SI Appendix

Materials and Methods

Expression and purification of Ets-1 constructs

Two constructs of murine Ets-1, termed EtsΔ138 and EtsΔ138cysless were expressed and purified using a modification of the protocols published previously (1). EtsΔ138 encodes the N-terminal 138 residues, of Ets-1 and includes the PNT domain and the canonical phosphorylation site (T38), and also carries an S26A mutation. In EtsΔ138cysless the 4 native Cys of EtsΔ138 were replaced by Ala generating the following site-specific mutations: C31A, C99A, C106A, C112A. Briefly, a pET28a vector encoding an N-terminal His$_6$-tag, a thrombin cleavage site and the desired Ets-1 sequence was transformed into BL21 Star (DE3) cells (ThermoFisher Scientific) and induced at 18 °C using 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Typically, for H$_2$O-based growths (25 g/L LB, or M9 media containing 1 g/L $^{15}$NH$_4$Cl and 4 g/L [U-$^{13}$C]-glucose as the only sources of nitrogen and carbon, respectively) cells were induced overnight starting from an OD$_{600}$ around 0.8; growths employing D$_2$O-based media (typically containing 1 g/L $^{15}$NH$_4$Cl and 3 g/L U-$[^2]$H, $^{13}$C]-glucose as the only sources of nitrogen and carbon, respectively) were induced for about 18 hours starting from an OD$_{600}$ around 0.6. In all cases, 40 mg/L of the antibiotic kanamycin was added to the growth media. For methyl-specific $^{13}$C, $^1$H labeling of Ile (δ1 only), Leu and Val residues (termed ILV) (2), in addition to uniform $^{15}$N, $^2$H labeling, D$_2$O-based M9 medium containing 1 g/L $^{15}$NH$_4$Cl and 2 g/L U-[$^2$H]-labeled glucose was supplemented with 50 mg/L of (methyl-$^{13}$C; 3,3-d$_2$) sodium α-ketoisobutyrate and 100 mg/L of (3- methyl-$^{13}$C; 3,4,4,4-d$_4$) sodium α-ketoisovalerate (all metabolic precursors were purchased from Cambridge Isotope Laboratories) 1 hour before induction. Finally, for methyl-specific $^{13}$C, $^1$H labeling of Ile (δ1 only) and Thr residues (termed IT) (3), in addition to uniform $^{15}$N, $^2$H labeling, 50 mg/L of (methyl-$^{13}$C; 3,3-d$_2$) sodium α-ketoisobutyrate was added 1 hour before induction, while 50 mg/L of (4-$^{13}$C; 2-3-d$_2$) L-threonine and 100 mg/L of $^2$H-labeled glycine were added to the media immediately before induction. Cells were lysed by sonication in a buffer containing 40 mM Tris, pH 7.5, 0.75 M NaCl, 0.1% Triton-X and a protease inhibitor cocktail (Roche), centrifuged at 16,000 g for 30 minutes and loaded onto a cobalt-based resin (Talon) by gentle agitation on a rotary agitator for one hour. After washing the beads with 4 column volumes (CVs) of lysis buffer, an additional 4 CVs of lysis buffer containing 6 M guanidinium chloride were passed through the column in two steps. This was followed by additional washes with lysis buffer, alone (6 CVs), and that containing 3 mM imidazole (2 CVs). The protein was eluted using 0.4 M imidazole (4 CVs) and dialyzed overnight at 4 °C against thrombin cleavage buffer (20 mM Tris, pH 8.3, 150 mM NaCl, 2.5 mM CaCl$_2$, 0.1% β-mercaptoethanol) in the presence of thrombin (Enzyme Research Laboratories; approximately 2 units per mg of protein). Cleavage of the His$_6$-tag was confirmed by electrophoresis on a 15 % SDS polyacrylamide gel. Thrombin was then removed by passing the cleavage solution through a benzamidine column (GE Healthcare), after which 10 mM EDTA, 1 mM AEBSF and 5 mM DTT (for EtsΔ138 only) were added. The protein was then concentrated using spin columns (Amicon) and injected onto a gel filtration column (Superdex 75HP, GE Healthcare) pre-equilibrated with NMR buffer (20 mM phosphate, pH 6.8, 150 mM KCl, 200 μM EDTA).
Expression and purification of Ets-1 positioning mutants

