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## *Monitoring Protein Kinases in Cellular Media with Highly Selective Chimeric Reporters*

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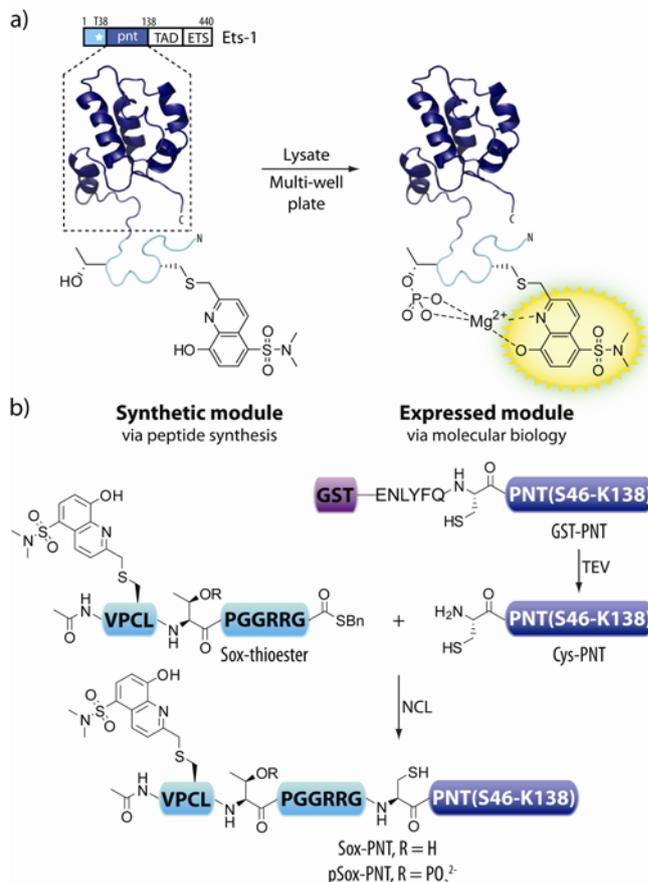
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# Monitoring Protein Kinases in Cellular Media with Highly Selective Chimeric Reporters\*\*

Elvedin Luković, Elizabeth Vogel Taylor, and Barbara Imperiali\*

Protein kinases are important regulators of cellular function, and the dynamics of their activities are critical indicators of the health or pathology of living systems.<sup>[1, 2]</sup> In particular, extracellular-signal regulated kinases 1 and 2 (ERK1/2) play a pivotal role in the mitogen-activated protein kinase (MAPK) signaling pathway responsible for regulated cell survival and proliferation.<sup>[3]</sup> The centrality of these enzymes in normal and diseased cell states underscores the need for high throughput, selective, and sensitive methods that accurately and directly diagnose kinase activities. The benchmark phosphorylation assays for ERK1/2 rely on transfer of radioactive  $\gamma$ -phosphate of [ $\gamma$ -<sup>32</sup>P]ATP to peptide or protein substrates.<sup>[4]</sup> While broadly employed, this approach has limitations, including the discontinuous nature of the radioactive assay and the non-native ATP concentrations that are utilized. Alternatively, for cellular imaging, genetically-encoded sensors that rely on phosphorylation-based changes in fluorescence resonance energy transfer (FRET) between fluorescent protein pairs<sup>[5, 6]</sup> have been constructed for several kinases, including ERK1/2.<sup>[7-10]</sup> These sensors are powerful because they can be expressed in cells, however, they cannot be used for high throughput screening of recombinant enzymes and unfractionated cell lysates due to the very limited fluorescence changes that accompany phosphorylation. As a complementary approach, probes based on small, organic fluorophores with direct readouts<sup>[6, 11]</sup> can give sensitive and robust signals under physiological conditions and are thus amenable to high throughput applications. For example, we have incorporated a sulfonamido-oxine (Sox) chromophore into peptides<sup>[12, 13]</sup> to report phosphorylation via chelation-enhanced fluorescence (CHEF) (Figure 1a). The weak binding affinity of the unphosphorylated substrate for  $Mg^{2+}$  increases significantly upon phosphorylation, resulting in robust (2- to 12-fold) fluorescence enhancements. This versatile peptide-based sensor design has been applied to monitor the activity of numerous Ser/Thr and Tyr kinases both *in vitro*<sup>[13]</sup> and in cell lysates.<sup>[12]</sup>

With more than 500 different kinases encoded in the human genome, sensor selectivity becomes paramount, particularly when studying enzymes under conditions that resemble their native environments, such as in unfractionated cell lysates or live cells.<sup>[6]</sup> While many protein kinases exploit linear recognition motifs comprising 4-8 residues that are proximal to the phosphorylation



**Figure 1.** Sox-based chemosensors. a) The ERK1/2 probe utilizes the PNT domain from Ets-1 for specific binding to the enzyme and senses phosphorylation via Sox-dependent CHEF ( $\lambda_{ex} = 360$  nm,  $\lambda_{em} = 485$  nm). b) The Sox-PNT sensor is synthesized via NCL.

site to drive specificity, a number of physiologically important kinases, including ERK1/2 and other MAPKs, phosphorylate substrates with short and ubiquitous consensus sequences. For these enzymes specificity is derived from extended recognition elements that include protein-protein interactions distal to the phosphorylation site. For example, ERK1 and 2 phosphorylate the transcription factor Ets-1 at Thr38 within a short ThrPro (TP) consensus motif.<sup>[14]</sup> Since this short sequence would be the target of multiple kinases, ERK recognition of Ets-1 depends on an adjoining N-terminal pointed (PNT) domain<sup>[15]</sup> to dock the substrate specifically to ERK1/2, which engages the phosphorylation machinery.<sup>[16]</sup> With this docking-domain strategy, PNT-based substrates demonstrate good affinity for ERK1/2 ( $K_M \sim 6-9 \mu M$ ),<sup>[15]</sup> in stark contrast to short peptide substrates derived only from the TP sequence ( $K_M > 200 \mu M$ ).<sup>[15, 17]</sup> Due to the size of the PNT domain (11 kDa) these docking-domain interactions cannot be exploited with the types of synthetic peptide-based sensors that have been previously reported. However, in light of its importance in cellular homeostasis and the prominence of ERK dysregulation in cancer, we set out to construct a chimeric sensor for ERK1/2 that combines the advantageous reporting properties of the Sox fluorophore with the outstanding specificity provided by the native protein domain-based recognition (Figure 1a). Most importantly, to ensure facile and high throughput analysis of ERK1/2 activity, our ultimate requirement is that the

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Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the author.

probe be highly selective in cell lysates where it would be exposed to hundreds of other active kinases.

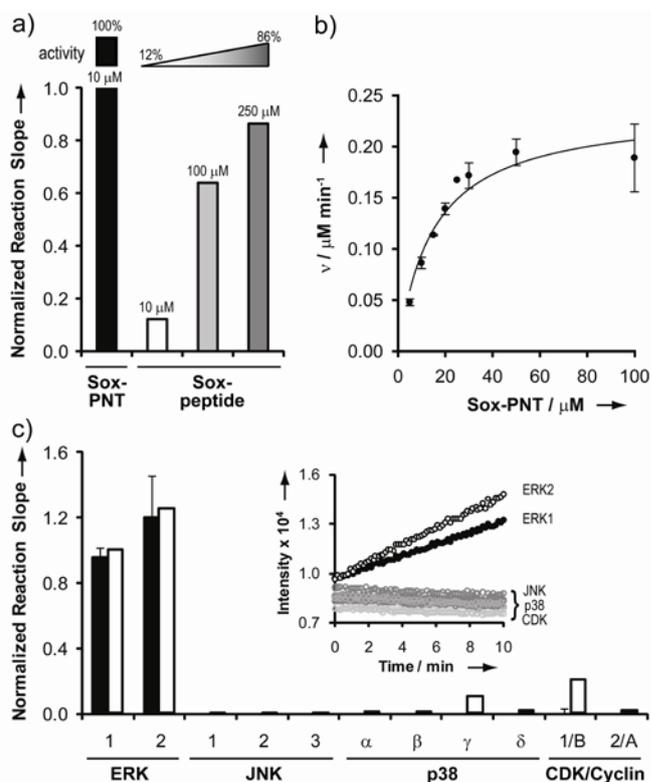
Herein we describe the semisynthesis of a chimeric Sox-based ERK1/2 sensor through a key native chemical ligation (NCL) reaction that efficiently conjugates the recombinant PNT domain of Ets-1 to a synthetic ERK1/2 consensus sequence including the Sox sensing module. The extended PNT recognition element confers the ERK1/2 sensor with excellent selectivity, as demonstrated by comparative quantitative analyses with a panel of related recombinant enzymes and in unfractionated lysates from four different cell lines. Most importantly, the docking domain-based sensor design should be generally applicable to the development of selective sensors for other medically important kinases.

