peptides were precipitated using cold ether. The crude peptide pellets were lyophilized and preparative HPLC was used to purify the peptides using a Shimadzu HPLC equipped with an Agilent PrepHT, Zorbax, SB-C18 (21.2 × 250mm,7uCrt). Mobile phases used were solvent A: H_2O 0.1% TFA (v/v); solvent B: CH_3CN 0.1% TFA (v/v). Flow rate: 10 mL min^{-1}, gradient 5 to 60% B over 60-90 min, monitored at 220 and 316 nm. Analytical HPLC data was obtained using a Shimadzu HPLC equipped with a column Agilent, Zorbax SB-C18 5.0 µm, (4.6 ×150mm, 80 Å). Mobile phases used solvent A: H_2O 0.1% TFA (v/v); solvent, B: CH_3CN 0.1% TFA (*v/v*). Rate flow: 1 mL min^{-1}, gradient: 5 to 90%

B over 15 min, monitored at 220 and 316 nm. HR electrospray ionization (ESI) mass spectra were recorded using an Agilent 6530 Accurate-Mass Q-TOF LC/MS with a nano LC and Orbitrap FT detector.

Expression, Purification and Activation of MAP Kinases - Activated full length ERK2 (Rattus norvegicus mitogen activated protein kinase 1, GenBank accession number NM_053842) was expressed and purified from bacteria then activated following our previously described protocol.[1] Activated full length ERK1 was expressed, purified and activated as described in Callaway et al.[2]

Preparation of cell lysates - Human non-small cell lung cancer cell line (A549-ATCC CCL-185) were purchased from the American type culture collection (ATCC; Manassas, VA). Human breast adenocarcinoma cell line (MDA-MB-231 – ATCC HTB-132) was a gift from Dr. Chandra Bartholomeusz, The University of Texas MD Anderson Cancer Center, Houston, TX, USA. Both cell lines were authenticated by genetic biomarkers (IDEXX BioResearch) and mycoplasma tests were performed monthly using MycoAlert™ Mycoplasma Detection Kit (Lonza). A549 cells were cultured in RPMI (Gibco) supplemented with 10% (*v/v*) Fetal Bovine Serum (FBS)-US grade (Gibco), 1× glutamax (Gibco), 100 U mL^{-1} penicillin (Gibco), and 100 μg mL^{-1} streptomycin (Gibco). MDA-MB-231 cells were maintained in DMEM/F12 (Gibco) with 10% (*v/v*) FBS-US grade (Gibco) in presence of 100 U mL^{-1} penicillin (Gibco) and 100 μg mL^{-1} streptomycin (Gibco). Cells were cultured in a humidified 5% CO_2 incubator at 37 °C. Cells were seeded in a 15 mm plate and incubated until 70-80% confluence. Cells were serum starved for 24 hours before incubation with U0126 (Tocris), JNK-IN-8 (Millipore) or DMSO for 2 hours. In some instances, after inhibitor or DMSO treatment, cells were induced by 100 nM EGF (Life technologies) for 30 min or 100 nM anisomycin (Cell signaling) for 30 minutes before lysis. A549 or MDA MB 231 cell lysates were prepared in M-PER Protein Extraction Reagent (Thermo) supplemented with protease and phosphatase inhibitors cocktail (Pierce) after washing in PBS (Gibco). The lysates were cleared by centrifugation, and Bradford assay reagent (Bio-Rad) was used to measure the protein concentration.

General Kinase assay protocol - Lysates (1-2 µl) were diluted into 15 µl of kinase assay buffer (25 mM HEPES pH 7.4, 50 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA with 2 mM DTT and 5 mM $MgCl_2$). Fluorescence was monitored using a BioTek Cytation 3 Plate Reader at 25 °C. In some cases, lysates were enriched with different concentrations of recombinant active ERK1 to construct a calibration regression model. The phosphorylation reaction was initiated upon the addition of SOX-peptide (5 µM final concentration) and ATP (500 µM final concentration) using an automatic BioTek dispenser (Rate 250 µL sec^{-1}). Assays were measured in 96-well, half-area, flat bottom, non-binding surface, black polystyrene plates. A final reaction volume of 30 µL was used in each well. Four experimental replicates were measured for each assay (λ_{ex} filter of 360 nm and λ_{em} filter of 485 nm, using a top mirror). Data were analyzed using the software OriginPro 8.5.1. The observed rate constants, corresponding errors and fluorescence values at F20, F30, F50 or F90 min were analyzed by linear discriminant analysis using the XLSTAT 2018 software package. The score factors from the LDA were used as input data for the multivariate analysis using support vector machine SVM (Solo 8.0). SVM was used to obtain the corresponding calibration regression models for each cell lysate and predictive values of ERK1 in each assay.

Kinase activity assay in cancer cell lysates - MDA-MB-231 and A549 cells were exposed to different treatments: serum starved control (n), inhibition of ERK phosphorylation by U0126 (U), induction of ERK phosphorylation by EGF (E), cells treated by U0126 then induced by EGF (E/U), inhibition of JNK pathway by JNK-IN-8 (J), induction of JNK phosphorylation by anisomycin (A), cells treated by JNK-IN-8 then induced by anisomycin (A/J). These were used to prepare different lysate solutions. To perform the assay, each lysate was diluted in kinase assay buffer in order to prepare lysate aliquots of 15 µL/well containing final protein content of 7 µg (high protein concentration assay) or 2 µg (low protein concentration assay). Peptide aliquots of 15 µL were added to the well plate to obtain a final concentration of 5 µM SOX-peptide and 500 µM ATP in the array.

Using recombinant active ERK1 to construct a calibration regression model for MDA-MB-231 and A549 cell lysates - Cell lysates were generated from MDA-MB-231 or A549 cells that were serum starved for 24 h then treated with U0126 (a MEK inhibitor) for 2 h before lysis. Lysates were enriched by different concentrations of recombinant ERK1 according to the following protocol. A 1 μM stock of active ERK1 was diluted into kinase assay buffer to obtain a final concentration of 0.1 μM. Individual 2× ERK1/lysate solutions were prepared in kinase assay buffer, containing the following concentrations of ERK1: 0 , 0.2, 0.4, 0.8 1.6, 3.2, 6.4 and 12.8 nM and final cell lysate content of 7 μg per 15 uL. 15 μL from each of these solutions were added to each well of the 96 well plate, to be mixed with 15 uL of the peptide aliquot to obtain a final concentration of 5 μM SOX-peptide and 500 μM ATP in the array.

U0126 Inhibition assays - Cell lysates from MDA-MB-231 cells treated with different doses (0, 0.2, 0.4, 1, 2.5, 6.25 and 15.65 μM) of the MEK inhibitor (U0126) were used to prepare final lysate solutions (7 μg/15 μL) using kinase assay buffer. Aliquots of 15 μL from each lysate were added to the array to obtain (7 μg lysate/well). Peptide aliquots of 15 μL were added to each well to obtain a final concentration of 5 μM SOX-peptide and 500 μM ATP in the array.

Animal Studies - Human A375 melanoma cell line (ATCC-CRL-1619) were purchased from the American type culture collection (ATCC; Manassas, VA). Cells were cultured in RPMI (Gibco) supplemented with 5% (*v/v*) Fetal Bovine Serum (FBS)-US grade (Gibco), 1× glutamax (Gibco), 100 U mL^{-1} penicillin (Gibco), and 100 μg mL^{-1} streptomycin (Gibco). 1×10^6 A375 cells were implanted *s.c.* into the right flank of twenty nude, athymic mice. Once tumors reached 150-200 mm^3 on average, mice were randomly assigned into two groups, and were intraperitoneally injected one time per day with either 15 mg kg^{-1} of BI-78D3 dissolved in (2.5% EtoH, 5% Tween-80, 1 × PBS vehicle) (treated gp.) or just the vehicle (control gp.). Tumor volumes and body weights were recorded each day. After 10 days of treatment, five mice from each group were euthanized, tumors were extracted, lysed and pooled into two

samples, one for the treated group and one for the control group. The lysates were cleared by centrifugation, and Bradford assay reagent (Bio-Rad) was used to measure the protein concentration. Cleared lysates were flash frozen in fractions with equal volumes and stored at -80 °C. The activated ERK content in each sample was estimated using the sensors array, western blot and immunoprecipitation-kinase assay protocols. The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas at Austin. All mice were allowed to acclimate for at least 1 week before use in experiments.

Kinase activity assay in the tumor extracts - Melanoma tumor extracts were used to prepare the following calibration regression model. Individual tumor solutions were prepared in assay buffer by adding the following 2× concentrations of recombinant active ERK1 or ERK2: 0, 0.8 1.6, 3.2, 6.4 and 12.8 nM, respectively. Tumor aliquots of 15 μL containing different 2× ERK1 concentrations and 7 μg of the tumor extract were then added to the array. Peptide aliquots of 15 μL were added to each well to obtain a final concentration of 5 μM SOX-peptide and 500 μM ATP in the array.

Western blot analysis - To estimate the amount of active ERK in each cell lysate using western blot, ERK1 or ERK2 calibration curve were constructed using different amounts of purified, recombinant, fully activated ERK1 or ERK2 (standard) that was generated essentially as described previously.[1] Five or six different known amounts of ERK standard were loaded to each gel side-by-side with lysates containing 65-70 μg of total protein. Proteins were fractionated on a 10% SDS polyacrylamide gel (Bio-Rad) and transferred to PVDF immunobilon-FL Membrane (Millipore). Primary antibodies were incubated overnight at 4 ºC using 1:2000 anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (E10) mouse mAb (Cell Signaling Technology) or 1:2000 anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (D13.14.4E) XP rabbit mAb (Cell Signaling Technology); 1:2000 anti-phospho-SAPK/JNK (Thr183/Tyr185) (G9) mouse mAb (Cat # 9255, Cell Signaling Technology); 1:1000 anti-JNK1 (2C6) mouse mAb (Cat # 3708, Cell Signaling Technology); (1:2000

anti-JNK2 (56G8) rabbit mAb (Cat # 9258, Cell Signaling Technology); 1:1000 anti-phospho-c-Jun (Ser-63) II rabbit polyclonal Ab (Cat # 9261, Cell Signaling Technology); 1:1000 anti-phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP rabbit mAb (Cat # 4511, Cell Signaling Technology); 1:1000 anti-p38 MAPK (D13E1) XP rabbit mAb (Cat # 8690, Cell Signaling Technology); 1:5000 anti-Vinculin (E1E9V) XP rabbit mAb (Cat # 13901, Cell Signaling Technology) and 1:5000 anti-actin, clone 4 mouse mAb (Millipore). Secondary anti-rabbit (Bio-Rad) or anti-mouse (Bio-Rad) horseradish peroxidase-conjugated secondary antibodies and Western Bright ECL Western Blotting Reagents (Advansta) were used to develop the blots. In the quantitative experiments, Li-Cor: IRDye 680RD goat anti-rabbit IgG and IRDye 800CW goat anti-mouse IgG secondary antibodies were used. Fluorescent western blot double staining was developed on the Odyssey fluorescent western blot system (Li-Cor). ERK calibration curve and unknown lysate concentrations were calculated using Image Studio Software. Precision Plus Protein™ Dual Color marker (Cat #1610374, Bio-Rad) was used in all the experiments. All western blot experiments were generally performed a minimum of two times.

Immune-complex protein-kinase assay - To immuno-precipitate ERK1/2, 2.5 µL of ERK1/2 antibody (anti p44/42 MAPK (ERK1/2) (137F5) rabbit mAb - Cell Signaling Technology) was added to 0.1-0.2 total µg of lysate and allowed to incubate overnight at 4 °C. Total protein was held constant across samples of each experiment and total volume was adjusted to 100 µl in all experiments. To develop phosphorylated ERK1 or ERK2 calibration curves, different amounts (1, 2, 4, 6 and 8 nM) of purified, recombinant, fully activated ERK1 or 2 (standard), expressed, purified and activated following our previous protocol,[1] were incubated with the same amount of the ERK1/2 antibody under the same conditions. Then, 20 µL of a 50% slurry of recombinant protein G agarose beads (Invitrogen # 15920010) was added to each sample and allowed to incubate for three more hours at 4 °C. Beads were then washed 5× in 500 µL lysis buffer (50 mM Tris HCl, pH 7.4, with 150 mM NaCl, 1 mM EDTA, and 1% Triton