First, a specific mutant of EtsΔ138 (C99A/C106A/C112A; EtsΔ138C31) was prepared as described earlier (4). Next, a T38A/P39A double mutant of EtsΔ138C31 was produced by a site directed mutagenesis according the following protocol - A single-step PCR reaction was carried out using 25 µL pFU DNA polymerase reaction buffer containing 200 µM each of the four deoxynucleoside triphosphates, 12.5 ng of template DNA (pET28a-His6-EtsΔ138C31), 50 ng of forward and reverse primers, and 0.5 µL of pFU DNA polymerase. The cycling parameters were 98 °C for 5 min, followed by 18 cycles at 98 °C for 50 s, 65 °C for 50 s, 72 °C for 7 s, with a final elongation step 72 °C for 10 min. The primers used were:
Forward – GCAGATGTCCGGCTGTAGCCGAGCAAGAAATGATG
Reverse – CATCATTTCTTGTGCAGCTGACACAGCGGACACATCTGC

After Digestion of parent template with Dpn1 (1 h), the reaction product was transformed into E. coli DH5α cells and the construct was verified by sequencing. Rest of the positioning mutants were generated as described above using EtsΔ138C31/T38A/P39A as the template. The N-terminal truncation construct Δ24EtsΔ138 (containing residues 24-138 of Ets-1) was generated from EtsΔ138 using site-directed mutagenesis and the corresponding position mutants were made using the same procedure as described above.

Expression and purification of spin-labeled ERK2 mutants

Detailed protocols for expressing ERK2 labeled using a variety of schemes and for activating the kinase (dual phosphorylation on Thr183 and Tyr185; ppERK2) using M KK1G7B (5) (a constitutively active mutant of MEK1) has been extensively described elsewhere (6, 7). The ERK2 mutants employed for the paramagnetic relaxation enhancement (PRE) measurements were constructed starting from the previously described vector (8) encoding a cysteine-less version of ERK2 - ERK2cysless: (pET28-His6-Cys125Ser/Cys159Ser/Cys252Leu/Cys63Ala/Cys38Ala/Cys164Ala/Cys214Ala-ERK2). The Gly240Cys mutant of ERK2cysless was generated using the QuikChange site-directed Mutagenesis kit (Agilent) using the following primers:
Forward – GTGATGGGAGATCCCAAGAATGCAACAGGTGTGGATTCTAGCTGG
Reverse – CCAGCTGAATCCTCAGCTGCGCATTCTTGAGATCTCCATCAC
The Leu252Cys mutant of ERK2cysless was engineered by GenScript. Both mutants were expressed at 18 °C and purified in a fashion identical to wild-type ERK2. Each mutant was individually site-specifically spin-labeled with 1-oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline-3-methyl methanethiosulfonate (MTSL) using the following procedure: The samples were dialyzed overnight against 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, 200 µM EDTA, 1 mM DTT. The following morning, an additional 1 mM of fresh DTT was added to the samples, which were then concentrated using spin columns and DTT was removed using PD-10 desalting columns (GE Healthcare) following manufacturer recommendations. The samples were then incubated with a 10-fold excess of MTS (Toronto Research Chemicals) at room temperature for one hour, then overnight at 4 °C. The samples were then further concentrated and unreacted MTS was removed by reloading the samples onto a PD-10 column. Finally, the samples were exchanged into the desired NMR buffer using multiple runs through a spin column until a 10000-dilution factor for the original buffer was achieved. The covalent attachment of a single MTS label was confirmed.
by mass spectroscopic analysis and by comparison of 1-dimensional $^1$H NMR spectra before and after reduction with 10 mM ascorbic acid.

**Resonance assignment of EtsΔ138**

All NMR experiments were carried out at 25 °C in NMR buffer (see above) using Bruker Avance spectrometers operating at $^1$H frequencies of 500 MHz and 800 MHz, or a Varian Inova spectrometer operating at 600 MHz. All spectrometers were equipped with cryogenic probes capable of applying pulsed field gradients along the z-axis.