The new sensor was assembled as illustrated in Figure 1, using NCL<sup>[18]</sup> to ligate the synthetic Sox-containing peptide thioester with the expressed PNT domain, comprising Ets-1 residues 46-138 (Figure 1b). The peptide thioester was synthesized using Fmoc-based solid phase peptide synthesis (SPPS) on highly acid-labile TGT resin, followed by an off-bead thioesterification of the protected peptide.<sup>[19]</sup> The Sox chromophore was introduced as the amino acid C-Sox.<sup>[13]</sup> An optimized phosphorylation motif based on the ERK2 phosphorylation sequence within the myelin basic protein (MBP)<sup>[17]</sup> (TPGGRR) was used in place of the phosphorylated region of Ets-1 (TPSSKE) to improve fluorescent properties of peptidyl probes (Table S1 in the Supporting Information). The distance between the TP recognition sequon and the PNT domain in the wild type protein was preserved in the sensor (Table S2 in the Supporting Information). This design introduced residue replacements in the unstructured N-terminal region of Ets-1, thereby minimizing perturbations to the overall secondary structure. Additionally, the C-terminal residue, Met44, was changed to Gly to eliminate the possibility of epimerization during thioesterification and to increase ligation efficiency.<sup>[20]</sup> The expressed C-terminal fragment of the sensor, GST-PNT, was proteolyzed to reveal Cys-PNT. After ligation of Cys-PNT to the peptide thioester in non-denaturing conditions, the ERK1/2 probe, Sox-PNT, was obtained with excellent conversion (80-90%) (Figure S1 in the Supporting Information). The corresponding phosphoprotein (pThr38), pSox-PNT, was constructed using analogous methods.

Initial spectroscopic studies with Sox-PNT and pSox-PNT revealed a robust 3-fold enhancement in fluorescence upon phosphorylation (Figure S2 in the Supporting Information). Subsequent *in vitro* assays determined Sox-PNT to be an efficient substrate for ERK2 when compared with the corresponding Sox-peptide (Ac-VP-CSox-LTPGGRRG-OH) (Figure 2a and Figure S3 in the Supporting Information). Furthermore, Sox-PNT demonstrated a  $K_M$  of 14.9  $\mu$ M for ERK2, which is comparable to the reported value for wild type PNT (9  $\mu$ M),<sup>[21]</sup> indicating that use of the MBP-derived TP sequence has minimal effect relative to the native protein (Figure 2b). In contrast, the MBPtide (APRTPGGRR), the basis of the Sox-PNT phosphorylation sequence, was reported to have a  $K_M$  of 2 mM for ERK2, underscoring the importance of the PNT domain in substrate kinetics.<sup>[17]</sup> Finally, Sox-PNT exhibited high selectivity for ERK1/2 when compared to related kinases from the JNK, p38 and CDK families (Figure 2c).

In order to demonstrate that the preference of Sox-PNT for ERK1/2 can translate to complex media, the probe was exposed to a panel of unfractionated cell lysates that contained varying levels of active ERK1/2. The activity of cellular ERK1/2 is linked to its phosphorylation state, which is modulated by the epidermal growth factor (EGF) signaling pathway (Figure 3a). In summary, EGF interacts with EGF receptors (EGFRs), which leads to activation of MEK1/2, which in turn phosphorylates and activates ERK1/2. This event can be regulated either by the upstream MEK1/2 inhibitor, U0126, or by a direct ERK1/2 inhibitor, PEA-15.<sup>[22]</sup> Summarized in Figure 3b are the results of ERK1/2 activity analyses on the crude lysates from four mammalian cell lines, which reveal the selectivity of the sensor in these complex media. In all cases, untreated lysates showed relatively low basal activity. Upon EGF stimulation, there

was a 4- to 10-fold increase in activity.<sup>[23]</sup> To demonstrate that Sox-PNT was specifically monitoring ERK1/2 activity, cells were exposed to the inhibitor U0126 and subsequently EGF-stimulated and lysed. Under these conditions, the ERK1/2 activity was returned to nearly basal levels. Western blot analysis was used to demonstrate that both stimulated and U0126 inhibitor-treated cells expressed ERK1/2 (Figure 3b), however, only EGF-stimulated samples showed enhanced levels of activated ERK1/2 as evidenced by analysis with the phospho-ERK1/2-specific antibody. In contrast, the Sox-peptide, lacking the PNT docking domain, showed promiscuous activity and signaled phosphorylation that could not be correlated with ERK1/2 activity (Figure 3c).



**Figure 2.** *In vitro* characterization of Sox-PNT. a) The efficiency of phosphorylation by recombinant ERK2 (11 ng) of the Sox-PNT probe was compared to that of the Sox-peptide under identical conditions. b) The kinetic parameters for Sox-PNT were obtained with ERK2 (10 ng) from a direct fit of  $v$  vs.  $[S]$  plots using the Briggs-Haldane equation. Plotted values indicate the mean  $\pm$  s.e.m. for triplicate measurements. c) Promiscuity of Sox-PNT (5  $\mu$ M) was tested with a panel of related kinases at 15 nM (black bars) and 150 nM (clear bars) of each enzyme. Inset: a representative plot of the change in the fluorescent signal over time obtained with 15 nM enzyme in the fluorescence plate reader. Plotted values for 15 nM of enzyme indicate the mean  $\pm$  s.e.m. for triplicate measurements.

Further evidence that Sox-PNT is selectively modified by ERK1/2 was obtained with PEA-15, a direct protein inhibitor of ERK1/2. Titration of PEA-15 into EGF-stimulated NIH-3T3 lysates created a dose-dependent response with a half inhibitory concentration of 40 nM and a  $K_i$  (30 nM) that reflected the reported  $K_i$  values (20 nM) (Figure 3d and the Supporting Information).<sup>[22]</sup>

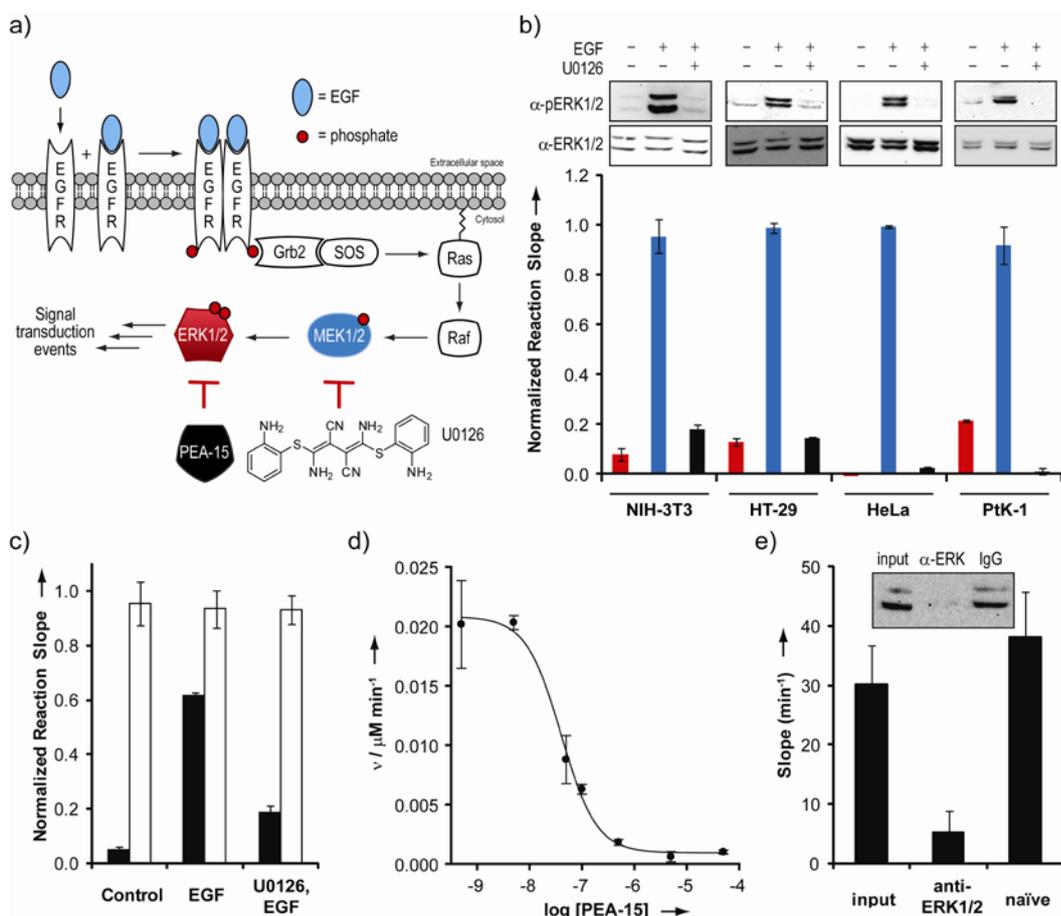
To directly correlate the observed fluorescent signal to the presence of ERK, we exposed our probe to ERK1/2-depleted lysates. Indeed, immunodepletion of ERK1/2 from EGF-stimulated HeLa lysate reduced activity by 7-fold compared to the input lysate or the sample that had been depleted with naïve rabbit IgG (Figure 3e). This indicates that the Sox-PNT signal is predominantly due to the ERK1/2-mediated phosphorylation. Immunodepletion was confirmed by western blot analysis with the immunodepleting antibody (Figure 3e, inset). Having validated the selectivity of Sox-PNT for ERK1/2, the sensor can be used to measure active ERK1/2