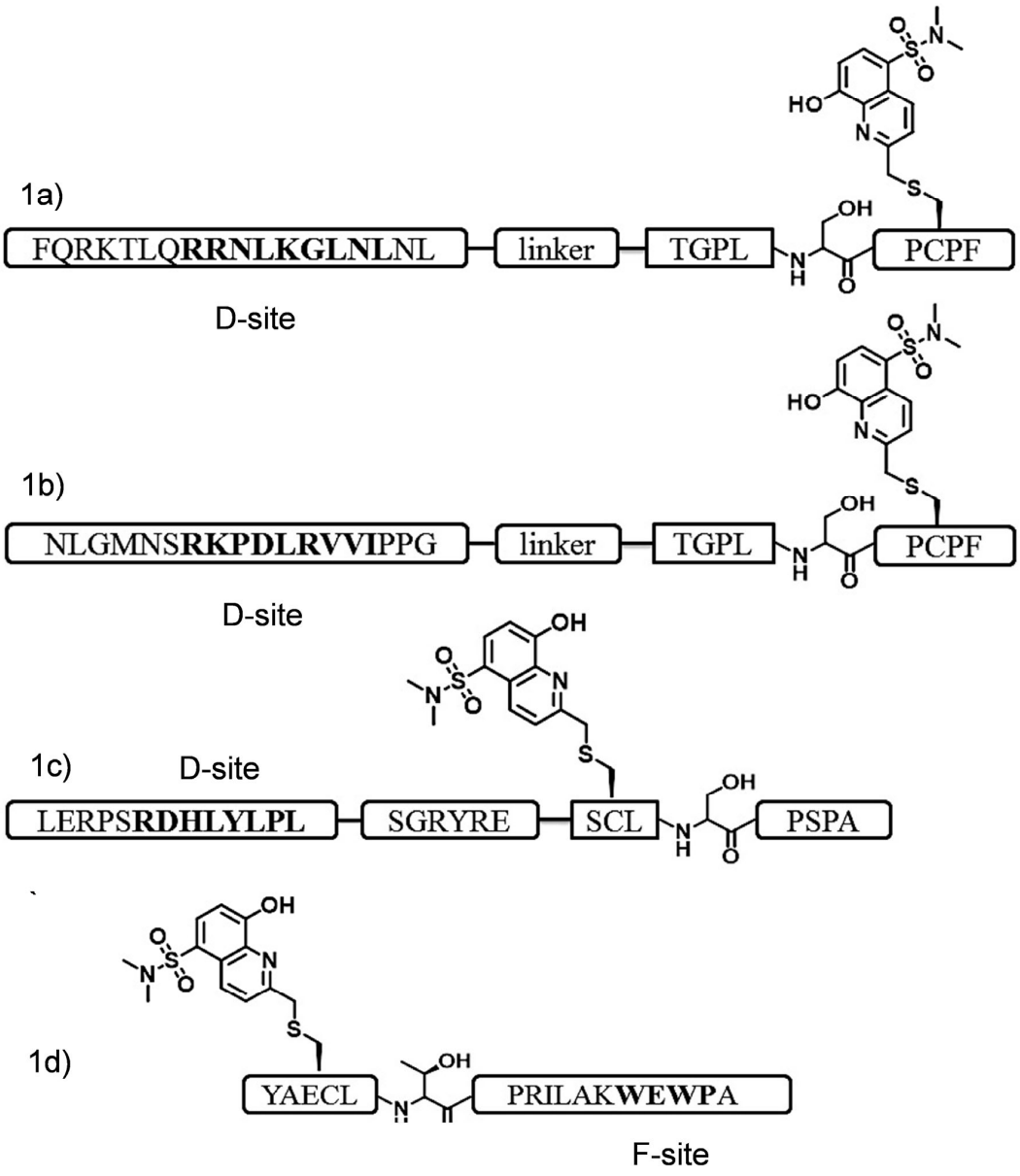
X-100), supplemented with protease and phosphatase inhibitors and 2× in 500 µL assay buffer (25 mM HEPES, pH 7.5, 10 µg mL^{-1} BSA, 10 mM $MgCl_2$, 50 mM KCl, 100 µM EGTA and 100 µM EDTA).

10 µL of beads per sample were suspended in kinase assay buffer (25 mM HEPES, pH 7.5, 2 mM DTT, 10 µg mL^{-1} BSA, 10 mM $MgCl_2$, 50 mM KCl, 100 µM EGTA, and 100 µM EDTA), containing 100 µM His-Ets-1 (1-138), expressed and purified following our previously published protocol.[3] Enzymatic reactions were initiated by the addition of 0.5 mM [γ-32P]-ATP and beads were kept in suspension by continuous shaking. Reaction progress curves were obtained by spotting 10 µL samples at 0.5, 1, 2, 4 and 8 minute intervals onto P81 paper, washed and quantified as described previously.[1]

SUPPORTING REFERENCES

1. Kaoud, T. S., Devkota, A. K., Harris, R., Rana, M. S., Abramczyk, O., Warthaka, M., Lee, S., Girvin, M. E., Riggs, A. F., and Dalby, K. N. (2011) Activated ERK2 is a monomer in vitro with or without divalent cations and when complexed to the cytoplasmic scaffold PEA-15, *Biochemistry 50*, 4568-4578.
2. Callaway, K., Abramczyk, O., Martin, L., and Dalby, K. N. (2007) The anti-apoptotic protein PEA-15 is a tight binding inhibitor of ERK1 and ERK2, which blocks docking interactions at the D-recruitment site, *Biochemistry 46*, 9187-9198.
3. Kaoud, T. S., Park, H., Mitra, S., Yan, C., Tseng, C. C., Shi, Y., Jose, J., Taliaferro, J. M., Lee, K., Ren, P., Hong, J., and Dalby, K. N. (2012) Manipulating JNK signaling with (--)-zuonin A, *ACS Chem Biol 7*, 1873-1883.

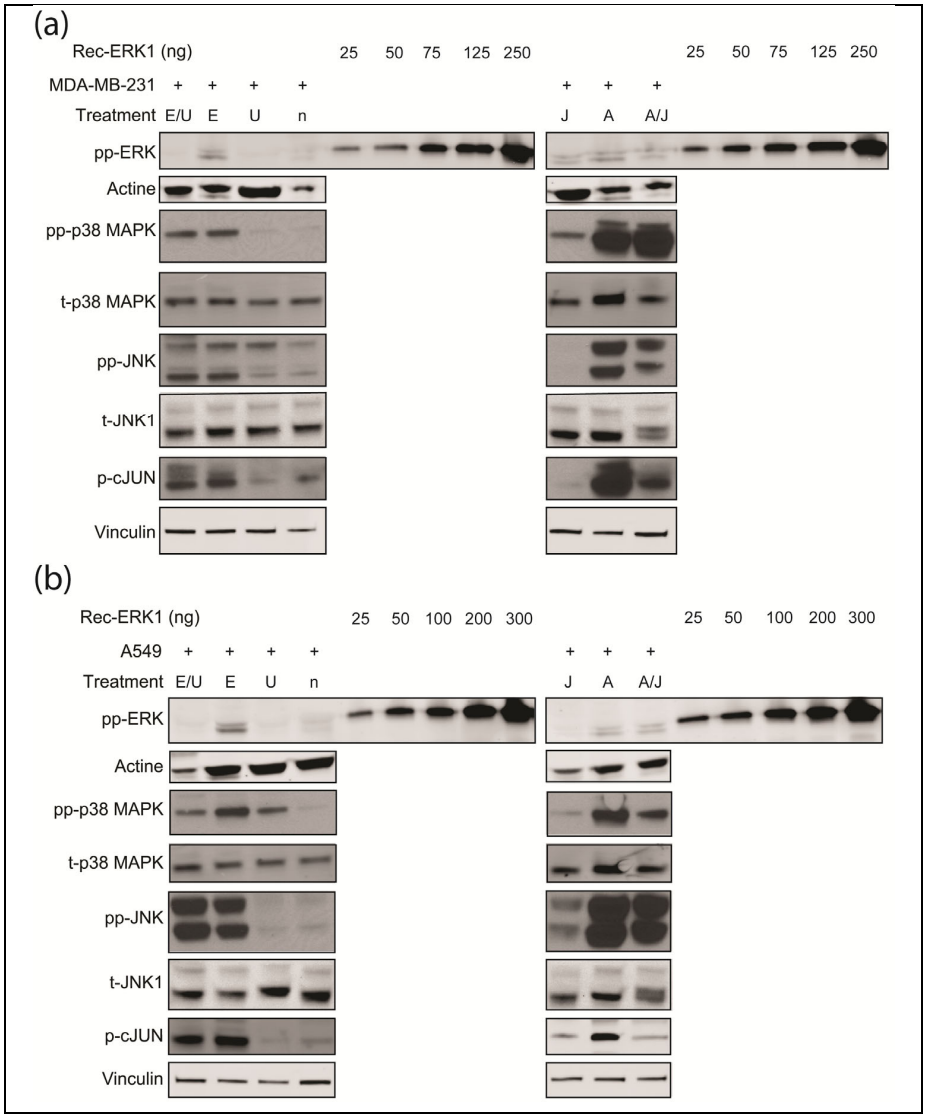
A. SUPPORTING SCHEMES



Scheme 1. SOX-peptide substrates presenting docking sites for MAPK recognition used for the sensing array. Linker = three 6-aminohexanoic acid group.