While resonance assignments are available for residues 29-138 (done for a construct termed Δ29EtsΔ138 in the nomenclature used here) (9, 10) of Ets–1, these assignments needed to be completed and validated under the conditions used in the present study. Assignment of the $^{13}$C, $^{15}$N and $^1$H resonances for free EtsΔ138 were obtained using canonical backbone-directed triple-resonance experiments (HNCACB, CBCA(CO)NH, HNCACO and HNCO) (11) at 800 MHz on a 1 mM U-$[^{15}$N, $^2$H,$^{13}$C]-labeled sample in NMR buffer (see above) in the presence of 2 mM DTT. An (H)C(CO)NH experiment with 20 ms mixing times was also collected at 600 MHz. Assignment of the resonances of EtsΔ138cysless were also obtained using standard backbone-directed approaches utilizing HNCACB, CBCA(CO)NH, (H)CONH, H(CC)NH, H(CC)H-TOCSY and (H)CCH-TOCSY (18.0 ms mixing time for all experiments that involved scalar mixing) collected on a 1 mM U-$[^{15}$N, $^{13}$C]-labeled sample at 500 MHz. Assignment of methyl resonances for all species was confirmed using 3-dimensional $^{13}$C-HMQC-NOESY-HMQC and $^{13}$C-edited NOESY-HSQC experiments (150 ms mixing time) at 600 MHz. A TROSY-based HNCO experiment was collected at 600 MHz on a sample containing 300 μM U-$[^{15}$N, $^2$H,$^{13}$C]-labeled EtsΔ138 in the presence of 250 μM inactive ERK2 in an NMR buffer containing 5 mM MgCl$_2$ and 10 mM DTT. Additionally a TROSY-based HNCA CB (600 MHz) experiment was acquired for 200 μM U-$[^{15}$N, $^2$H,$^{13}$C]-labeled EtsΔ138 in the presence of 250 μM U-$^2$H-labeled inactive ERK2. A TROSY-based HNCO (600 MHz) experiment was also acquired for 200 μM U-$[^{15}$N, $^2$H,$^{13}$C]-labeled EtsΔ138 in the presence of 250 μM ppERK2. Details of these, and additional triple-resonance experiments are provided in Table S1.

**NMR-based titrations**

All NMR titrations were performed by progressively diluting the NMR inactive species in the complex with NMR buffer containing 10 mM DTT (except in the case of 3. below) and only the NMR-active species.

A series of 2-dimensional $^{15}$N, $^1$H TROSY experiments (600 MHz) were acquired for:

1. A 200 μM U-$[^{15}$N, $^2$H,$^{13}$C]-labeled EtsΔ138 sample containing 0, 25 μM, 50 μM, 100 μM, 150 μM, 200 μM and 250 μM inactive ERK2 in the presence of 5.0 mM MgCl$_2$.

2. A 200 μM U-$[^{15}$N, $^2$H,$^{13}$C]-labeled EtsΔ138 in the presence of 0, 10 μM, 20 μM, 40 μM, 60 μM, 90 μM, 123 μM, 154 μM, 205 μM, 237 μM, 303 μM, 337 μM ppERK2 in the presence of 5.0 mM MgCl$_2$.

3. A 180 μM U-$[^{15}$N, $^2$H]-labeled EtsΔ138cysless in the presence 400 μM inactive ERK2cysless was titrated with 50 μM, 100 μM, 200 μM, 400 μM, 1 mM MTSL. This was done to test for non-specific binding of free MTSL by the EtsΔ138cysless• ERK2cysless complex.
A series of 2-dimensional $^{13}$C, $^1$H HMBC experiments were acquired in D$_2$O-based NMR buffer for:

4. A 171 µM ILV, U-[$^{15}$N, $^2$H]-labeled EtsΔ138cysless sample containing 0, 25 µM, 50 µM, 90 µM, 130 µM, 180 µM, 250 µM, 250 µM, 376 µM inactive ERK2. All experiments were acquired at 900 MHz except for the 50 µM point that was acquired at 800 MHz.

5. A 40 µM IT, U-$^{15}$N, $^2$H]-labeled EtsΔ138 sample was titrated with 0, 5 µM, 10 µM, 20 µM, 30 µM, 40 µM, 55 µM, 72 µM, 90 µM of U-$^2$H-labeled ppERK2 in the presence of 6 mM MgCl$_2$, and 400 µM of the non-hydrolysable ATP-analog, AMPPCP. All experiments were acquired at 800 MHz.

Chemical shift perturbations (CSP) were calculated using the following formula (7):

$$
\Delta \delta_j = \sqrt{0.5 \sum_{i=1}^{J} (w_{ik} \Delta \delta_i)^2}
$$

(S1)

Where the index $j$ identifies a particular residue, $i$ identifies a nucleus ($^1$H, $^{15}$N or $^{13}$C), and $w_{ik}$ is a weighting factor defined as $w_{ik}=1/\sigma_{ik}$, with $\sigma_{ik}$ defined as the standard deviation for atom type $i$ and amino acid type $k$ obtained from the Biological Magnetic Resonance Databank. A corrected standard deviation $\sigma_0^{corr}$ to use as threshold in order to discriminate between perturbed and unperturbed residues was calculated using the iterative procedure described earlier (7).