(13 ng) in EGF-stimulated lysate (40  $\mu$ g) (Figure S5 in the Supporting Information), which will be an important tool for quantifying ERK1/2 levels in tissue samples.

The MAPK signaling pathways are composed of numerous kinases intricately regulated by stress responses and extracellular signals. Deconvoluting the specific functions of individual enzymes has been challenging, partly due to the difficulty of creating probes that exclusively target a kinase of interest. Here we have presented a selective ERK1/2 activity chemosensor that comprises both a chemical sensing motif and recombinant enzyme docking domain. Thereby, the Sox-based kinase-sensing strategy has been extended beyond the realm of enzymes that recognize linear peptide substrates. Quantitative studies with the probe indicate that the PNT domain confers exquisite selectivity toward ERK1/2, which was impossible to achieve with simple peptide probes. Moreover, the docking-domain approach now allows us to target a wider set of kinases (such as other members of the MAPK family, JNK and p38) that have thus far been elusive due to their complex substrate recognition mechanisms. The chimeric protein probe also offers distinct advantages for solution-based analyses that can be carried out with simple equipment. The reliable semisynthesis of multimilligram (6 mg) quantities of Sox-PNT allows at least 5000 assays to be performed in 384-well plates. Moreover, Sox-based sensors exhibit large dynamic ranges with excellent  $Z'$  factor values,<sup>[13]</sup> which are a measure of the fidelity of assay data.<sup>[24]</sup> Currently, in light of the efficient semisynthesis, excellent selectivity and robustness in high throughput analysis, the Sox-PNT sensor can be broadly applied for quantifying ERK1/2 activities in applications ranging from drug discovery to diagnostics.

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**Figure 3.** Specificity of the Sox-PNT sensor toward ERK1/2 in unfractionated cell lysates. a) The EGF signalling pathway results in stimulation of ERK1/2 activity. U0126, an inhibitor of an upstream kinase, MEK1/2, and PEA-15, a direct inhibitor of ERK1/2 can regulate the activity of ERK1/2. b) Sox-PNT (5  $\mu$ M) was used to measure enzyme activity in 40  $\mu$ g of untreated lysates (red bars), EGF-stimulated lysates (blue bars), or U0126-treated and then EGF-stimulated lysates (black bars). Inset: western blot for pERK1/2 (top) and ERK1/2 (bottom). c) The ability of Sox-PNT (black bars, 5  $\mu$ M) to report the different phosphorylation states of ERK1/2 in HeLa lysates (40  $\mu$ g) was directly compared to the Sox-peptide (clear bars, 5  $\mu$ M). d) To obtain the  $IC_{50}$  value for inhibition of ERK1/2 by PEA-15, EGF-stimulated NIH-3T3 lysates (40  $\mu$ g) were treated with various concentrations of PEA-15 and enzyme activity was measured with Sox-PNT (5  $\mu$ M). e) Kinase activity was measured with Sox-PNT (5  $\mu$ M) from an EGF-stimulated HeLa lysate (40  $\mu$ g) before (input) and after immunodepletion of this lysate with anti-ERK1/2 or naïve rabbit IgG. Inset: western blot for ERK1/2 in the measured samples. The top band in all western blots is ERK1 (44 kDa) and the bottom band ERK2 (42 kDa). Plotted values indicate the mean  $\pm$  s.e.m. for triplicate measurements.

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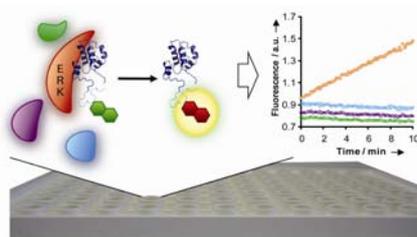
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Monitoring Protein Kinases in Cellular  
Media with Highly Selective Chimeric  
Reporters



**The best of both worlds:** By combining a recombinant docking domain, PNT, with a chemical sensing module, Sox, a highly selective ERK kinase sensor was synthesized. This probe displays excellent *in vitro* characteristics and, moreover, can be used to exclusively monitor ERK1/2 activity in unfractionated cell lysates without any off-target kinase inhibitors. Strong selectivity and robust biophysical parameters allow ERK1/2 activity to be analyzed in a high throughput manner, without time-consuming enzyme purification.

## Supporting Information

# Monitoring Protein Kinases in Cellular Media with Highly Selective Chimeric Receptors

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## I. General information

Unless otherwise noted, all solvents and reagents were obtained commercially and used without further purification. *N*<sup>α</sup>-Fmoc-protected amino acids [Fmoc-Arg(Pbf)-OH, Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Thr(PO(OBn)OH)-OH, Fmoc-Val-OH]<sup>§</sup> were purchased from Novabiochem. Fmoc-C(Sox[TBDPS])-OH was synthesized according to published procedures.<sup>1</sup> Anhydrous CH<sub>2</sub>Cl<sub>2</sub> was distilled from calcium hydride. Doubly deionized water was degassed by bubbling argon for 20 min at room temperature. Organic solutions were concentrated *in vacuo* by rotary evaporation at ~10 Torr (house vacuum) at 25-40 °C, then at ~0.5 Torr (vacuum pump), unless otherwise indicated. Peptides were purified via preparative reverse-phase HPLC employing a gradient of solvents A (H<sub>2</sub>O with 0.1% v/v TFA) and B (CH<sub>3</sub>CN with 0.1% v/v TFA). Peptide purity was determined by analytical reverse-phase HPLC. FPLC protein purification was done in 20 mM Tris (pH 8.0 at 25 °C) and 200 mM NaCl. Protein purity was confirmed by SDS-PAGE with Coomassie blue staining or by western blots visualized using appropriate antibodies.

## II. Instrumentation

HPLC: HPLC was carried out on Waters Prep LC 4000 System or Waters Delta 600 System equipped with Waters 2487 dual wavelength absorbance detectors. Columns used: C<sub>18</sub> analytical (flow rate = 1 mL/min), Beckman Ultrasphere ODS, 5 μm, 150 x 4.6 mm; C<sub>18</sub> preparatory (flow rate = 15 mL/min), YMC-Pack Pro, 5 μm, 250 x 20 mm.

FPLC: FPLC was carried out on Pharmacia Biotech system equipped with Pharmacia Biotech UV-MII absorbance detector and Pump P-500. Columns used: Pharmacia Biotech Superdex<sup>TM</sup> 75 analytical; Pharmacia Biotech HiLoad<sup>TM</sup> 16/60 Superdex<sup>TM</sup> 75 prep grade. Flow rate = 1 mL/min for both.

ESI-MS: Applied Biosystems Mariner mass spectrometer.