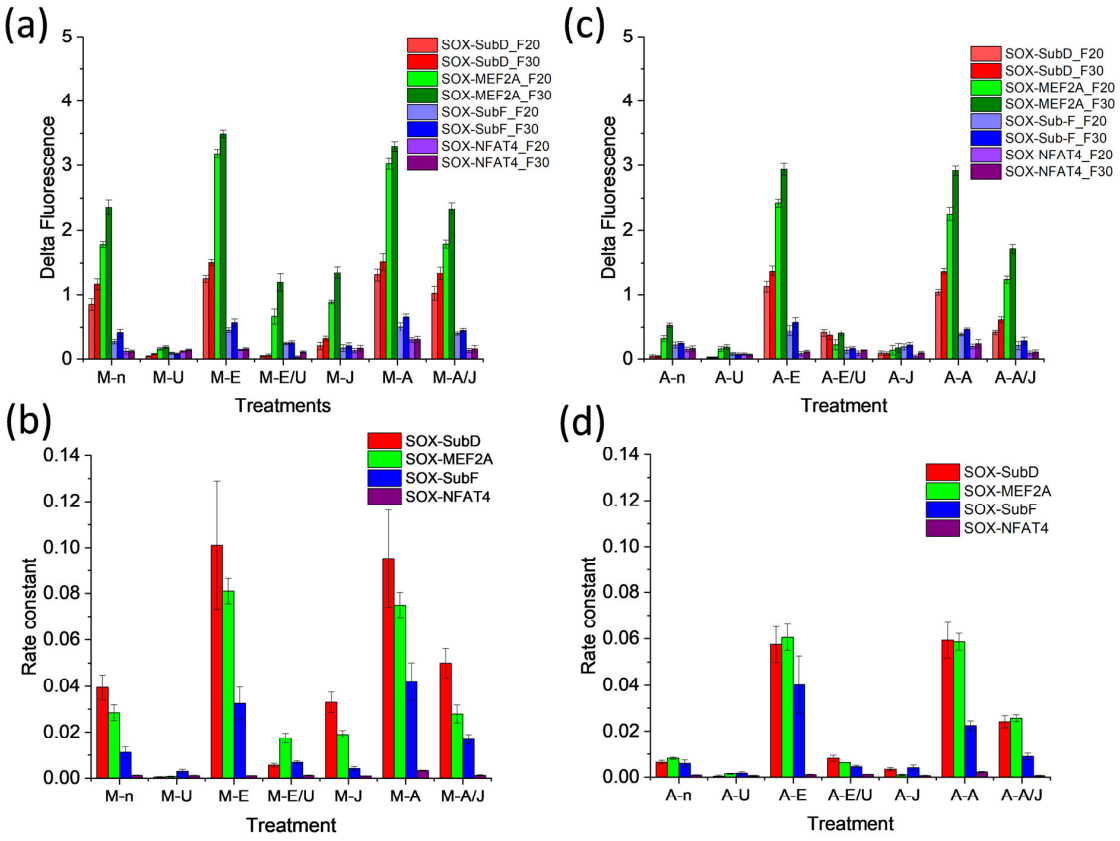
B. SUPPORTING FIGURES

Figure S1. Western blots of MDA-MB-231 and A549 cell lysates



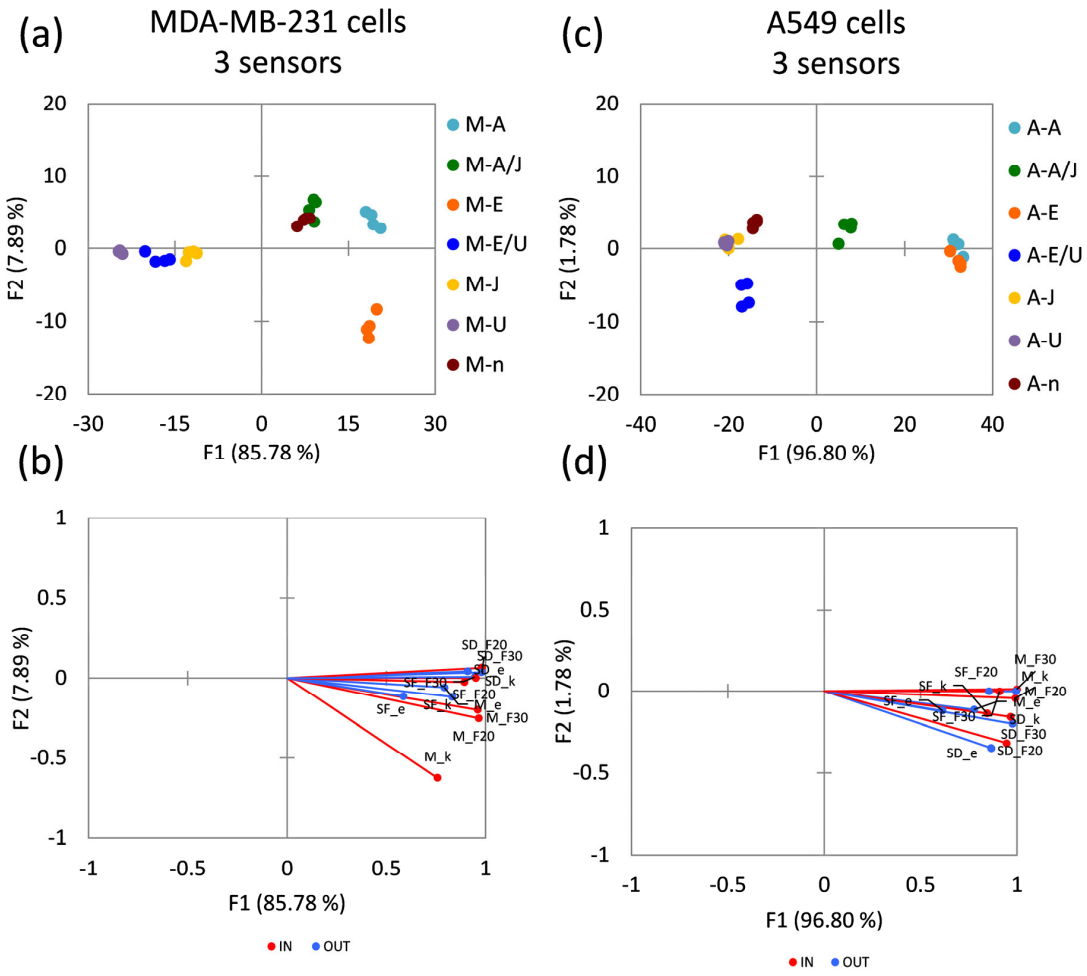
Western blot analysis of different MDA-MB-231 (a) and A549 (b) cell lysates used in this study. The cells were serum starved for 24 hours before incubation with 10 μM U0126 (ERK pathway inhibitor), 10 μM JNK-IN-8 (JNK inhibitor) or DMSO for 2 hours. Wherever mentioned, cells were treated with 100 nM epidermal growth factor (EGF) for 30 min or 100 nM anisomycin (which activates JNK) for 15-20 minutes before lysis. (E) Represents cells stimulated by EGF or (A) by anisomycin. (U) Represents cells treated with the MEK inhibitor U0126 or (J) with JNK-IN-8. (E/U) represents cells treated with the MEK inhibitor and then induced by EGF. (A/J) represents cells treated with JNK-IN-8 then induced by anisomycin. (n) represents serum starved non-treated cells. Lysates containing 65 μg of total protein and different known amounts of purified, recombinant, fully phosphorylated ERK1 were fractionated by SDS PAGE (10% gel) and subjected to western blot analysis using a LI-COR Odyssey in order to quantify the phosphorylated forms of ERK1/2. In each gel, the calculated signals of recombinant ERK1 were employed to build a calibration curve and actin signals were used as a loading control for the cell lysates (the results were similar in two different experiments). To study the activity of JNK and p38 MAPK in the tested lysates, the lysates were subjected to western blot and analyzed for pp-p38 MAPK, p38 MAPK, pp-JNK, JNK1, p-cJUN and vinculin (loading control).

Figure S2. Fingerprint of cell lysates with different MAPK expression



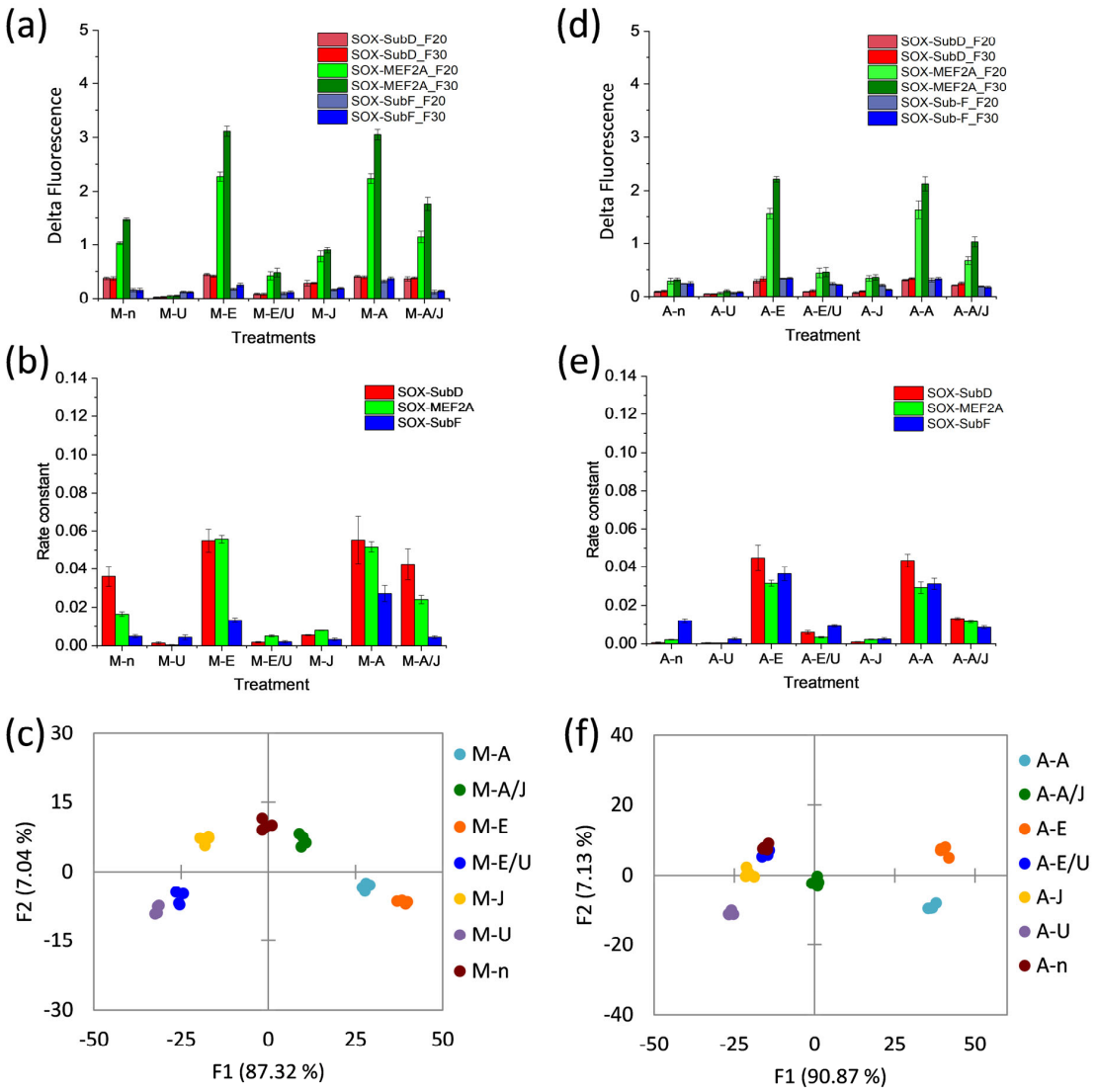
First experiment at high protein concentration: Fingerprint of MDA-MB-231 and A549 cell lines presenting differing kinase expression. (a, c) Fluorescence and (b, d) rate constant patterns generated by peptides upon phosphorylation by MDA-MB-231 (left column) and A549 (right column) cell lysates using 7 µg/well of total protein concentration. Serum starved control (n), Cells treated by MEK inhibitor U0126 (U), Cells induced by EGF (E), Cells treated by U0126 then induced by EGF (E/U), Cells treated by JNK inhibitor JNK-IN-8 (J), Cells induced by anisomycin (A), Cells treated by JNK-IN-8 then induced by anisomycin (A/J). Data represents the average of four experimental replicates, $\Delta F \times 1000$ was calculated by subtracting the fluorescence background from the observed fluorescence. The rate constant unit is min^{-1}.

Figure S3.



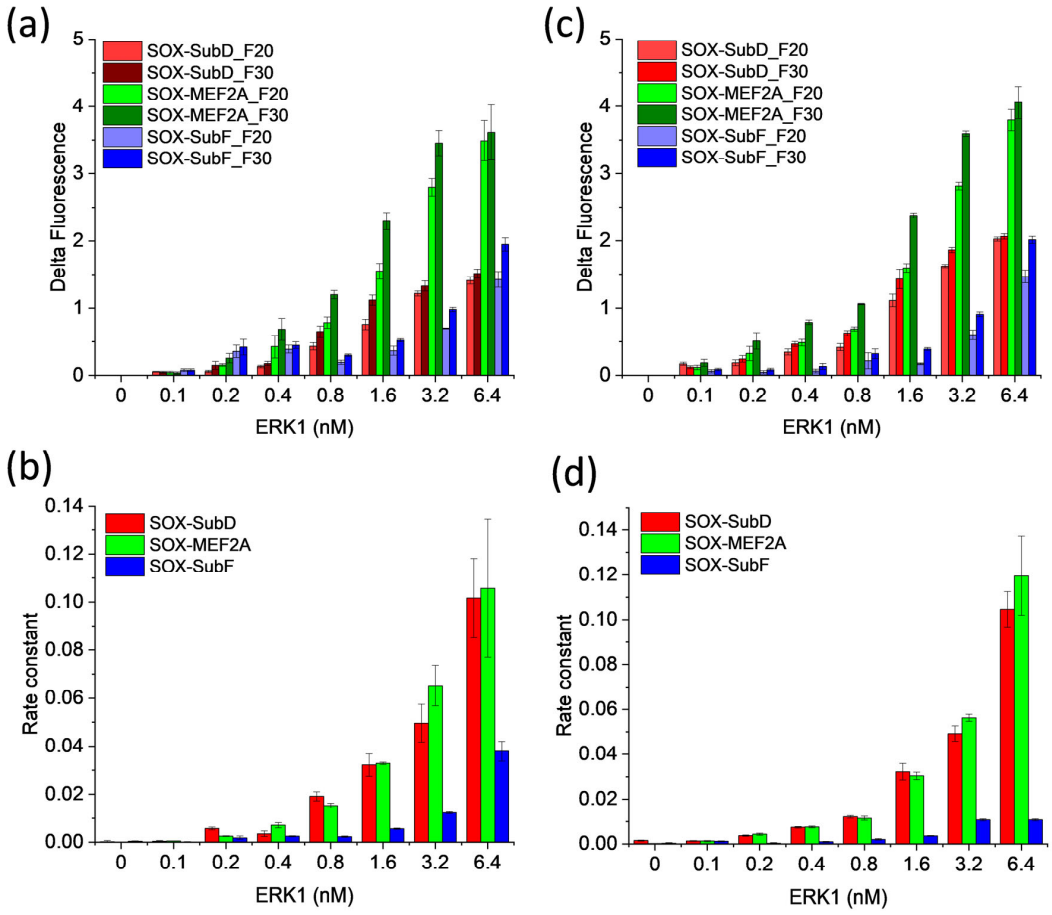
First experiment at high protein concentration: LDA score plots and loading plots of different MDA-MB-231 and A549 cell lysates, classifying differing kinase expression using 3 peptide sensors. (a, c) LDA score plots and (b, d) loading plots of the response from the sensing array showing differentiation of sets of lysates of serum starved, inhibited and/or stimulated MDA-MB-231 (left column) and A549 (right column) cell lines with 100% and 82.14% cross-validation using 3 sensors. Vectors corresponding to the contribution of SOX-SubD (SD_F30,F20,k,e), SOX-MEF2A (M_F30,F20,k,e) and SOX-SubF (SF_F30,F20,k,e) to the differentiation of different cell lysates are colored in blue and red. Serum starved control (n), Cells treated by MEK inhibitor U0126 (U), Cells induced by EGF (E), Cells treated by U0126 then induced by EGF (E/U), Cells treated by JNK inhibitor JNK-IN-8 (J), Cells induced by anisomycin (A), Cells treated by JNK-IN-8 then induced by anisomycin (A/J). F30, F20, k, e correspond to ΔF (30 min), ΔF (20 min), rate constant and error, respectively.

Figure S4.



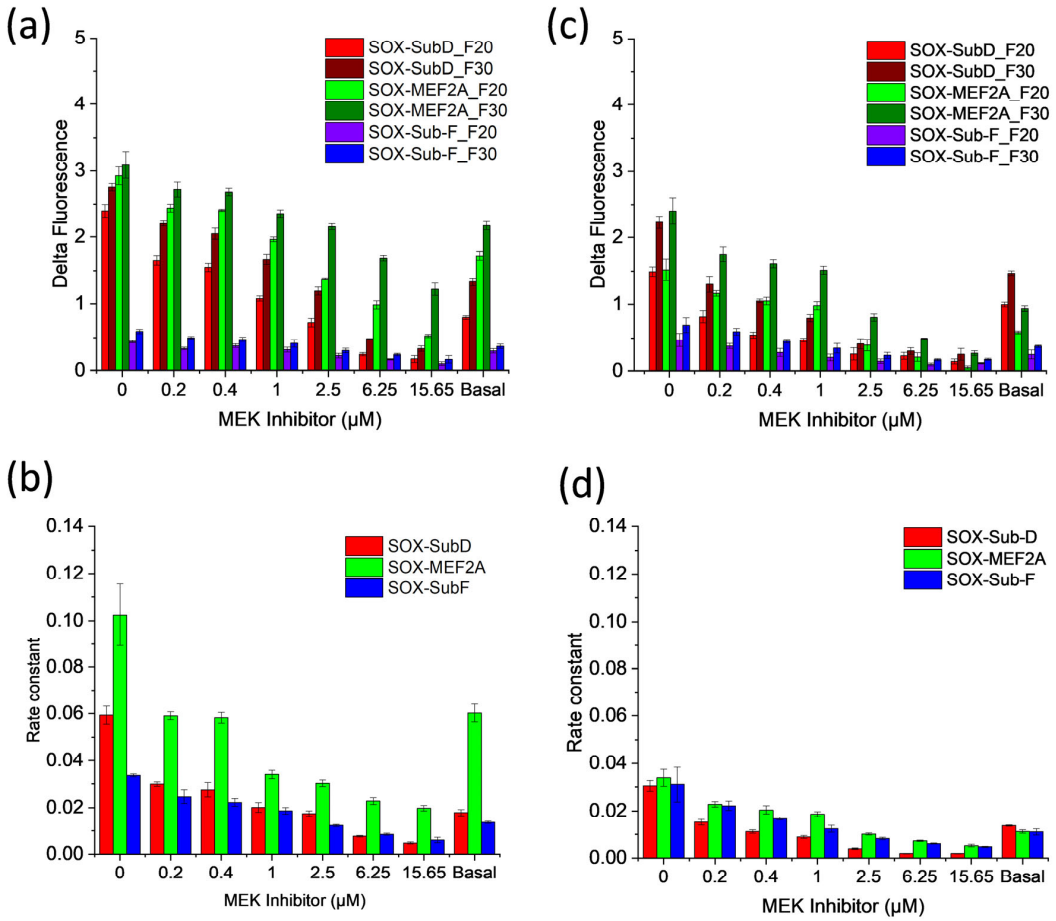
Second experiment at low protein concentration: Fingerprints and LDA score plots of different MDA-MB-231 and A549 cell lysates classifying differing kinase expression. (a, d) Fluorescence and (b, e) rate constant patterns generated by three peptide sensors upon phosphorylation by MDA-MB-231 (left) and A549 (right) cell lysates using 2 µg/well total protein. (c, f) LDA score plots of the response from the sensing array showing differentiation of sets of lysates of serum starved, inhibited and/or stimulated MDA-MB-231 (left) and A549 (right) cell lines with 100% and 85.71% cross-validation, respectively. Serum starved control (n), Cells treated by MEK inhibitor U0126 (U), Cells induced by EGF (E), Cells treated by U0126 then induced by EGF (E/U), Cells treated by JNK inhibitor JNK-IN-8 (J), Cells induced by anisomycin (A), Cells treated by JNK-IN-8 then induced by anisomycin (A/J). Data represents the average of four experimental replicates, $\Delta F \times 1000$ was calculated by subtracting the fluorescence background from the observed fluorescence. The rate constant unit is min^{-1}.

Figure S5. Increasing ERK concentration in cell lysates



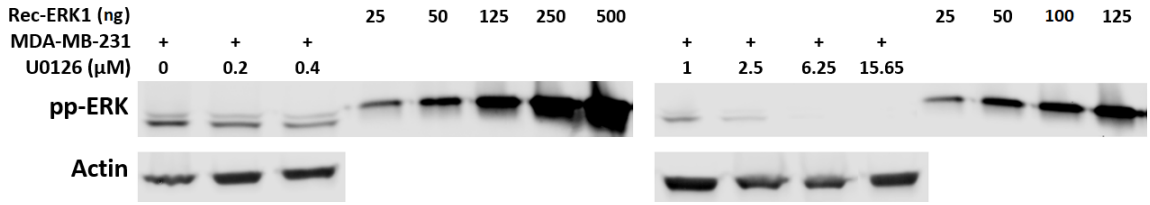
Fingerprint of increasing concentration of recombinant, fully phosphoryated ERK1 in MDA-MB-231 and A549 cell lysates. (a, c) Fluorescence and (b, d) rate constant patterns generated by peptides upon phosphorylation by 7 µg cell lysates contain known amounts of recombinant, activated ERK1. Cell lysates MDA-MB-231 (left column) and A549 (right column) were generated from cells serum starved for 24 hrs then treated by U0126 (MEK inhibitor) for 2 hours before lysis. Each ERK1 concentration represents the average of four experimental replicates. ΔF ×1000 was calculated by subtracting the fluorescence background from the observed fluorecence. The rate constant unit is min^{-1}.

Figure S6. Cells treated by different doses of the MEK inhibitor (U0126)



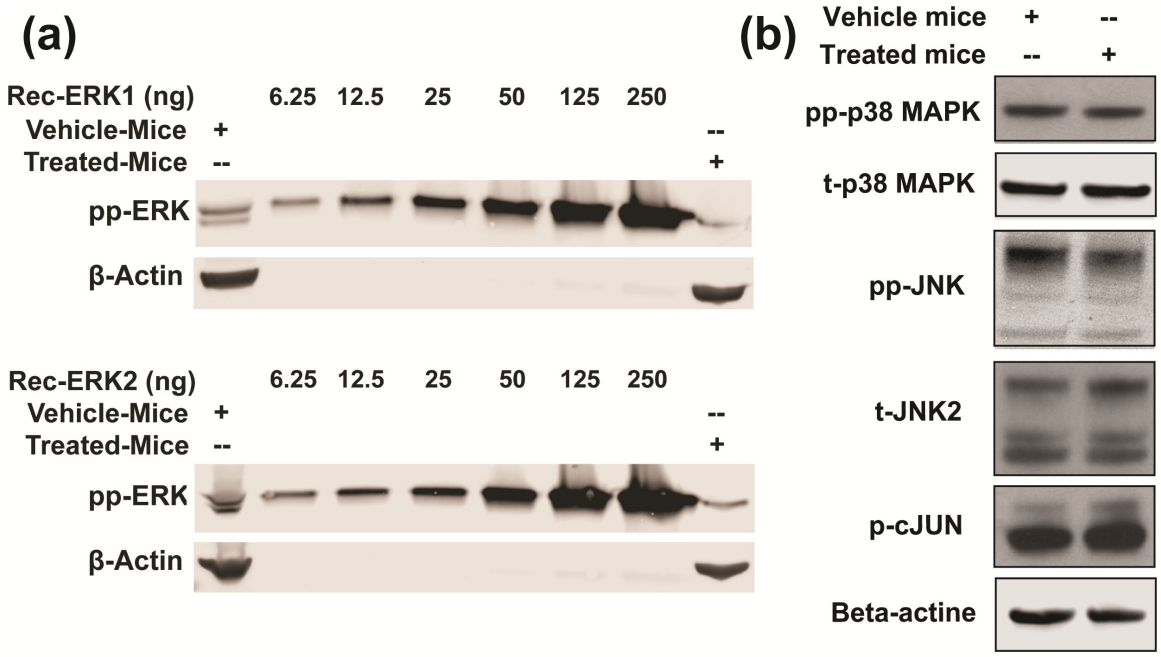
Two experimental replicates of the fingerprints of different MDA-MB-231 lysates produced by the peptide array. Cells were treated by increasing the concentration of the MEK inhibitor (U0126) before induction by EGF and lysis. (a, c) Fluorescence and (b, d) rate constant patterns generated by peptides upon phosphorylation by 7 µg of each lysate. Data represents the average of four experimental replicates, ΔF ×1000 was calculated by subtracting the fluorescence background from the observed fluorescence. The rate constant unit is min^{-1}.

Figure S7. Western blots of MDA-MB-231 cell lysates with U0126 inhibitor



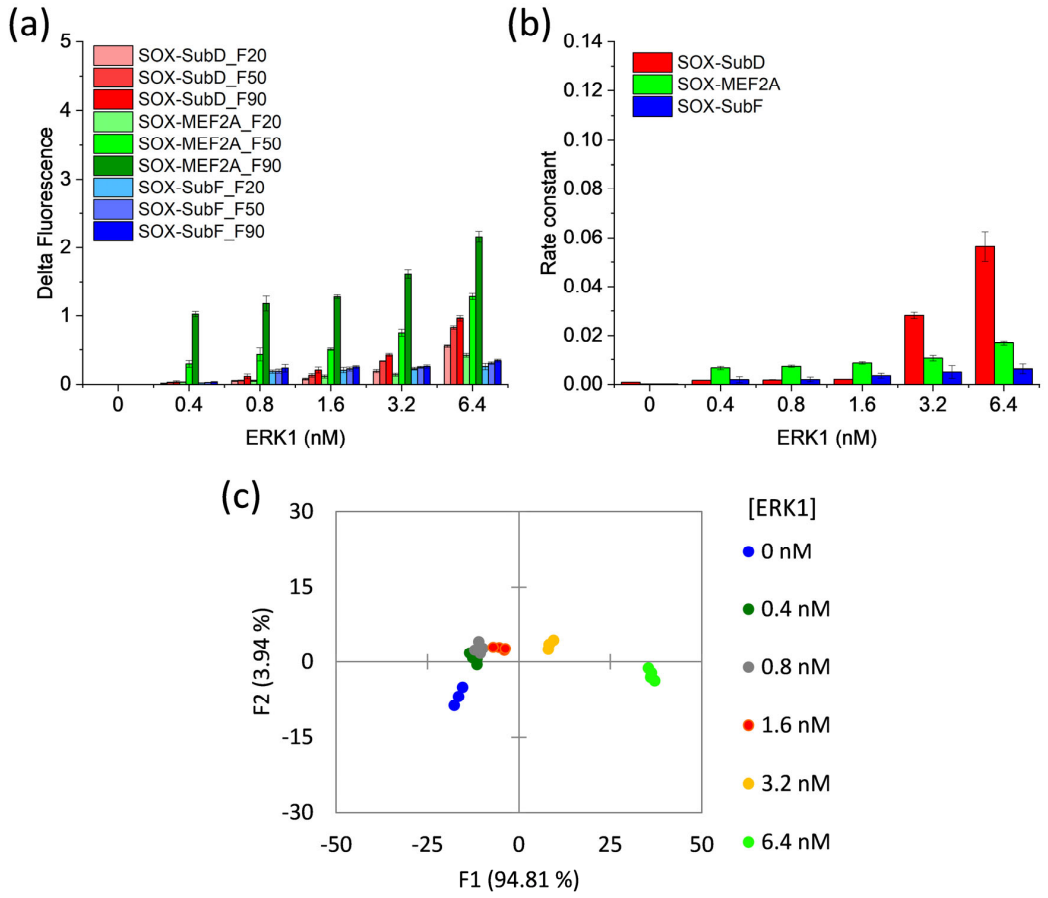
Western blot analysis of MDA-MB-231 cells treated with different doses of U2016. The cells were serum starved for 24 hours before incubation with different concentrations of the ERK pathway inhibitor U0126 (0-15.65 µM) for 2 hours. Then cells were stimulated by 100 nM EGF for 30 minutes and lysed. Lysates containing 70 µg of total protein and different known amounts of purified, recombinant, fully phosphorylated ERK1 were fractionated by SDS PAGE (10% gel) and subjected to western blot analysis using a LI-COR Odyssey, in order to quantify the phosphorylated form of ERK in lysates. In each gel, the calculated signals of recombinant ERK1 were employed to build a calibration curve and actin signals were used as a loading control for the cell lysates.

Figure S8. Western blots of the tumor samples



Western blot analysis of the tumor samples extracted from five nude mice bearing melanoma A375 xenografts treated with control vehicle or 15 mg kg^{-1} of BI-78D3. Extracts containing 70 µg total protein and different known amounts of purified, recombinant, fully phosphorylated ERK1 or ERK2 were fractionated by SDS PAGE (10% gel) and subjected to western blot analysis using a LI-COR Odyssey. (a) In order to quantify the phosphorylated form of ERK in each extract, the calculated signals of recombinant ERK1 or ERK2 at concentrations of 6.25, 12.5, 25 and 50 ng were employed to build a calibration curve (the signal reached saturation at 125 and 250 ng, respectively). β-actin signals were employed as a loading control for the tumor extracts. (b) To study the activity of JNK and p38 MAPK in the tumor samples, tumor extracts were subjected to western blot and analyzed for pp-p38 MAPK, p38 MAPK, pp-JNK, JNK2, p-cJUN and beta-actine (loading control).

Figure S9. Increasing concentration of ERK in tumor lysates derived from BI-78D3-treated mice

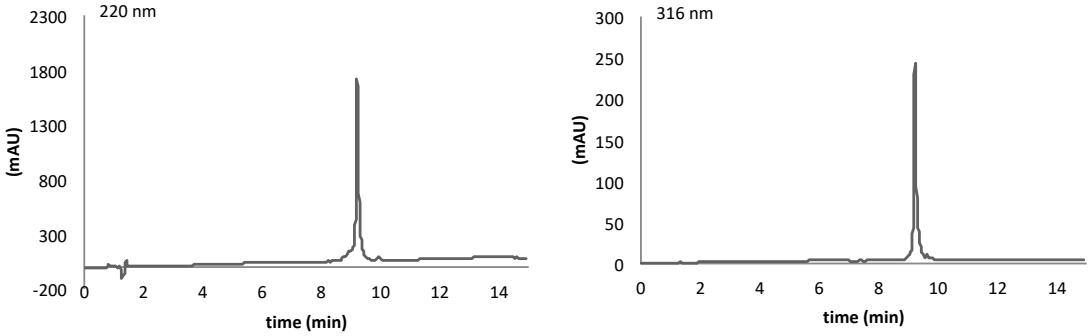


Fingerprints and LDA score plot of tumor samples that were enriched by increasing concentrations of recombinant, fully phosphorylated ERK1. (a) Fluorescence and (b) rate constant patterns generated by peptides upon phosphorylation by 7 µg of tumor lysates contain known amounts of recombinant, activated ERK1. The tumor samples were extracted from five nude mice bearing melanoma A375 xenografts treated with 15 mg kg^{-1} BI-78D3 (methods section). Extracts from the five mice were pooled into one sample and enriched by different concentrations of recombinant ERK1. Each ERK1 concentration represents the average of four experimental replicates. ΔF ×1000 was calculated by subtracting the fluorescence background from the observed fluorescence. The rate constant units are min^{-1} (c) LDA score plot showing the response of the biosensors to increasing recombinant ERK1 concentrations that were added to the tumor sample.