MALDI-TOF MS: PerSeptive Biosystems Voyager MALDI-TOF instrument.

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<sup>§</sup> Abbreviations: ATP: adenosine triphosphate, BME: β-mercaptoethanol, Bn: benzyl, *t*Bu: *t*-butyl, Boc: *t*-butoxycarbonyl, BSA: bovine serum albumin, CDK: cyclin-dependent kinase, DIEA: diisopropylethylamine, DMEM: Dulbecco's Modified Eagle Medium, DMF: *N,N*-dimethylformamide, DMSO: dimethyl sulfoxide, DTT: dithiothreitol, EDTA: ethylenediaminetetraacetic acid, EGTA: glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid, ERK1/2: extracellular signal-regulated kinase 1/2, ESI-MS: Electrospray Ionization Mass Spectrometry, Fmoc: 9-fluorenylmethoxycarbonyl, FPLC: fast protein liquid chromatography, FPR: fluorescence plate reader, HEPES: 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid, HOAt: 7-aza-1-hydroxybenzotriazole, HOBt: 1-hydroxybenzotriazole, HPLC: high performance liquid chromatography, JNK: c-Jun N-terminal kinase, MALDI-TOF MS: Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry, Pbf: 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl, PyAOP: (7-azabenzotriazol-1-yl)oxytripyrrolidinophosphonium hexafluorophosphate, PyBOP: Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate, SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis, SPPS: solid-phase peptide synthesis, TBDPS: *t*-butyldiphenylsilyl, TFA: trifluoroacetic acid, TIS: triisopropylsilane, TNBS: 2,4,6-trinitrobenzene sulfonic acid, Tris: 2-amino-2-hydroxymethyl-propane-1,3-diol, UV: Ultraviolet.

UV-Vis Spectrophotometer: Shimadzu UV-2401PC.

Fluorometer: Fluoromax 3 from Jobin Yvon. Cuvette: Starna Cells (16.100F-Q-10) 100  $\mu$ L sub-micro cuvette, 1 cm path length.

Fluorescence Plate Reader: HTS 7000 Bio Assay Reader from Perkin Elmer. Plate: Corning (3992) assay plate, 96-well, half area, no lid, flat bottom, non-binding surface, non-sterile, white polystyrene (120  $\mu$ L reaction volume) or MatriCal (MP101-1-PP) 384-well MatriPlate, 50  $\mu$ L, black polystyrene (20  $\mu$ L reaction volume).

Chemilluminescence Reader: Bio-Rad with Quantity One 1-D Analysis Software.

### III. Stock solutions

Due to the affinity of the phosphorylated probes for  $Mg^{2+}$ , by analogy with previously reported probes,<sup>1,2</sup> the reagents with the highest purity and lowest metal content were used where indicated to avoid the necessity of removing metal ion impurities after preparations.

1. Stock solutions of the Sox-containing peptides and proteins were prepared in doubly deionized water (Sox-peptides) or TBS (solution 7 for Sox-PNT), and concentrations were determined by UV-Vis (based on the determined extinction coefficient of the fluorophore unit, 5-(*N,N*-dimethylsulfonamido)-8-hydroxy-2-methylquinoline,  $\epsilon_{355} = 8247 \text{ M}^{-1} \text{ cm}^{-1}$  at 355 nm in 0.1 M NaOH with 1 mM  $Na_2EDTA$ ). An average of the values from three separate solutions, each prepared using a different volume of the stock solution, was read on UV-Vis spectrophotometer. Purified peptide stock solutions can be stored at 4  $^{\circ}C$  for at least 6 months or -20  $^{\circ}C$  for longer periods. Protein stock solutions were flash-frozen and stored at -80  $^{\circ}C$ .

2. Magnesium chloride stock solution was prepared from Alfa Aesar's Puratronic grade salt and stored at room temperature. Most commercially available salts contain  $Zn^{2+}$  as significant impurities and should not be used due to the high affinity of the phosphorylated peptides for  $Zn^{2+}$ . The  $Mg^{2+}$  concentration was determined by titration with a standardized solution of EDTA (Aldrich) in the presence of an Eriochrome Black T (Aldrich) as described previously.<sup>2</sup>

3. 500 mM HEPES (SigmaUltra) was prepared and adjusted to pH 7.4 with NaOH (99.998+%, Aldrich) solution and stored at 4  $^{\circ}C$ .

4. 1 M Tris (Calbiochem, ULTROL grade) was prepared and adjusted to pH 7.5 with NaOH (99.998+%, Aldrich) solution and stored at 4  $^{\circ}C$ .

5. 2 M NaCl (SigmaUltra) was prepared in doubly deionized water and stored at room temperature.

6. 500 mM and 10 mM dithiothreitol (DTT, Biotechnology grade, Mallinckrodt) solutions were prepared in degassed doubly deionized water and stored at -80  $^{\circ}C$ .

7. 10x TBS Buffer (200 mM Tris-HCl [pH 8 at 4 °C], 2 M NaCl) was prepared from Tris-HCl (Calbiochem, ULTROL grade, 15.76 g for 1 L solution) and NaCl (SigmaUltra, 58.453 g for 1 L solution) and adjusted to pH 8 at 4 °C with NaOH (99.998+%, Aldrich) solution.

8. 20 mg/mL BSA (Heat Shock Fraction V, Roche) was prepared in ultrapure water, filtered through a 0.45 micron syringe filter to remove particulates and stored at 4 °C.

9. 0.1% Brij-35 P (Fluka) was prepared in doubly deionized water and stored at room temperature.

10. 20% Triton X-100 (SigmaUltra) was prepared in doubly deionized water and stored at 4 °C.

11. 100 mM ATP (Disodium salt, Low Metals Grade, Calbiochem) was prepared in ultrapure water and stored in aliquots at -80 °C.

12. 500 mM EGTA was prepared from ethylene glycol-bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (SigmaUltra) dissolved in 2 M NaOH and stored at 4 °C.

13. 10x Assay Buffer I: 500 mM Tris-HCl (pH 7.5 at 25 °C), 100 mM MgCl<sub>2</sub>, 10 mM EGTA, 20 mM DTT and 0.1% Triton X-100.

14. 10x Assay Buffer II: 500 mM Tris-HCl (pH 7.5 at 25 °C), 100 mM MgCl<sub>2</sub>, 10 mM EGTA, 20 mM DTT and 0.1% Brij 35 P.

15. Lysis Buffer I: PBS, 1 mg/mL lysozyme, 1 mM DTT, 1% NP-40 Alternative and protease inhibitor cocktail III (Calbiochem: 100 μM AEBSF, 80 nM aprotinin, 5 μM bestatin, 1.5 μM E-64, 2 μM leupeptin, 1 μM pepstatin A).

16. Lysis Buffer II: 50 mM Tris (pH 7.5 at 25 °C), 150 mM NaCl, 50 mM β-glycerophosphate (pH 7.3), 10 mM sodium pyrophosphate, 30 mM NaF, 1% Triton X-100, 1 mM benzamidine, 2 mM EGTA, 100 μM Na<sub>3</sub>VO<sub>4</sub>, 1 mM DTT, protease inhibitor cocktail III (10 μL/mL, Calbiochem: 10 mM AEBSF, 8 μM aprotinin, 0.5 mM bestatin, 0.15 mM E-64, 0.2 mM leupeptin, 0.1 mM pepstatin A), and phosphatase inhibitor cocktail 1 (10 μL/mL, Sigma, P2825). The buffer can be stored at -20 °C for up to 3 months; without protease and phosphatase inhibitors, it can be stored at -20 °C indefinitely.

17. HeLa Medium: DMEM (Gibco, 11995) supplemented with 10% (v/v) heat-deactivated FBS (fetal bovine serum, warmed to 56 °C for 30 min, inverted several times every 10 min), 100 U/mL penicillin (Gibco, 15140) and 100 μg/mL streptomycin (Gibco, 15140). Serum-free medium: DMEM, 2 mM L-Gln (Gibco, 25030), 100 U/mL penicillin and 100 μg/mL streptomycin. All cell media were filtered through a 1 L filter flask (0.22 μm PES sterilizing low protein binding membrane, non-pyrogenic, polystyrene, Corning 431098).

18. NIH-3T3 Medium: DMEM (Gibco, 11995) supplemented with 10% (v/v) FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. Serum-free medium: DMEM, 2 mM L-Gln, 100 U/mL penicillin and 100 μg/mL streptomycin.