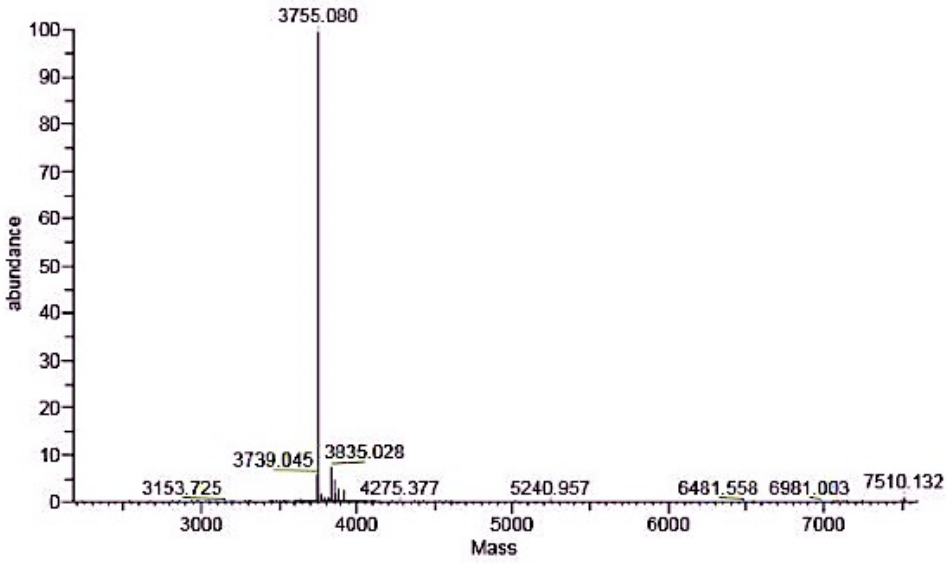
D. SUPPORTING CHARACTERIZATION OF SYNTHESIZED PEPTIDES

Figure S10: SOX-Sub-D peptide

(a)



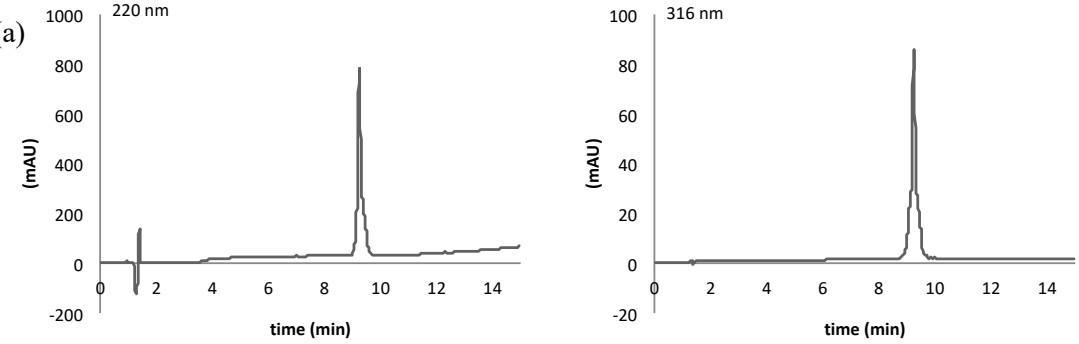
(b)



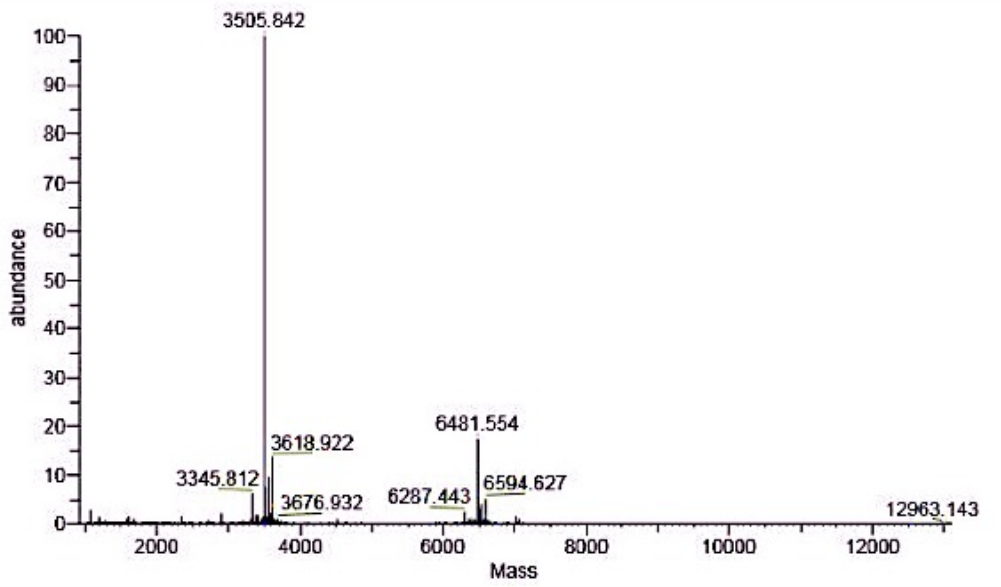
HPLC chromatogram and MS data of SOX-Sub-D peptide. (a) HPLC gradient: 5 to 90% B over 15 min, r.t. = 9.20 min at 220 nm and 316 nm correspondingly. (b) ESI obsd. m/z $[M]^+$ = 3755.080, calc. m/z $[M]^+$ = 3755.06.

Figure S11: SOX-MEF2A peptide

(a)



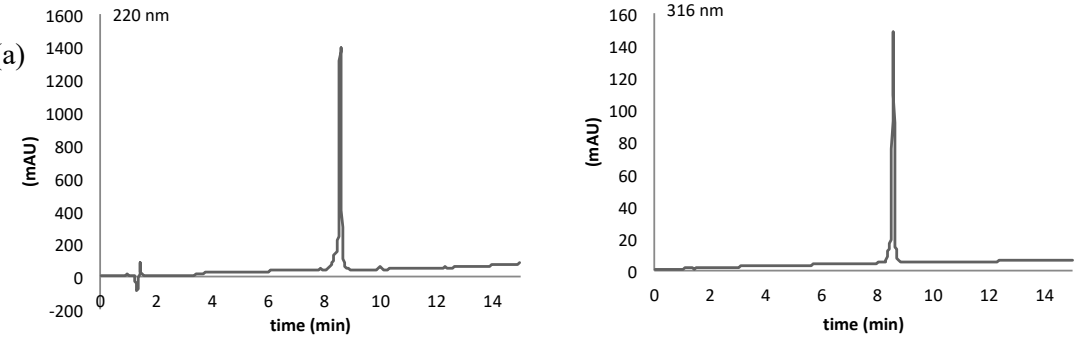
(b)



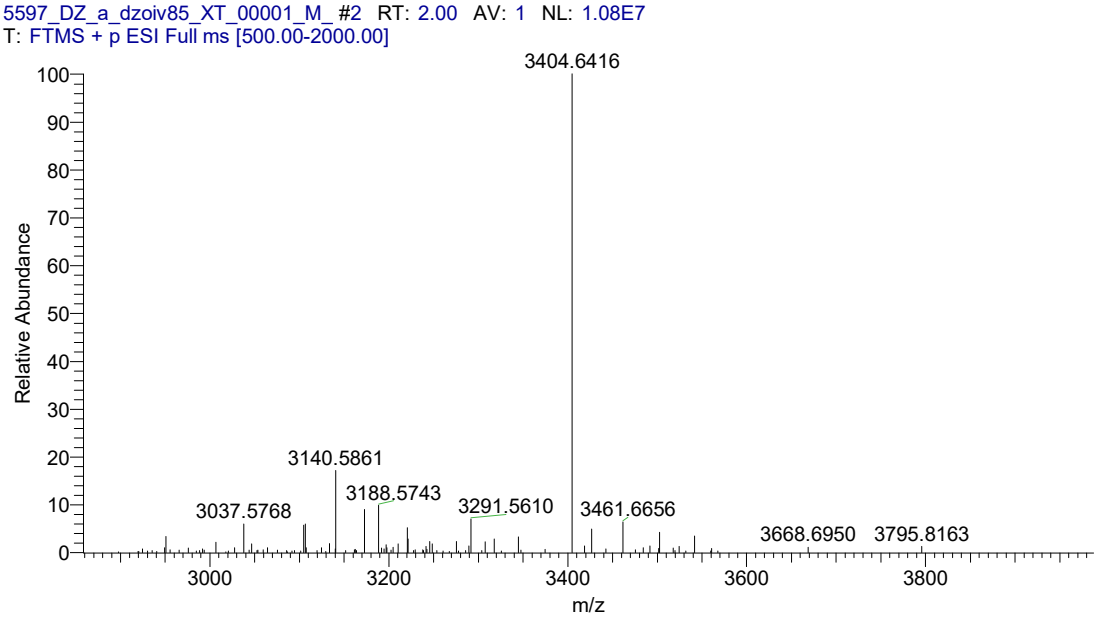
HPLC chromatogram and MS data of SOX-MEF2A peptide. (a) HPLC gradient: 5 to 90% B over 15 min, r.t. = 9.22 min at 220 nm and 316 nm correspondingly. (b) ESI obsd. m/z $[M]^+$ = 3505.842, calc. m/z $[M]^+$ = 3505.84.

Figure S12: SOX-NFAT4 peptide

(a)

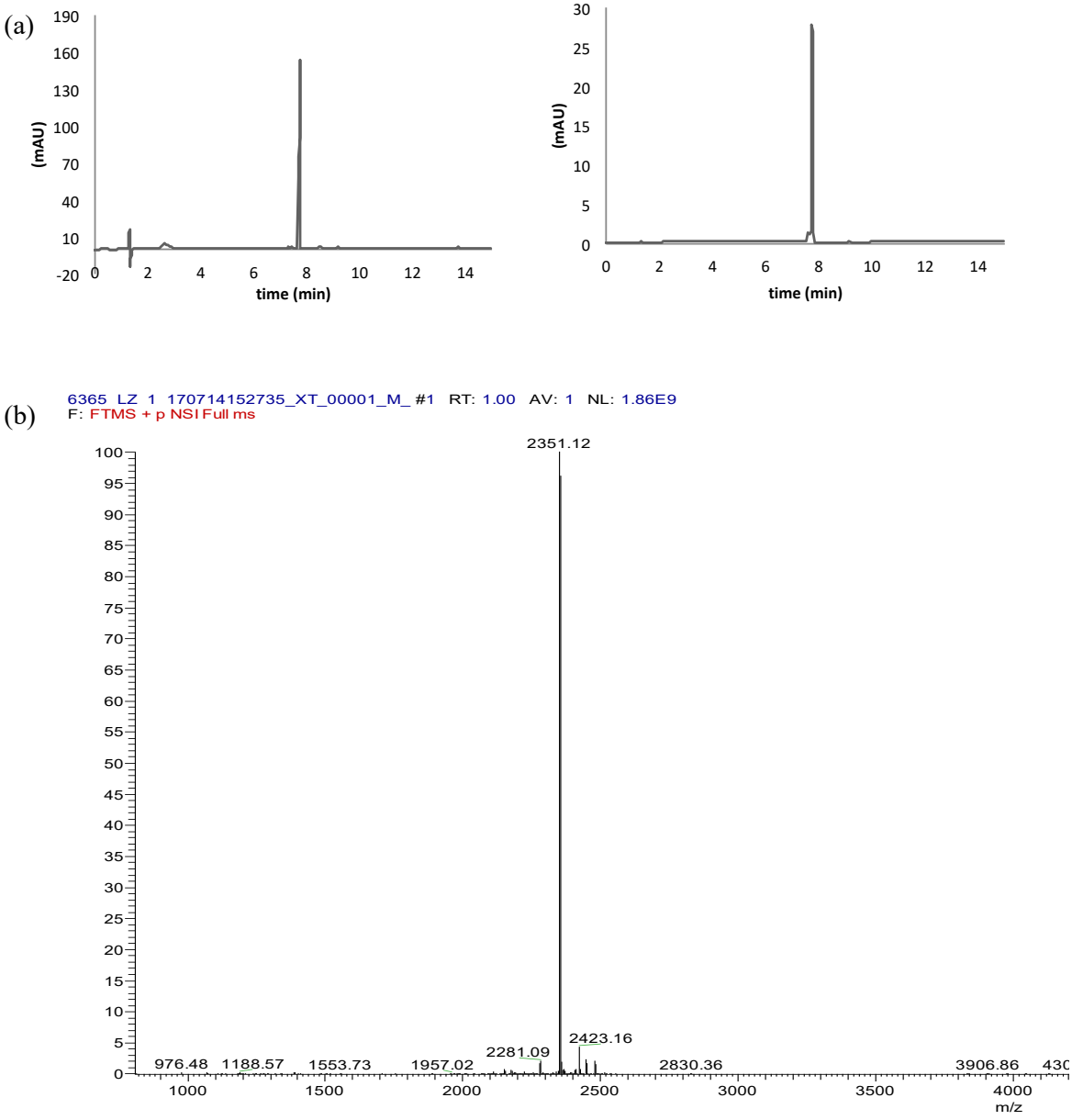


(b)



HPLC chromatogram and MS analysis of SOX-NFAT4 peptide. (a) HPLC gradient: 5 to 90% B over 15 min, r.t. = 8.55 min at 220 nm and 316 nm correspondingly. (b) ESI obsd. m/z $[M]^+$ = 3404.6416, calc. m/z $[M]^+$ = 3404.64.