19. HT-29 Medium: McCoy's 5A Medium (Gibco, 1660) supplemented with 10% (v/v) FBS, 2 mM L-Gln, 100 U/mL penicillin and 100 µg/mL streptomycin. Serum-free medium: McCoy's 5A Media, 4 mM L-Gln, 100 U/mL penicillin and 100 µg/mL streptomycin.

20. PtK-1 Medium: Ham's F-12 Medium (Sigma, N8641) supplemented with 10% (v/v) FBS, 2 mM L-Gln, 1 mM sodium pyruvate (Gibco, 11360), 100 U/mL penicillin and 100 µg/mL streptomycin. Serum-free medium: Ham's F12 Medium, 4 mM L-Gln, 1 mM sodium pyruvate, 100 U/mL penicillin and 100 µg/mL streptomycin.

#### IV. Semisynthesis of (p)Sox-PNT

##### *a. Peptide synthesis*

Peptides were synthesized using standard Fmoc amino acid protection chemistry on Fmoc-Gly-Novasyn-TGT resin (Novabiochem, 0.2 mmol/g). The resin was swelled in CH<sub>2</sub>Cl<sub>2</sub> (5 min.) and then DMF (5 min.) prior to synthesis. All the amino acids except for Fmoc-C(Sox[TBDPS])-OH were attached according to the following procedure: Fmoc deprotection (20% 4-methylpiperidine in DMF, 3 x 5 min.), rinsing step (DMF, 5 x), coupling step (amino acid/PyBOP/HOBt/DIEA, 6:6:6:6, 0.15 M in DMF, 30-45 min.), rinsing step (DMF, 5 x; DCM, 5 x). Fmoc-C(Sox[TBDPS])-OH was coupled in the following manner: amino acid/PyAOP/HOAt/2,4,6-collidine, 2:2:2:5, 0.15 M in DMF, 2-12 hr. The coupling was repeated if necessary (amino acid/PyAOP/HOAt/2,4,6-collidine, 1:1:1:3, 0.15 M in DMF, 2-12 hr) as determined by the TNBS test for free amines. It is important to wash the resin rigorously (DMF followed by CH<sub>2</sub>Cl<sub>2</sub>) to remove excess amino acid before performing any tests for free amines. This is particularly necessary after coupling of Fmoc-C(Sox[TBDPS])-OH due to its deep red color, which does not affect its coupling efficiency. At the end of the synthesis, the Fmoc group was removed with 20% 4-methylpiperidine in DMF (3 x 5 min.) and the resin was rinsed with DMF (5 x). The resin-attached free amines were capped by exposure to Ac<sub>2</sub>O (20 equiv.) and pyridine (20 equiv.) in DMF for 30 min. The resin was rinsed with DMF (5 x), CH<sub>2</sub>Cl<sub>2</sub> (5 x) and subjected to 20% 4-methylpiperidine in DMF (3 x 5 min.) to remove any C-Sox aryl esters that might have formed during acetylation. The resin was finally washed with DMF, CH<sub>2</sub>Cl<sub>2</sub>, MeOH (5 x each) and dried under vacuum.

##### *b. Thioesterification of Sox-containing peptides*

Following SPPS the peptide (10 µmol from 50 mg of resin) was cleaved from the resin with side-chain protection intact by agitating with 0.5% TFA in CH<sub>2</sub>Cl<sub>2</sub> for 1.5 h. The resin was removed by filtration and rinsed with CH<sub>2</sub>Cl<sub>2</sub>. The solvent was mostly evaporated under a stream of nitrogen, and the peptide was triturated with cold hexanes. The hexanes were removed *in vacuo*, and the resulting white powder was dissolved in 16 mL of anhydrous CH<sub>2</sub>Cl<sub>2</sub> and treated with HATU (30 mg, 80 µmol) and HOAt (10.8 mg, 80 µmol) dissolved in 0.5 mL dry DMF, followed by addition of 2,4,6-collidine (21 µL, 160 µmol) and benzylmercaptan (19 µL, 160 µmol). The reaction was stirred under N<sub>2</sub> overnight. The solvent was removed *in vacuo*, and the peptide was deprotected with TFA/H<sub>2</sub>O/TIS (95:2.5:2.5% v/v) for 3 h. The resulting solution was concentrated under a stream of N<sub>2</sub> and precipitated by addition of cold Et<sub>2</sub>O. The pellet was

trituated with cold Et<sub>2</sub>O (3 x), redissolved in water, filtered and lyophilized. The peptides were purified by preparative reverse-phase HPLC using UV detection at 228 nm (amide bond absorption) and 316 nm (C-Sox absorption). Only fractions showing a single peak by analytical HPLC and with a correct mass were used in further experiments.

*c. Characterization data for peptides*

Name	Peptide Sequence	Mol. Formula	HPLC t <sub>R</sub> (min.) <sup>a</sup>	[M] Calcd.	[M+H] <sup>+</sup> found <sup>b</sup>
<b>Sox-peptide</b>	Ac-VPC(Sox)LTPGGRRG-CO <sub>2</sub> H	C <sub>67</sub> H <sub>101</sub> N <sub>19</sub> O <sub>16</sub> S <sub>3</sub>	25.7	1418.6	1419.3
<b>Sox-thioester</b>	Ac-VPC(Sox)LTPGGRRG-COSBn	C <sub>64</sub> H <sub>96</sub> N <sub>17</sub> O <sub>16</sub> PS	27.2	1523.7	1524.8
<b>pSox-peptide</b>	Ac-VPC(Sox)LpTPGGRRG-CO <sub>2</sub> H	C <sub>60</sub> H <sub>96</sub> N <sub>19</sub> O <sub>20</sub> PS <sub>2</sub>	24.4	1498.6	1499.2
<b>pSox-thioester</b>	Ac-VPC(Sox)LpTPGGRRG-COSBn	C <sub>44</sub> H <sub>82</sub> N <sub>17</sub> O <sub>15</sub> PS	27.2	1603.7	1604.9

<sup>a</sup> Peptides were purified according to the following method: 5% B (5 min.) followed by a linear gradient 5–95% B (30 min.). <sup>b</sup> The data was collected on a MALDI-TOF mass spectrometer.

*d. Plasmid construction for GST-ENLYFQC-PNT(46-138)-His<sub>6</sub> (GST-PNT)*

The gene fragment encoding residues 46-138 of Ets-1 (including the PNT domain) was amplified from an Ets-1 plasmid using primers to insert 5' EcoR1 and 3' Not1 restriction sites for incorporation into a pGEX-4T-2 vector. The primers also encoded an amino-terminal TEV protease cleavage site (ENLYGQ<del>C</del>) and a carboxy-terminal hexahistidine tag. For this amplification the following PCR primers were used:

5'- GCC GGA ATT CGT GAA AAC CTG TAT TTT CAG TGC TCC CAA GCC TTG AAA GCT -3'

3'- GCC CCC TTT TGC GGC CGC CTA GTG ATG GTG ATG GTG ATG ACC TTT CAC ATC CTC TTT CTG -5'

The PCR-amplified fragments were digested with Not1 and EcoR1 and ligated to Not1/EcoR1-digested and CIP-treated pGEX-4T-2. The ligation mixture was transformed into DH5α cells and grown on carbenicillin-resistant plates. Plasmid DNA was isolated from selected colonies and confirmed by sequencing.

*e. Expression of GST-PNT*

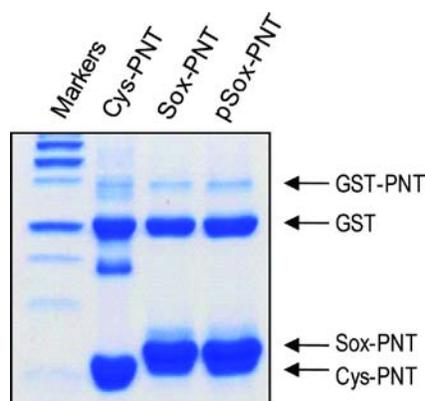
The PNT domain plasmid was transformed into BL21(DE3) competent cells (Stratagene) and grown at 37 °C to midlog phase in 1 L of LB media with carbenicillin. The culture was cooled to 30 °C, and the cells were induced with 0.2 mM IPTG and grown for an additional 4.5 h. Cells were harvested by centrifugation and frozen at -80 °C. For cell lysis, the pellet from a 1 L expression was thawed and resuspended in 40 mL of freshly prepared lysis buffer I and incubated for 20 min at 4 °C. The cells were then sonicated and subjected to centrifugation at 12,429 x g for 25 min, followed by filtration through a 0.2 micron filter.