Figure S13: SOX-Sub-F peptide



HPLC chromatogram and MS analysis of SOX-Sub-F peptide. (a) HPLC gradient: 5 to 90% B over 15 min, r.t. = 7.75 min at 220 nm and 316 nm correspondingly. (b) ESI obsd. m/z $[M]^+$ = 2351.12, calc. m/z $[M]^+$ = 2351.12.

E. SUPPORTING TABLES

Table S1. Characterization data of peptides

Peptide	Peptide sequence	Molecular Formula	ESI $[M]^+$ Calcd. *m/z*	ESI $[M]^+$ Found *m/z*	HPLC r.t. (min)
1a	Ac- FQRKTLQRRNLKGLNLNLXXXTGPLSPC(SOX)PF-NH_2	$C_{171}H_{279}N_{49}O_{42}S_2$	3755.06	3755.080	9.20
1b	Ac-NLGMNSRKPDLRVVIPPGXXXTGPLSP**CSOX**PF-NH_2	$C_{159}H_{256}N_{42}O_{41}S_3$	3505.84	3505.842	9.22
1c	Ac-LERPSRDHLYLPLSGRYRES**CSOX**LSPSPA-OH	$C_{149}H_{233}N_{43}O_{45}S_2$	3404.64	3404.641	8.55
1d	YAE**CSOX**LTPRILAKWEWPA	$C_{111}H_{157}N_{25}O_{28}S_2$	2351.12	2351.12	7.75

Table S2. The jack-knife classification matrix of different treatments in MDA-MB-231 cell lines. (for LDA score plot in Figure 2a).

Confusion matrix for the cross-validation results:

from \ to	M-A	M-A/J	M-E	M-E/U	M-J	M-U	M-n	Total	%correct
M-A	4	0	0	0	0	0	0	4	100.00%
M-A/J	0	4	0	0	0	0	0	4	100.00%
M-E	0	0	4	0	0	0	0	4	100.00%
M-E/U	0	0	0	4	0	0	0	4	100.00%
M-J	0	0	0	0	4	0	0	4	100.00%
M-U	0	0	0	0	0	4	0	4	100.00%
M-n	0	0	0	0	0	0	4	4	100.00%
Total	4	4	4	4	4	4	4	28	100.00%

Table S3. The jack-knife classification matrix of different treatments in A549 cell lines. (for LDA score plot in Figure 2b).

Confusion matrix for the cross-validation results:

from \ to	A-A	A-A/J	A-E	A-E/U	A-J	A-U	A-n	Total	% correct
A-A	4	0	0	0	0	0	0	4	100.00%
A-A/J	0	4	0	0	0	0	0	4	100.00%
A-E	0	0	4	0	0	0	0	4	100.00%
A-E/U	0	0	0	4	0	0	0	4	100.00%
A-J	0	0	0	0	2	2	0	4	50.00%
A-U	0	0	0	0	0	4	0	4	100.00%
A-n	0	0	0	0	0	0	4	4	100.00%
Total	4	4	4	4	2	6	4	28	92.86%

Table S4. The jack-knife classification matrix of ERK inhibition in MDA-MB-231 cell lines (for LDA score plot in Figure 5a).

Confusion matrix for the cross-validation results:

from \ to	0 μM	0.2 μM	0.4 μM	1 μM	15.65 μM	2.5 μM	6.25 μM	Total	% correct
0 μM	3	0	0	0	0	0	0	3	100.00%
0.2 μM	0	3	0	0	0	0	0	3	100.00%
0.4 μM	0	0	3	0	0	0	0	3	100.00%
1 μM	0	0	0	3	0	0	0	3	100.00%
15.65 μM	0	0	0	0	3	0	0	3	100.00%
2.5 μM	0	0	0	0	0	3	0	3	100.00%
6.25 μM	0	0	0	0	0	0	3	3	100.00%
Total	3	3	3	3	3	3	3	21	100.00%

Table S5. The jack-knife classification matrix of ERK inhibition in MDA-MB-231 cell lines (for LDA score plot in Figure 5b).

Confusion matrix for the cross-validation results:

from \ to	0 μM	0.2 μM	0.4 μM	1 μM	15.65 μM	2.5 μM	6.25 μM	Total	% correct
0 μM	4	0	0	0	0	0	0	4	100.00%
0.2 μM	0	4	0	0	0	0	0	4	100.00%
0.4 μM	0	0	4	0	0	0	0	4	100.00%
1 μM	0	0	0	4	0	0	0	4	100.00%
15.65 μM	0	0	0	0	4	0	0	4	100.00%
2.5 μM	0	0	0	0	0	4	0	4	100.00%
6.25 μM	0	0	0	0	0	0	4	4	100.00%
Total	4	4	4	4	4	4	4	28	100.00%

Table S6. Values of activated ERK concentration predicted by the regression model, western blot and immunoprecipitation-kinase assay protocols, in lysates of MDA-MB-231 cells treated with different concentrations of the MEK inhibitor U0126.

Cells	Treatment U0126 (μM)	pp-ERK (ng μg^{-1} Lysate)				
		Sensors		Western blot	Kinase assay	
		Mean (n=4)	±SD		Value (n=1)	±SE*
MDA-MB-231	0	0.529	0.023	0.665	0.490	0.077
	0.2	0.397	0.025	0.371	0.422	0.096
	0.4	0.271	0.012	0.272	0.244	0.091
	1	0.268	0.041	0.214	0.224	0.085
	2.5	0.039	0.010	0.115	0.049	0.031
	6.25	0.038	0.008	0.033	0.040	0.042
	15.65	0.050	0.008	0.020	0.040	0.021
IC50 (μM)**		0.33±0.09		0.31±0.035	0.31±0.035	

Recombinant ERK1 calibration curves were employed to generate the data in this table.
*Standard Error of Regression Slope.
**IC50 of the ERK pathway inhibition by U0126 was calculated using the values generated by each used protocol. Data were fitted to equation 1: $C_0 = C_{00} - \left(C_{00} \frac{i}{i + (K_{50})} \right) + C'$

The parameters used in deriving equation 1 are defined as follows; i, concentration of inhibitor I; C_o, observed pp-ERK concentration; C_{oo}, is the observed pp-ERK concentration in the absence of inhibitor, C' is the observed pp-ERK concentration at saturating inhibitor, I; K_{50} is the concentration that leads to half the maximal change in C_o.

Table S7. Comparison of the predicted values of activated ERK in the tumor samples that were extracted from five nude mice bearing melanoma A375 xenografts treated with control vehicle or 15mg kg^{-1} of the covalent ERK inhibitor BI-78D3. The values were obtained using the sensor array/calibration regression model, western blot and immune-complex protein-kinase assay protocols.

Treatment	pp-ERK (ng μg^{-1} Lysate)						
	Sensors		Western blot*		*p*-value (two-tailed)** $p \leq 0.05$	Kinase assay	
	Mean (n=4)	±SD	Mean (n=2)	±SD		Value (n=1)	±SE***
Treated Mice	0.004	0.001	0.007	0.005	0.260	0.007	0.007
Vehicle Mice	0.119	0.012	0.143	0.028	0.178	0.135	0.054

*Average of calculated pp-ERK values using Rec-ERK1 and Rec-ERK2 calibration experiments.
**Unpaired *t* test and *p-value* calculations for the sensors and the western blot means were done using GraphPad Prism 7 software.
***Standard Error of Regression Slope.

SVM analysis

a) Input and output data sets of the quantitative analysis of ERK1 in MDA-MB-231 cell lysates. Tables S8-S13.

Table S8. Input calibration data set of the quantitative analysis of MDA-MB-231 cell lysates that were enriched by different amounts of recombinant active ERK1. The number of score factors utilized corresponded to predictions that gave the lowest error and best R^2.

ERK1 Concentration (nM)	Score Factors		
	F1	F2	F3
0	-22.106	-2.677	-6.808
0	-21.264	-3.417	-5.556
0	-21.707	-3.028	-6.214
0	-21.425	-3.276	-5.796
0.1	-16.797	-1.139	-1.450
0.1	-17.732	-3.964	-2.939
0.1	-17.161	-3.068	-3.077
0.1	-16.048	-3.100	-1.065
0.2	-13.534	-3.098	4.099
0.2	-11.882	-5.965	4.281
0.2	-14.486	-3.193	2.741
0.2	-12.853	-5.835	3.088
0.8	-4.251	4.125	0.244
0.8	-3.696	4.037	3.806
0.8	-3.994	3.999	1.198
0.8	-3.161	6.617	2.097
3.2	20.752	6.018	-5.973
3.2	22.319	7.278	-3.627
3.2	20.581	7.337	-4.649
3.2	22.314	6.841	-5.094
6.4	35.414	-12.178	0.862
6.4	36.657	-10.250	0.048
6.4	38.083	-10.866	0.748
6.4	36.026	-10.318	0.205

Table S9. Input test data set of the quantitative analysis of MDA-MB-231 cell lysates that were enriched by different amounts of recombinant active ERK1. The number of score factors utilized corresponded to predictions that gave the lowest error and best R^2.

ERK1 Concentration (nM)	Score Factors		
	F1	F2	F3
0.4	-7.994	-2.292	3.490
0.4	-10.143	-0.854	5.165
0.4	-9.885	-1.295	5.163
0.4	-9.756	-1.997	5.029
1.6	6.544	11.524	3.271
1.6	8.772	12.479	0.390
1.6	4.849	11.033	2.936
1.6	7.565	10.522	3.386

Table S10. Output calibration and test data set of the quantitative analysis of MDA-MB-231 cell lysates that were enriched by different amounts of recombinant active ERK1.

Calibration data set		Test data set	
Actual ERK (nM)	Predicted ERK (nM)	Actual ERK (nM)	Predicted ERK (nM)
0.00	0.02	0.40	0.56
0.00	-0.02	0.40	0.47
0.00	0.00	0.40	0.47
0.00	-0.02	0.40	0.45
0.10	0.08	1.60	1.39
0.10	0.08	1.60	1.99
0.10	0.11	1.60	1.31
0.10	0.12	1.60	1.39
0.20	0.23		
0.20	0.21		
0.20	0.18		
0.20	0.22		
0.80	0.82		
0.80	0.78		
0.80	0.79		
0.80	0.82		
3.20	3.18		
3.20	3.18		
3.20	3.18		
3.20	3.26		
6.40	6.38		
6.40	6.42		
6.40	6.42		
6.40	6.37		

Table S11. Report generated by the SVM analysis for the output calibration and test data set of the quantitative analysis of MDA-MB-231 cell lysates that were enriched by recombinant active ERK1.

Preprocessing: Autoscale	SVM: number of SVs: 17
X-block compression: PCA with 3 components	Cross validation: custom (user) split
SVM type: epsilon-SVR	RMSEC: 0.0247514
SVM kernel type: radial basis function	RMSECV: 0.0818216
SVM optimal parameters:	RMSEP: 0.171027
cost = 100	R^2 Cal: 0.999888
epsilon = 0.01	R^2 CV: 0.998778
gamma = 0.1	R^2 Pred: 0.91944

Table S12. Input data set for the prediction of unknown pp-ERK in treated MDA-MB-231 cell lysates using the calibration data set in Table S8. The number of score factors utilized corresponded to predictions that gave the lowest error and best R^2.

Treatment	Score factors			Unknown pp-ERK (nM)		
	F1	F2	F3	Predicted values	Average	±SD
M-n	7.295	3.928	-2.994	2.043		
	6.130	3.049	-2.402	1.889		
	7.677	4.123	-2.934	2.068		
	8.320	4.165	-4.990	2.323	2.081	0.155
M-U	-24.693	-0.417	0.577	0.193		
	-24.275	-0.602	1.470	0.137		
	-24.062	-0.749	1.819	0.115		
	-24.507	-0.289	1.030	0.156	0.150	0.029
M-E	18.681	-10.634	-1.477	5.052		
	18.199	-11.127	-0.571	4.953		
	19.886	-8.360	-0.617	4.752		
	18.540	-12.337	-1.198	5.219	4.994	0.169
M-E/U	-16.723	-1.676	3.228	0.128		
	-18.349	-1.787	1.676	0.005		
	-15.882	-1.482	1.373	0.116		
	-20.156	-0.386	-0.416	-0.087	0.040	0.088
M-J	-11.757	-0.431	-1.860	0.421		
	-12.594	-0.555	-0.782	0.322		
	-13.047	-1.711	0.164	0.283		
	-11.224	-0.673	-1.333	0.439	0.366	0.065
M-A	18.037	5.078	6.215	1.816		
	18.966	4.642	8.213	1.742		
	19.311	3.282	5.428	2.046		
	20.538	2.784	5.081	2.199	1.951	0.182
M-A/J	9.013	6.758	-3.977	2.399		
	8.230	5.330	-3.213	2.168		
	9.363	6.433	-3.258	2.302		
	9.084	3.645	-4.252	2.306	2.294	0.082

Table S13. Input data set for the prediction of unknown pp-ERK in treated MDA-MB-231 lysates with different MEK-inhibitor concentrations using the calibration data set in Table S8. The number of score factors utilized corresponded to predictions that gave the lowest error and best R^2.