*f. Isolation of GST-PNT and on-resin TEV proteolysis to yield Cys-PNT(46-138)-His<sub>6</sub> (Cys-PNT)*

The filtered supernatant was incubated with 5 mL of Glutathione Sepharose 4 Fast Flow resin (Amersham Biosciences) for 1.5 hours at 4 °C, following the manufacturer's protocol. After extensive washes with PBS (20 column volumes) the resin-bound GST-PNT was proteolyzed overnight with TEV (prepared in house) in 5 mL of PBS, 5 mM EDTA and 5 mM BME at 25 °C with slight agitation. Upon completion of the reaction (ca. 12 hours), the soluble fraction was filtered and the resin was washed with PBS (4 x 2 mL). The filtrate and PBS washes were combined and concentrated in a swinging bucket centrifuge using 3.5 kDa MWCO Amicon Ultra-15 centrifugal filters (Millipore) to give 24 mg of Cys-PNT (MW = 11,672.34 g/mol) as determined by Bio-Rad's Protein Assay. The protein was analyzed by SDS-PAGE and visualized with Coomassie blue dye and by western blot with a mouse anti-hexahistidine primary antibody. Purified protein was stored at 4 °C.

*g. Native Chemical Ligation to obtain Sox-PNT*

To a 2 mL centrifuge tube was added Cys-PNT (21.7 mg, 1.86 μmol, 1 equiv.), followed by MESNa and Tris (pH 8.0 at 25 °C) so that the final concentrations were: 1.4 mM Cys-PNT, 150 mM MESNa and 50 mM Tris in 1.3 mL total reaction volume. Lastly, Sox-thioester (4.2 mg, 2.79 μmol, 1.5 equiv.) dissolved in 40 μL of water was added and the reaction was gently mixed for 24-48 h at 25 °C. The progress of the reaction was analyzed by SDS-PAGE, and visualized with Coomassie blue dye (Figure S1).



**Figure S1.** Coomassie-stained gel of the ligation reaction indicated 80-90% conversion of the Cys-PNT expressed segment to the products, Sox-PNT and pSox-PNT.

*h. Purification of Sox-PNT*

The crude mixture was immediately purified on the FPLC using the Superdex 75 prep column in 20 mM Tris (pH 8 at 25 °C) and 200 mM NaCl. The protein was analyzed by SDS-PAGE, and visualized with Coomassie blue dye. The fractions with the desired product were combined and concentrated in a swinging bucket centrifuge using 3.5 kDa MWCO Amicon Ultra-15 centrifugal filters. The final concentration of Sox-PNT was determined using the Sox chromophore absorbance in the UV-Vis. The overall yield from NCL to final purification of Sox-PNT (6.2 mg,

MW = 13,016.73 g/mol) was 24%. The protein was aliquoted, flash-frozen and stored at -80 °C where it is stable for at least 1 year.

## V. *In vitro* evaluations of Sox-based probes

### a. *Ets-1- and MBP-derived peptide probes for ERK1/2*

Early peptide probes were based on the  $\beta$ -Turn Focused (BTF) design developed in our lab using the Sox amino acid as the sensing moiety.<sup>2</sup> Two peptides, one derived from the Ets-1 phosphorylation sequence (entry 1 in Table S1) and one from the MBP phosphorylation sequence (entry 2), were synthesized and their fluorescence difference measured. The main differences lie in the underlined region.

In fluorescence increase measurements the concentration of the substrate was held constant while the concentration of ATP was varied. No enzyme was present. A solution of the substrate (or phosphopeptide) was prepared (20 mM HEPES [pH 7.4], 10 mM MgCl<sub>2</sub>, 10  $\mu$ M peptide at 30 °C), and fluorometer readings were obtained without any ATP present. ATP (stock solution 11) was then added to a final concentration of 1 mM. At each ATP concentration a fluorescence emission spectrum was recorded (slit widths: Em = 5 nm, Ex = 5 nm;  $\lambda_{ex}$  = 360 nm,  $\lambda_{em}$  = 380-650 nm). Fluorescence increase was obtained by dividing the fluorescence of the phosphopeptide by the fluorescence of the substrate at 485 nm. The values reported are averages of at least three separate measurements.

**Table S1.** Fold fluorescence increase of BTF and RDF chemosensors in the presence and absence of ATP

Entry	Peptide Sequence	Fold fluorescence increase	
		0 mM ATP	1 mM ATP
1	H <sub>2</sub> N-DDK-Sox-PL-(p)T-P <u>SS</u> KEG-CO <sub>2</sub> H	1.7	1.5
2	H <sub>2</sub> N-DDK-Sox-PL-(p)T-P <u>G</u> GRRG-CO <sub>2</sub> H	2.1	2.3
3	Ac-VPLL-(p)T-P-CSox- <u>G</u> RRG- CO <sub>2</sub> H	5.4	5.1
4	Ac-VPLL-(p)T-P <u>G</u> -CSox- <u>R</u> RG- CO <sub>2</sub> H	6.3	6.2
5	Ac-VP-CSox-L-(p)T-P <u>G</u> GRRG- CO <sub>2</sub> H	6.9	8.1

MBP-based probe offered better fluorescence enhancements than the Ets-1-based sensor. Because of these results the eventual Recognition-Domain Focused (RDF) peptides incorporated the MBP-derived phosphorylation sequence and the optimal one was ligated to the PNT domain of Ets-1.

RDF probes (entries 3-5) incorporate the C-Sox amino acid and generally have significantly higher fluorescence increases compared to the BTF probes. The peptide sensor with the highest fluorescence increase (entry 5) preserves the entire MBP-based sequence C-terminal to the TP recognition site and gives a fluorescence enhancement of ca. 7-fold under these conditions.

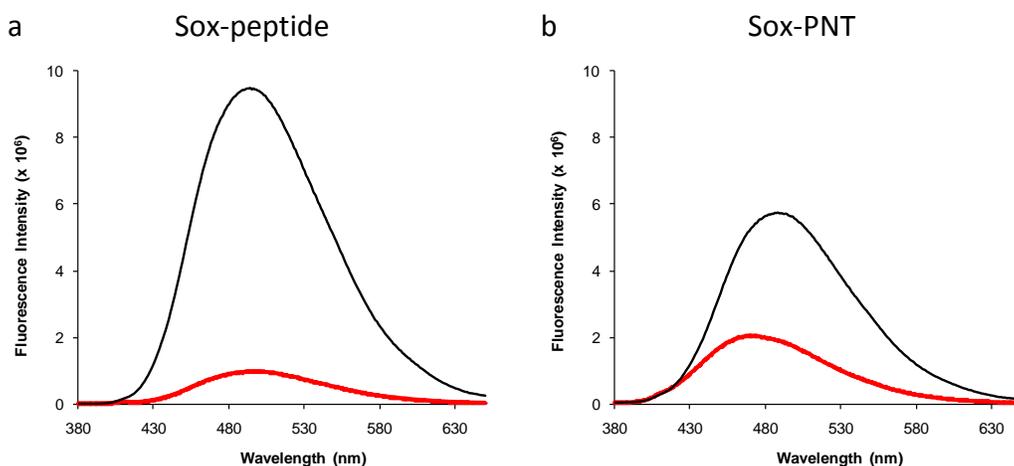
**Table S2.** Sequences surrounding the TP recognition region (red residues) of the wild type Ets-1, MBP and Sox-PNT.

				38							46	138			
Ets-1(32-138)	V	P	L	L	T	P	S	S	K	E	<u>M</u>	<u>M</u>	S	...	K
MBPtide			A	P	R	T	P	G	G	R	R				
Sox-PNT	V	P	C-Sox	L	T	P	G	G	R	R	<u>G</u>	<u>C</u>	S	...	K

The Met44-Met45 site in Ets-1 was changed to Gly44-Cys45 (underlined residues) in Sox-PNT due to requirements for NCL.

*b. Fluorescence increases of Sox-peptide and Sox-PNT chemosensors*

The fluorescence increases of the Sox-peptide (Figure S1a) and Sox-PNT (Figure S1b) were measured in the fluorometer (slit widths:  $E_m = 5$  nm,  $E_x = 5$  nm;  $\lambda_{ex} = 360$  nm,  $\lambda_{em} = 380-650$  nm) in a quartz microcuvette (total volume of 120  $\mu$ L) under the following conditions: 10  $\mu$ M unphosphorylated (red) or phosphorylated (black) sample, 50 mM Tris (pH 7.5), 10 mM  $MgCl_2$ , 1 mM EGTA, 2 mM DTT and 0.01% Brij 35 P at 30 °C.



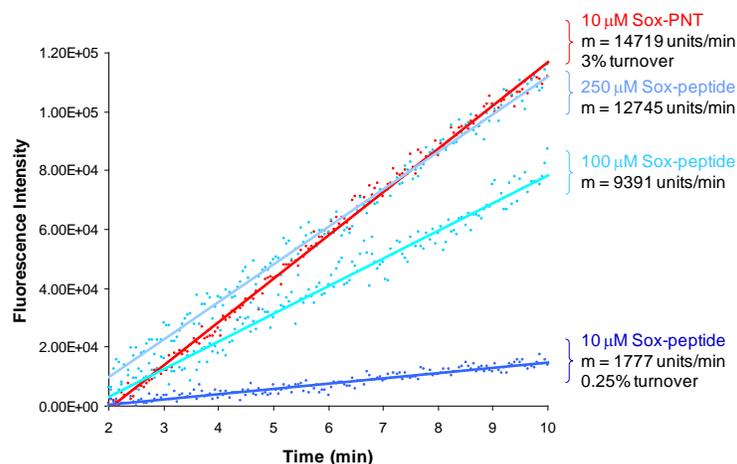
**Figure S2.** Fluorescence spectra of phosphorylated (black) and unphosphorylated (red) versions of (a) Sox-peptide and (b) Sox-PNT sensors.

While there is a drop in fluorescence enhancement in the Sox-PNT probe, a 3-fold change observed with Sox-PNT is still a robust increase for use in vitro, cell lysate and in vivo experiments.

*c. Affinity of ERK2 for Sox-PNT and Sox-peptide*

The activity of recombinant ERK2 (NEB) was measured over time with Sox-PNT and Sox-peptide in the fluorometer (slit widths:  $E_m = 5$  nm,  $E_x = 5$  nm;  $\lambda_{ex} = 360$  nm,  $\lambda_{em} = 485$  nm) in a quartz microcuvette (total volume of 120  $\mu$ L) under the following conditions: 50 mM Tris (pH 7.5 at 25 °C), 10 mM  $MgCl_2$ , 1 mM EGTA, 2 mM DTT, 0.01% Brij 35 P, 10  $\mu$ M Sox-PNT or 10-250  $\mu$ M Sox-peptide and 11 ng ERK2 at 30 °C. The fluorescence slopes (m) of the reactions

(Figure S3) were determined by a least-squares fit using Microsoft Excel, then normalized to the highest slope (obtained with 10  $\mu$ M Sox-PNT) and plotted in the bar graph form (Figure 2a in the main text) for clearer visualization.



**Figure S2.** Fluorescence intensity over 10 minutes of 10  $\mu$ M Sox-PNT (red) and 10–250  $\mu$ M Sox-peptide (shades of blue) with 11 ng ERK2.

*d. Determination of kinetic constants for ERK2 with Sox-PNT*

Recombinant ERK2 (Invitrogen, 5 nM, 42 ng, MW = 69,700 g/mol) was added to initiate each reaction. The kinetic assays were performed in the FPR ( $\lambda_{\text{ex}} = 360$  nm,  $\lambda_{\text{em}} = 485$  nm) in a 96-well plate (120  $\mu$ L per reaction) containing varying chemosensor concentrations (0.2–5  $K_M$ ) at 30  $^{\circ}$ C. Fluorescence slopes were determined by a least-squares fit using Microsoft Excel. Slopes were then converted to a rate using published procedures.<sup>1</sup> The plots of  $v$  vs.  $[S]$  were fit using SigmaPlot 9.01<sup>3</sup> in order to obtain  $K_M$  and  $V_{\text{max}}$  values. The assay conditions were as follows: 50 mM Tris (pH 7.5 at 25  $^{\circ}$ C), 10 mM  $\text{MgCl}_2$ , 1 mM EGTA, 2 mM DTT, 0.01% Triton X-100, 1 mM ATP at 30  $^{\circ}$ C. Alternatively, 384-well plates can be used (20  $\mu$ L reaction volume) with, otherwise, the same conditions.

*e. Selectivity of Sox-PNT in a panel of MAPKs*

The assays were performed in the FPR ( $\lambda_{\text{ex}} = 360$  nm,  $\lambda_{\text{em}} = 485$  nm) in a 96-well plate (120  $\mu$ L per reaction) containing 5  $\mu$ M chemosensor at 30  $^{\circ}$ C. Recombinant ERK1 and 2, JNK1, 2, and 3, p38 $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  and CDK1/Cyclin B and CDK2/Cyclin A (all enzymes were obtained from Invitrogen) were appropriately diluted with 50 mM Tris (pH 7.5 at 25  $^{\circ}$ C), 10 mM  $\text{MgCl}_2$ , 1 mM ATP, 1 mM EGTA, 2 mM DTT, 0.01% Triton X-100. Addition of 15 or 150 nM of enzyme initiated reactions. Fluorescence slopes were determined by a least-squares fit using Microsoft Excel. The slopes obtained with 15 and 150 nM of enzyme were normalized to the slope with ERK1 and plotted on the same graph for easy visualization (Fig. 2c in the main text). Due to the high 150 nM concentration of enzymes, these experiments could only be performed once. The assays with 15 nM enzyme are reported with s.e.m. for triplicate measurements. The final assay

conditions were as follows: 5  $\mu$ M Sox-PNT, 50 mM Tris (pH 7.5 at 25 °C), 10 mM MgCl<sub>2</sub>, 1 mM EGTA, 2 mM DTT, 0.01% Triton X-100, 1 mM ATP and 15 or 150 nM enzyme at 30 °C.

## **VI. Experiments in crude cell lysates**

### *a. Crude cell lysate preparation*

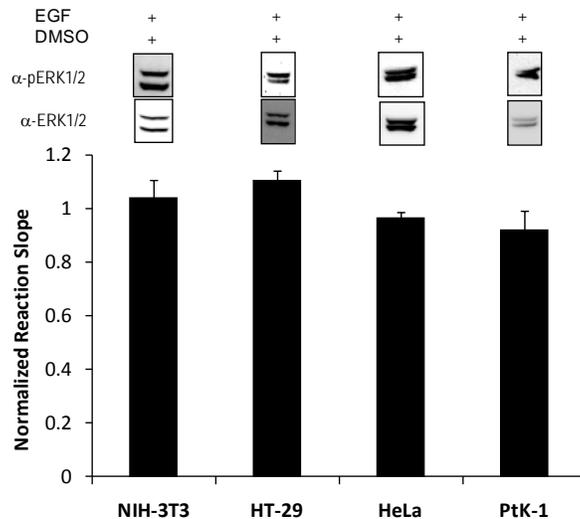
Cell cultures were maintained according to manufacturer's recommendations using the appropriate media (FBS-containing solutions 17-20) that were stored at 4 °C in the dark and warmed to 37 °C for 30 min before use. Prior to lysis, cells were serum-starved on 150 mm tissue culture dishes for 15-18 h in serum-free media supplemented with an additional 2 mM L-Gln (Serum-free media solutions 17-20). Cells were either 1. untreated, 2. stimulated with EGF (100 ng/mL) for 5 min at 37 °C, 3. preincubated with U0126 (5  $\mu$ M delivered in 2.5  $\mu$ L DMSO) for 1 h and then stimulated with EGF (100 ng/mL) for 5 min at 37 °C, or 4. treated with DMSO (2.5  $\mu$ L) for 1 h followed by stimulation with EGF (100 ng/mL) for 5 min at 37 °C. The media was aspirated and cells washed with cold PBS (2 x 20 mL). The cells were kept on ice and lysed by addition of Lysis Buffer II (~ 100  $\mu$ L/dish) directly on the plate followed by scraping and passing the mixture through a 22-gauge needle attached to a 1 mL syringe. The lysed solution was clarified at 14,000 x g for 10 min at 4 °C. The supernatant was collected and stored in aliquots at -80 °C. Total protein concentration was determined using Bio-Rad's Protein Assay.