MEK Inhibitor	Score factors			Unknown pp-ERK (nM)		
	F1	F2	F3	Predicted values	Average	±SD
0 μM	11.321	4.890	-7.366	2.67		
	9.512	8.121	-7.570	2.92		
	10.633	6.099	-9.279	2.73		
	9.504	5.224	-9.181	2.60	2.73	0.12
0.2 μM	14.206	3.087	0.900	2.22		
	14.053	4.066	2.683	1.94		
	15.034	0.470	4.161	2.12		
	13.727	4.466	2.533	1.90	2.05	0.13
0.4 μM	7.188	6.367	2.931	1.36		
	4.384	5.749	0.443	1.40		
	3.521	4.381	0.578	1.34		
	6.505	4.988	1.129	1.49	1.40	0.06
1 μM	-1.338	6.046	-2.516	1.40		
	-0.259	4.832	-1.529	1.29		
	-1.201	5.036	-0.785	1.14		
	0.810	4.589	-4.134	1.70	1.38	0.21
2.5 μM	-15.404	2.006	-1.333	0.21		
	-15.805	-0.688	-3.069	0.20		
	-16.009	-1.130	-1.759	0.13		
	-14.700	-0.660	-2.688	0.26	0.20	0.05
6.25 μM	-20.771	-3.422	-1.789	0.15		
	-21.320	-3.113	-1.713	0.19		
	-22.678	-3.136	-1.807	0.25		
	-21.592	-2.790	-0.619	0.20	0.20	0.04
15.65 μM	-23.546	-3.608	2.540	0.22		
	-24.037	-5.077	3.188	0.30		
	-23.448	-4.248	3.445	0.22		
	-24.366	-4.803	2.920	0.30	0.26	0.04

b) Input and output data sets of the quantitative analysis of ERK1 in A549 cell lysates. Tables S14-S18.

Table S14. Input calibration data set of the quantitative analysis of A549 cell lysates that were enriched by different amounts of recombinant active ERK1. The number of score factors utilized corresponded to predictions that gave the lowest error and best R^2.

ERK1 Concentration (nM)	Score Factors					
	F1	F2	F3	F4	F5	F6
0	-49.929	-9.439	27.010	5.427	-0.517	-0.490
0	-49.512	-9.671	24.831	3.998	-0.430	-0.803
0	-48.090	-7.765	24.487	3.620	1.190	0.844
0	-48.252	-8.367	27.566	5.005	0.539	0.084
0.1	-45.807	-3.666	-6.015	-4.421	2.399	2.646
0.1	-44.859	-3.463	-6.753	-3.103	4.562	2.205
0.1	-45.902	-3.212	-5.032	-3.969	4.757	5.145
0.1	-47.409	-5.559	-6.129	-4.101	3.057	4.480
0.4	-35.154	-4.152	-6.295	-3.009	-5.657	-0.311
0.4	-35.429	-2.469	-7.576	-3.683	-4.347	0.057
0.4	-34.662	-3.774	-4.675	-1.775	-4.475	0.073
0.4	-33.931	-3.700	-6.200	-3.334	-3.897	-0.207
0.8	-17.741	0.908	-11.934	4.258	4.354	-4.238
0.8	-19.534	3.409	-9.431	0.869	3.613	-3.523
0.8	-16.565	0.281	-10.906	2.123	4.657	-3.769
0.8	-18.621	2.380	-10.503	3.827	2.365	-2.812
3.2	64.468	28.627	10.020	-4.678	-0.553	1.874
3.2	62.410	28.943	11.666	-6.323	1.169	-1.419
3.2	63.216	28.103	11.398	-3.805	1.191	-1.238
3.2	64.108	27.031	11.288	-7.853	0.348	-0.784
6.4	109.079	-27.557	-0.994	0.135	-0.123	0.443
6.4	108.814	-27.140	-1.149	0.195	-0.173	0.740
6.4	108.850	-27.792	-1.016	-0.425	0.322	-0.501
6.4	108.497	-26.929	-1.310	0.236	-0.028	0.090

Table S15. Input test data set of the quantitative analysis of A549 cell lysates that were enriched by different amounts of recombinant active ERK1. The number of score factors utilized corresponded to predictions that gave the lowest error and best R^2.

ERK1 Concentration (nM)	Score Factors					
	F1	F2	F3	F4	F5	F6
0.2	-44.527	-3.547	-4.544	-3.475	-3.618	-1.568
0.2	-42.075	-1.463	-4.298	-4.009	-2.966	-2.316
0.2	-43.758	-4.346	-3.022	-4.388	-1.082	-1.312
0.2	-44.606	-4.397	-4.364	-3.709	-0.096	-0.569
1.6	19.431	16.683	-9.340	8.532	-1.913	3.775
1.6	17.789	18.484	-9.372	9.915	-0.167	1.405
1.6	20.921	16.859	-7.229	9.096	-2.372	1.467
1.6	18.777	16.699	-10.180	8.825	-2.112	0.535

Table S16. Output calibration and test data set of the quantitative analysis of A549 cell lysates that were enriched by different amounts of recombinant active ERK1.

Calibration data set		Test data set	
Actual ERK (nM)	Predicted ERK (nM)	Actual ERK (nM)	Predicted ERK (nM)
0.00	-0.02	0.20	0.09
0.00	0.02	0.20	0.13
0.00	0.02	0.20	0.17
0.00	0.01	0.20	0.14
0.10	0.10	1.60	1.70
0.10	0.12	1.60	1.56
0.10	0.11	1.60	1.73
0.10	0.11	1.60	1.66
0.40	0.42		
0.40	0.38		
0.40	0.42		
0.40	0.47		
0.80	0.82		
0.80	0.76		
0.80	0.93		
0.80	0.78		
3.20	3.18		
3.20	3.15		
3.20	3.18		
3.20	3.26		
6.40	6.40		
6.40	6.38		
6.40	6.40		
6.40	6.36		

Table S17. Report generated by the SVM analysis for the output calibration and test data set of the quantitative analysis of A549 cell lysates that were enriched by different amounts of recombinant active ERK1.

Preprocessing: Autoscale	SVM: number of SVs: 16
SVM type: epsilon-SVR	Cross validation: custom (user) split
SVM kernel type: radial basis	RMSEC: 0.0391808
function	RMSECV: 0.0553884
SVM optimal parameters:	RMSEP: 0.0811417
cost = 31.6228	R^2 Cal: 0.999746
epsilon = 0.01	R^2 CV: 0.999457
gamma = 0.0031623	R^2 Pred: 0.995937

Table S18. Input data set for the prediction of unknown pp-ERK in treated A549 cell lysates using the calibration data set in Table S14. The number of score factors utilized corresponded to predictions that gave the lowest error and best R^2.

Treatment	Score factors						Unknown pp-ERK (nM)		
	F1	F2	F3	F4	F5	F6	Predicted values	Average	±SD
A-n	14.486	3.593	1.141	0.045	0.117	0.340	0.965		
	14.518	2.772	2.172	1.682	0.025	0.559	0.962		
	13.633	4.006	1.607	0.662	0.761	0.326	0.978		
	13.613	3.693	0.626	0.476	0.423	0.267	0.990	0.974	0.011
A-U	20.095	1.026	0.129	3.433	0.300	0.159	0.876		
	21.067	0.851	1.343	2.232	0.343	0.091	0.829		
	20.873	0.576	1.083	1.984	0.192	0.069	0.842		
	20.364	0.442	0.285	2.613	0.265	0.036	0.873	0.855	0.020
A-E	32.688	2.451	2.653	0.450	0.047	3.196	2.871		
	30.367	0.336	0.491	0.148	0.583	0.237	2.728		
	32.202	1.635	2.616	0.878	4.970	0.053	2.839		
	32.723	2.006	2.475	0.304	0.627	2.681	2.848	2.822	0.056
A-E/U	17.007	7.942	1.640	0.869	0.095	0.318	1.193		
	17.147	4.952	0.795	0.583	0.188	0.316	1.120		
	15.785	4.771	0.802	1.438	0.018	0.073	1.138		
	15.447	7.389	2.552	0.016	0.000	0.145	1.248	1.174	0.050
A-J	20.023	0.048	2.055	2.586	0.031	0.290	0.827		
	17.869	1.308	0.629	1.368	0.064	0.535	0.888		
	20.116	0.320	2.174	2.212	0.149	0.373	0.821		
	20.822	1.195	2.120	0.080	0.459	0.191	0.799	0.834	0.033
A-A	33.329	1.128	1.037	0.635	1.323	1.231	2.855		
	31.040	1.234	2.304	0.241	1.433	0.009	2.692		
	32.198	0.642	0.355	0.193	2.348	1.262	2.748		
	31.371	0.502	2.275	1.256	0.916	0.229	2.718	2.753	0.062
A-A/J	6.262	3.374	5.128	1.078	0.280	0.166	1.753		
	7.769	2.895	4.854	1.149	0.221	0.195	1.823		
	7.918	3.470	4.289	2.603	0.583	0.116	1.758		
	4.999	0.665	4.964	0.043	0.678	0.279	1.771	1.776	0.028

Table S19. Predicted unknown values of pp-ERK (ng/µg Lysate)

Cells	Code	pp-ERK (nM)	±SD	pp-ERK (ng)	pp-ERK (ng μg^{-1} Lysate)
MDA-MB-231	M-n	2.080	0.160	2.621	0.374
	M-U	0.150	0.030	0.189	0.027
	M-E	4.990	0.170	6.287	0.898
	M-E/U	0.040	0.090	0.050	0.007
	M-J	0.370	0.070	0.466	0.067
	M-A	1.950	0.180	2.457	0.351
	M-A/J	2.290	0.080	2.885	0.412
A549	A-n	0.970	0.010	1.222	0.175
	A-U	0.860	0.020	1.084	0.155
	A-E	2.820	0.060	3.553	0.508
	A-E/U	1.170	0.050	1.474	0.211
	A-J	0.830	0.030	1.046	0.149
	A-A	2.750	0.060	3.465	0.495
	A-A/J	1.780	0.030	2.243	0.320
MDA-MB-231 MEK-Inhibitor	0 µM	2.730	0.120	3.440	0.529
	0.2 µM	2.046	0.130	2.578	0.397
	0.4 µM	1.399	0.060	1.763	0.271
	1 µM	1.381	0.210	1.740	0.268
	2.5 µM	0.201	0.050	0.254	0.039
	6.25 µM	0.199	0.040	0.250	0.038
	15.65 µM	0.260	0.040	0.328	0.050

c) Input and output data sets of the quantitative analysis of ERK1 in treated tumor samples. Tables S20-S23.

Table S20. Input calibration data set of the quantitative analysis of the treated tumor samples that were enriched by recombinant active ERK1. The number of score factors utilized corresponded to predictions that gave the lowest error and best R^2.

ERK1 Concentration (nM)	Score Factors				
	F1	F2	F3	F4	F5
0	-15.393	-5.048	-0.584	0.258	0.005
0	-17.675	-8.752	-2.183	-0.800	-0.009
0	-16.532	-6.902	-1.376	-0.271	0.004
0	-16.532	-6.902	-1.376	-0.271	0.004
0.4	-13.502	1.756	2.306	-0.590	0.023
0.4	-11.478	-0.596	1.344	3.172	0.007
0.4	-11.485	0.548	1.590	2.267	-0.018
0.4	-12.620	0.777	1.856	1.334	-0.009
1.6	-4.003	2.304	-0.523	-0.226	-0.022
1.6	-5.379	2.826	-0.183	-1.519	-0.026
1.6	-7.054	2.919	1.028	0.738	-0.021
1.6	-3.750	2.640	-0.626	-0.944	-0.022
6.4	35.399	-1.285	-1.135	0.631	2.160
6.4	37.189	-3.748	3.814	-0.387	1.893
6.4	36.280	-2.229	1.048	0.704	-2.901
6.4	36.031	-3.018	1.521	-1.207	-1.153

Table S21. Input test data set of the quantitative analysis of the treated tumor samples that were enriched by recombinant active ERK1.

ERK1 Concentration (nM)	Score Factors				
	F1	F2	F3	F4	F5
0.8	-9.889	2.697	1.121	-2.192	0.003
0.8	-10.571	1.678	1.101	-1.417	0.021
0.8	-10.950	4.077	2.728	-0.081	-0.006
0.8	-12.120	2.368	2.287	-0.541	0.038
3.2	8.308	3.512	-3.614	-0.499	-0.002
3.2	8.227	3.523	-3.242	0.827	-0.001
3.2	9.488	4.350	-3.511	-0.089	-0.022
3.2	8.013	2.504	-3.390	1.104	0.056

Table S22. Output calibration and test data set of quantitative analysis of the treated tumor samples that were enriched by recombinant active ERK1.

Calibration data set		Test data set	
Actual ERK (nM)	Predicted ERK (nM)	Actual ERK (nM)	Predicted ERK (nM)
0.00	0.03	0.80	1.06
0.00	-0.03	0.80	0.86
0.00	-0.02	0.80	0.80
0.00	-0.02	0.80	0.61
0.40	0.43	3.20	3.32
0.40	0.37	3.20	3.20
0.40	0.45	3.20	3.45
0.40	0.36	3.20	3.13
1.60	1.63		
1.60	1.57		
1.60	1.17		
1.60	1.73		
6.40	6.37		
6.40	6.37		
6.40	6.37		
6.40	6.37		

Table S23. Report generated by the SVM analysis for the output calibration and test data set of quantitative analysis of the treated tumor samples that were enriched by recombinant active ERK1.

Preprocessing: Autoscale SVM type: epsilon-SVR SVM kernel type: radial basis function SVM optimal parameters: cost = 100 epsilon = 0.01 gamma = 0.0031623	SVM: number of SVs: 14 Cross validation: custom (user) split RMSEC: 0.115701 RMSECV: 0.337862 RMSEP: 0.153783 R^2 Cal: 0.998057 R^2 CV: 0.986024 R^2 Pred: 0.986619

d) Input and output data sets of the quantitative analysis of ERK1 in treated and control tumor samples. Tables S24-S26.

Table S24. LDA score factors used for the prediction of unknown pp-ERK in vehicle (control) tumor samples. The number of score factors utilized corresponded to predictions that gave the lowest error and best R^2.

ERK1	Score Factors				
Concentration (nM)	F1	F2	F3	F4	F5
0	-10.816	-3.696	-2.485	-0.285	-0.154
0	-11.427	-5.014	-2.279	0.180	0.225
0	-11.865	-3.551	-2.547	-0.126	-0.023
0	-12.397	-3.239	-2.581	-0.230	0.041
0.4	-8.746	1.676	-0.565	0.421	-0.173
0.4	-9.200	0.733	-0.783	0.038	-0.130
0.4	-9.679	0.949	-0.455	0.451	0.175
0.4	-10.413	2.972	-0.582	0.872	-0.051
0.8	-5.662	2.177	1.114	-0.899	0.113
0.8	-6.471	4.099	0.500	-0.166	-0.286
0.8	-5.616	4.600	1.686	1.344	0.226
0.8	-8.178	4.178	0.904	-0.051	0.177
1.6	-4.678	-0.085	-0.668	-0.090	-0.718
1.6	-3.480	3.249	0.577	0.934	-0.588
1.6	-5.865	0.852	0.881	-1.184	0.165
1.6	-5.447	1.944	2.948	-1.724	0.993
3.2	7.472	-4.842	2.946	0.302	0.101
3.2	8.699	-3.781	2.649	0.008	-0.250
3.2	7.460	-4.605	3.503	-0.213	0.429
3.2	5.190	-3.703	2.068	0.456	-0.275
6.4	25.281	1.430	0.028	-0.958	-2.888
6.4	24.967	1.424	-3.877	-0.351	-0.547
6.4	26.129	2.193	-2.362	-1.436	2.404
6.4	24.741	0.040	-0.620	2.707	1.034

Table S25. Input data set for the prediction of unknown pp-ERK in treated and control tumor samples using the calibration data set in Table S20. The number of score factors utilized corresponded to predictions that gave the lowest error and best R^2.

Treatment	Score factors					Unknown pp-ERK (nM)		
	F1	F2	F3	F4	F5	Predicted values	Average	±SD
Treated	-15.393	-5.048	-0.584	0.258	0.005	0.027		
(0 nM	-17.675	-8.752	-2.183	-0.800	-0.009	0.026		
ERK1)	-16.532	-6.902	-1.376	-0.271	0.004	0.021		
	-16.532	-6.902	-1.376	-0.271	0.004	0.021	0.024	0.003
Control	-10.816	-3.696	-2.485	-0.285	-0.154	0.774		
(0 nM	-11.427	-5.014	-2.279	0.180	0.225	0.629		
ERK1)	-11.865	-3.551	-2.547	-0.126	-0.023	0.643		
	-12.397	-3.239	-2.581	-0.230	0.041	0.599	0.661	0.067

Table S26. Predicted unknown values of pp-ERK (ng μg^{-1} tumor Lysate)

Treatment	pp-ERK (nM)	±SD	pp-ERK (ng)	pp-ERK (ng μg^{-1} Lysate)
Treated Mice	0.024	0.003	0.030	0.004
Control Mice	0.661	0.067	0.833	0.119

e) Input and output data sets of the quantitative analysis of ERK1 in treated cell and tumor samples using general model. Tables S27-S32.

Table S27. The jack-knife classification matrix of MDA-MB-231 cell lysates (U0126 treated) and tumor samples (BI-78D3 treated) that were enriched by different amounts of recombinant active ERK1.

Confusion matrix for the cross-validation results:

from \ to	0 nM	0.1 nM	0.2 nM	0.4 nM	0.8 nM	1.6 nM	3.2 nM	6.4 nM	Total	% correct
0 nM	4	4	0	0	0	0	0	0	8	50.00%
0.1 nM	0	4	0	0	0	0	0	0	4	100.00%
0.2 nM	0	0	4	0	0	0	0	0	4	100.00%
0.4 nM	0	0	0	8	0	0	0	0	8	100.00%
0.8 nM	0	0	0	0	8	0	0	0	8	100.00%
1.6 nM	0	0	0	0	0	8	0	0	8	100.00%
3.2 nM	0	0	0	0	0	0	8	0	8	100.00%
6.4 nM	0	0	0	0	0	0	0	8	8	100.00%
Total	4	8	4	8	8	8	8	8	56	92.86%

Table S28. LDA score factors used as input data set for the general regression model of MDA-MB-231 cell lysates (U0126 treated) and tumor samples (BI-78D3 treated) that were enriched by different amounts of recombinant active ERK1. The number of score factors utilized corresponded to predictions that gave the lowest error and best R^2.

sample type	ERK concentration (nM)	Score Factors		
		F1	F2	F3
MDA-MB-231 cell lysates	0	-12.355	-0.522	-1.746
MDA-MB-231 cell lysates	0	-12.797	-0.264	-1.733
MDA-MB-231 cell lysates	0	-12.802	-0.714	-1.953
MDA-MB-231 cell lysates	0	-12.329	-0.241	-1.631
MDA-MB-231 cell lysates	0.1	-11.999	0.325	-0.999
MDA-MB-231 cell lysates	0.1	-12.774	0.383	-1.263
MDA-MB-231 cell lysates	0.1	-12.773	-0.069	-1.399
MDA-MB-231 cell lysates	0.1	-12.247	0.108	-1.183
MDA-MB-231 cell lysates	0.2	-10.437	0.977	-0.597
MDA-MB-231 cell lysates	0.2	-10.433	2.105	-0.045
MDA-MB-231 cell lysates	0.2	-10.868	0.736	-0.875
MDA-MB-231 cell lysates	0.2	-10.435	-0.079	-1.074
MDA-MB-231 cell lysates	0.4	-8.028	1.401	0.862
MDA-MB-231 cell lysates	0.4	-8.366	1.918	0.848
MDA-MB-231 cell lysates	0.4	-8.625	0.961	0.685
MDA-MB-231 cell lysates	0.4	-8.263	1.366	0.941
MDA-MB-231 cell lysates	0.8	-2.086	1.239	2.150
MDA-MB-231 cell lysates	0.8	-2.628	2.128	2.494
MDA-MB-231 cell lysates	0.8	-3.263	1.662	2.567
MDA-MB-231 cell lysates	0.8	-2.439	1.588	1.846
MDA-MB-231 cell lysates	1.6	3.110	-2.528	0.540
MDA-MB-231 cell lysates	1.6	3.928	-1.346	0.732
MDA-MB-231 cell lysates	1.6	1.177	-2.553	-0.155
MDA-MB-231 cell lysates	1.6	3.129	-1.656	0.453
MDA-MB-231 cell lysates	3.2	8.101	-3.796	-0.286
MDA-MB-231 cell lysates	3.2	9.090	-2.547	-0.670
MDA-MB-231 cell lysates	3.2	8.483	-2.027	-1.292
MDA-MB-231 cell lysates	3.2	8.055	-3.809	-0.649
MDA-MB-231 cell lysates	6.4	21.118	2.310	-1.227
MDA-MB-231 cell lysates	6.4	25.305	3.210	-0.360
MDA-MB-231 cell lysates	6.4	24.852	3.119	-2.644
MDA-MB-231 cell lysates	6.4	23.845	-0.398	1.226

Table S28 Continued

Tumor samples	0	-13.217	2.512	-0.058
Tumor samples	0	-13.207	-0.948	-5.286
Tumor samples	0	-13.261	0.882	-2.670
Tumor samples	0	-13.283	0.910	-2.666
Tumor samples	0.4	-7.258	0.070	2.048
Tumor samples	0.4	-6.258	0.493	-1.512
Tumor samples	0.4	-7.896	1.380	0.406
Tumor samples	0.4	-6.442	-0.203	0.946
Tumor samples	0.8	-0.441	0.819	3.877
Tumor samples	0.8	-3.968	2.875	3.685
Tumor samples	0.8	-2.806	1.506	2.697
Tumor samples	0.8	-4.921	0.867	3.794
Tumor samples	1.6	0.806	-3.722	-0.099
Tumor samples	1.6	2.438	-2.743	1.036
Tumor samples	1.6	1.480	-1.996	2.879
Tumor samples	1.6	3.697	-1.991	1.344
Tumor samples	3.2	8.977	-4.851	0.594
Tumor samples	3.2	7.709	-2.754	0.207
Tumor samples	3.2	9.808	-1.564	0.963
Tumor samples	3.2	7.623	-3.936	0.603
Tumor samples	6.4	22.923	1.868	-1.586
Tumor samples	6.4	25.150	2.758	-1.175
Tumor samples	6.4	23.251	4.315	-2.169
Tumor samples	6.4	24.849	0.466	-1.420

Table S29. Report generated by the SVM analysis of the output for calibration data (tumor sample) and test data (MDA-MB-231 cell lysates) using general model. Input for calibration/test data were the score factors in Table S28.

Preprocessing: Autoscale	SVM: number of SVs: 20
SVM type: epsilon-SVR	Cross validation: custom (user) split
SVM kernel type: radial basis	RMSEC: 0.190278
function	RMSECV: 0.227255
SVM optimal parameters:	RMSEP: 0.31384
cost = 10	R^2 Cal: 0.992811
epsilon = 0.01	R^2 CV: 0.989556
gamma = 0.031623	R^2 Pred: 0.980339

Table S30. Output for calibration data (tumor sample) and test data (MDA-MB-231 cell lysates) using general model. Input for calibration/test data were the score factors in Table S28.

Calibration data set (tumor sample)		Test data set (MDA-MB-231 cell lysates)			
Actual ERK (nM)	Predicted ERK (nM)	Actual ERK (nM)	Predicted ERK (nM)	Average (nM)	±SD (nM)
0.00	0.06	0.00	0.01	0.00	0.02
0.00	0.55	0.00	-0.02		
0.00	0.29	0.00	-0.02		
0.00	0.29	0.00	0.03		
0.40	-0.17	0.40	0.31	0.28	0.08
0.40	1.01	0.40	0.38		
0.40	0.45	0.40	0.18		
0.40	0.25	0.40	0.25		
0.80	0.46	0.80	0.83	0.80	0.14
0.80	0.45	0.80	0.83		
0.80	0.60	0.80	0.61		
0.80	-0.19	0.80	0.94		
1.60	1.51	1.60	1.76	1.79	0.13
1.60	1.48	1.60	1.93		
1.60	0.74	1.60	1.62		
1.60	1.65	1.60	1.84		
3.20	2.79	3.20	2.88	3.06	0.17
3.20	2.68	3.20	3.18		
3.20	2.90	3.20	3.22		
3.20	2.53	3.20	2.96		
6.40	6.03	6.40	5.72	6.01	0.47
6.40	6.42	6.40	6.38		
6.40	6.25	6.40	6.42		
6.40	6.28	6.40	5.50		

Table S31. Report generated by the SVM analysis of the output for calibration data (MDA-MB-231 cell lysates) and test data (tumor sample) using general model. Input for calibration/test data were the score factors in Table S28.

Preprocessing: Autoscale	SVM: number of SVs: 30
SVM type: epsilon-SVR	Cross validation: custom (user) split
SVM kernel type: radial basis	RMSEC: 0. 238527
function	RMSECV: 0.296601
SVM optimal parameters:	RMSEP: 0.432443
cost = 31.6228	R^2 Cal: 0.989813
epsilon = 0.01	R^2 CV: 0.980637
gamma = 0.01	R^2 Pred: 0.967016

Table S32. Output for calibration data (MDA-MB-231 cell lysates) and test data (tumor sample) using a general model. Input for calibration/test data were the score factors in Table S28.

Calibration data set (MDA-MB-231 cell lysates)		Test data set (tumor sample)			
Actual ERK (nM)	Predicted ERK (nM)	Actual ERK (nM)	Predicted ERK (nM)	Average (nM)	±SD (nM)
0.00	0.05	0.00	0.08	0.35	0.24
0.00	0.02	0.00	0.67		
0.00	0.02	0.00	0.33		
0.00	0.06	0.00	0.33		
0.10	0.02	0.40	-0.17	0.38	0.49
0.10	-0.01	0.40	1.02		
0.10	-0.04	0.40	0.45		
0.10	0.00	0.40	0.24		
0.20	0.26	0.80	0.48	0.35	0.35
0.20	0.36	0.80	0.48		
0.20	0.22	0.80	0.60		
0.20	0.21	0.80	-0.16		
0.40	0.31	1.60	1.54	1.34	0.41
0.40	0.38	1.60	1.47		
0.40	0.18	1.60	0.73		
0.40	0.25	1.60	1.63		
0.80	0.82	3.20	2.81	2.72	0.15
0.80	0.83	3.20	2.68		
0.80	0.61	3.20	2.88		
0.80	0.93	3.20	2.53		
1.60	1.76	6.40	6.03	6.25	0.16
1.60	1.91	6.40	6.42		
1.60	1.63	6.40	6.23		
1.60	1.83	6.40	6.30		
3.20	2.89				
3.20	3.18				
3.20	3.23				
3.20	2.98				
6.40	5.72				
6.40	6.38				
6.40	6.42				
6.40	5.49				