### *b. Western blot visualization of ERK1/2 by chemiluminescence*

ERK1/2 expression was analyzed by western blots that were probed with anti-ERK1/2 antibody (Upstate) or anti-pERK1/2 antibody (Cell Signaling Tech.) according to the manufacturer's protocols. Incubation with secondary antibody (goat anti-rabbit antibody conjugated to HRP from Pierce) was followed by exposure of the blot to SuperSignal West Dura Extended Duration Substrate (Pierce) and visualized by enhanced sensitivity chemiluminescence (Bio-Rad, Quantity One 1-D Analysis Software).

### *c. Assays with crude cell lysates and Sox-PNT*

The assays were performed in the FPR ( $\lambda_{\text{ex}} = 360$  nm,  $\lambda_{\text{em}} = 485$  nm) in a 96-well plate (120  $\mu$ L per reaction) containing 5  $\mu$ M Sox-PNT at 30 °C. Untreated, EGF-stimulated, U0126-treated then EGF-stimulated or DMSO-treated then EGF-stimulated lysates from NIH-3T3, HT-29, HeLa or PtK-1 cells were added to start the reactions. The reactions were monitored for 15 min. Fluorescence slopes were determined by a least-squares fit using Microsoft Excel then normalized to the highest slope and plotted in the bar graph form (Figure 3b in the main text and Figure S4) for clearer visualization. The final assay conditions were as follows: 5  $\mu$ M Sox-PNT, 50 mM Tris (pH 7.5 at 25 °C), 10 mM MgCl<sub>2</sub>, 1 mM EGTA, 2 mM DTT, 0.01% Triton X-100, 1 mM ATP and 40  $\mu$ g lysate at 30 °C.



**Figure S4.** Effects of DMSO on ERK1/2 activation. DMSO-treated and then EGF-stimulated lysates were assayed with Sox-PNT to show that DMSO does not have an effect on ERK1/2 activity. This was also confirmed by western blots (inset) probed with anti-pERK1/2 (top) and anti-ERK1/2 (bottom) antibodies. Plotted values indicate the mean  $\pm$  s.e.m. for triplicate measurements.

*d. Determination of  $IC_{50}$  and  $K_i$  with PEA-15 in EGF-stimulated NIH-3T3 lysates*

The assays were performed in the FPR ( $\lambda_{ex} = 360$  nm,  $\lambda_{em} = 485$  nm) in a 96-well plate (120  $\mu$ L per reaction). Sox-PNT (5  $\mu$ M) was preincubated for 5 min with PEA-15 (7 concentrations in the 0.0005-50  $\mu$ M range) at 30  $^{\circ}$ C. The reactions were started by addition of EGF-stimulated NIH-3T3 lysate and monitored for 15 min. Fluorescence slopes were determined by a least-squares fit using Microsoft Excel and converted to rates ( $v$  in  $\mu$ M/min) using published procedures.<sup>1</sup> The plots of  $v$  vs.  $\log$  [PEA-15] were fit using SigmaPlot 9.01<sup>3</sup> in order to obtain the  $IC_{50}$  value (Figure 3d in the main text). The final assay conditions were as follows: 5  $\mu$ M Sox-PNT, 50 mM Tris (pH 7.5 at 25  $^{\circ}$ C), 10 mM  $MgCl_2$ , 1 mM EGTA, 2 mM DTT, 0.01% Brij 35 P, 1 mM ATP and 40  $\mu$ g lysate at 30  $^{\circ}$ C.

The  $K_i$  value was derived from the above-determined  $IC_{50}$  using the following equation,<sup>4</sup>

$$IC_{50} = \left( 1 + \frac{[S]}{K_M} \right) K_i \quad (1)$$

where  $S$  is the concentration of Sox-PNT in the assays. Upon rearranging, eq. (1) can be solved for  $K_i$ .

*e. Immunodepletions of ERK1/2 from EGF-stimulated HeLa lysate*

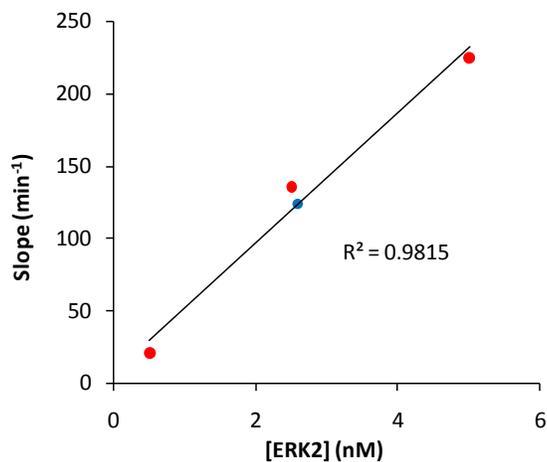
EGF-stimulated HeLa lysate (900  $\mu\text{g}$ ) was precleared first by incubation with Protein A sepharose beads (50  $\mu\text{L}$ , Amersham) for 1 hr on a rotating shaker at 4  $^{\circ}\text{C}$ . The precleared EGF-stimulated HeLa lysate (300  $\mu\text{g}$ ) was incubated with anti-ERK1/2 antibody (5  $\mu\text{g}$ , Upstate) or naïve rabbit IgG (5  $\mu\text{g}$ , Santa Cruz) for 1 hr on a rotating shaker at 4  $^{\circ}\text{C}$ . The immune complexes were precipitated with 50  $\mu\text{L}$  Protein A sepharose beads for 1 hr with mixing at 4  $^{\circ}\text{C}$ . In total, three rounds of immunodepletions were carried out to ensure removal of all ERK1/2. The immunodepletions were analyzed by western blots (probed with anti-ERK1/2 antibody from Upstate) that were visualized using chemilluminescence, as described above.

*f. Estimation of active ERK1/2 in EGF-stimulated HeLa lysates*

The assays were performed in the FPR ( $\lambda_{\text{ex}} = 360 \text{ nm}$ ,  $\lambda_{\text{em}} = 485 \text{ nm}$ ) in a 96-well plate (120  $\mu\text{L}$  per reaction) containing 5  $\mu\text{M}$  Sox-PNT at 30  $^{\circ}\text{C}$ . The standard curve was obtained by incubating Sox-PNT with 0.5, 2.5 or 5 nM recombinant ERK2 and then plotting the average slope of each reaction as a function of ERK2 concentration (Figure S5). Using a least-squares fit in Microsoft Excel the following equation was obtained,

$$y = 45.07x + 7.47 \quad (2)$$

where  $y$  is the fluorescence intensity at a given concentration of ERK2 ( $x$ ) in the 0.5-2.5 nM range. At the same time, Sox-PNT was incubated with EGF-stimulated NIH lysates and the reaction slope was determined (124  $\text{min}^{-1}$ ) by a least-squares fit using Microsoft Excel. When substituted in eq. (2) and solved for  $x$ , the concentration of ERK2 in EGF-stimulated NIH lysates is obtained (2.6 nM in 40  $\mu\text{g}$  of lysate) that can also be converted to amount of enzyme (13 ng) because of the known reaction volume (120  $\mu\text{L}$ ) and ERK2 molecular weight (42,000 g/mol). However, since lysates contain both ERK1 and 2, the concentration (and amount) is representative of both enzymes. The final assay conditions were as follows: 5  $\mu\text{M}$  Sox-PNT, 50 mM Tris (pH 7.5 at 25  $^{\circ}\text{C}$ ), 10 mM  $\text{MgCl}_2$ , 1 mM EGTA, 2 mM DTT, 0.01% Brij 35 P, 1 mM ATP and 40  $\mu\text{g}$  lysate, 0.5, 2.5 or 5 nM recombinant ERK2 at 30  $^{\circ}\text{C}$ .



**Figure S5.** Determination of [ERK1/2] in lysate. Standard curve with a range of ERK2 concentrations (red circles, 0.5-5 nM) was first obtained. The standard curve was then used to measure the concentration (blue circle, 2.6 nM) and amount (13 ng) of ERK1/2 in 40  $\mu$ g of EGF-stimulated NIH-3T3 lysates.

## VI. References

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