**Backbone $^{15}$N relaxation experiments**

TROSY-based $^{15}$N relaxation experiments (12) (600 MHz) were carried out for EtsΔ138 under various conditions using the following relaxation delays (experiments were performed in interleaved fashion): for $R_1$ - 10 ms, 50 ms, 100 ms ($\times$ 2), 200 ms, 400 ms, 800 ms, 1.0 s, 1.2 s; for $R_2$ - 10 ms, 30 ms ($\times$ 2), 50 ms, 70 ms, 90 ms, 110 ms. Both experiments were performed using recycle delays of 3 s. $^1$H-$^{15}$N steady-state NOE experiments (13, 14) were performed in interleaved fashion using a 5 s period with or without $^1$H saturation. Experiments were collected on a 200 µM U-$^{15}$N, $^2$H, $^{13}$C]-labeled sample of EtsΔ138 in NMR buffer containing 5 mM MgCl$_2$ in the presence of – (1) 200 µM inactive ERK2, (2) 200 µM ppERK2 and (3) 200 µM ppERK2 in the presence of 600 µM AMPPCP. These correspond to the following states (1) inactive complex, (2) active complex and (3) pre-chemistry complex. Additionally, another set of data were acquired for a 300 µM sample of U-$^{15}$N, $^2$H, $^{13}$C]-labeled EtsΔ138 in NMR buffer containing 5 mM MgCl$_2$ in the presence of 250 µM inactive ERK2.

The $R_1$ and $R_2$ rates were obtained from single exponential fits of the relaxation data using routines provided within the NMRView (15) package. Errors in the $^1$H-$^{15}$N steady-state NOEs were estimated by simple error propagation using the following equation:

$$
\sigma_{NOE} = \frac{I_{sat}}{I_{ref}} \sqrt{\left(\frac{\sigma_{sat}}{I_{sat}}\right)^2 + \left(\frac{\sigma_{ref}}{I_{ref}}\right)^2}
$$

(S2)
where $I_{\text{sat/ref}}$ are the resonance intensities measured in the presence/absence of $^1\text{H}$ saturation, while $\sigma_{\text{sat/ref}}$ represent the corresponding standard deviations of the noise measured in regions devoid of signal.

**Reduced spectral density analysis**

The reduced spectral density functions (16) at 0, $\omega_N$ and 0.87$\omega_H$ frequencies were determined utilizing the measured $R_1$, $R_2$ and $\{^1\text{H}\}-^1\text{H}$ steady-state NOEs using the following equations:

\[
J(0) = \frac{2R_1 - R_2'}{4(c^2 + d^2)}
\]

\[
J(\omega_N) = \frac{R_1'}{3(c^2 + d^2)}
\]

\[
J(0.87\omega_H) = \frac{HF}{d^2}
\]

The dipolar and CSA strengths (d, c, respectively) are given by

\[
d = -\frac{\mu_0}{4\pi} \left( \frac{\gamma_N \gamma_H h}{2r_{NH}^3} \right);
\]

\[
c = \frac{\gamma_N B_0 \Delta \sigma}{3}
\]

where the various symbols have their usual meanings. The modified relaxation rates are given by (17)

\[
R_1' = R_1 - 7 \left( \frac{0.870}{0.921} \right)^2 HF
\]

\[
R_2' = R_2 - 13 \left( \frac{0.870}{0.955} \right)^2 HF
\]

\[
HF = -\frac{\gamma_N}{5\gamma_H} (1 - \text{NOE}) R_1
\]

**Sidechain relaxation measurements**

Intra-methyl $^1\text{H}-^1\text{H}$ dipole-dipole cross-correlation rates ($\eta$) (18) were measured for the following samples of IT, U-$[^{15}\text{N}, ^2\text{H}]$-labeled EtsΔ138 bound to ppERK2; two-samples were used with EtsΔ138:ppERK2 ratios of 200:250 µM and 389:198 µM EtsΔ138 in the presence of 10 mM DTT, 5 mM MgCl$_2$ and 600 µM AMPCP. This corresponds to the pre-chemistry state discussed above. Two distinct datasets for the “allowed” and “forbidden” transitions were collected in series at 600 MHz using the following relaxation delays: 1 ms, 5 ms, 10 ms, 15 ms, 22 ms, 30 ms ($\times 2$) 40 ms, 50 ms, 70 ms. Reference experiments were collected on 415 µM IT, U-$[^{15}\text{N}, ^2\text{H}]$-labeled EtsΔ138 alone using the following relaxation delays: 1 ms, 5 ms, 10 ms, 20 ms, 30 ms, 45 ms, 60 ms, 80 ms ($\times 2$), 110 ms, 150 ms, 190 ms, 240 ms. For each individual time point the number of collected transients was varied to optimize the resulting signal-to-noise ratio and the intensities of the corresponding experiments were normalized accordingly.
The normalized intensities for the allowed (I_A) and forbidden (I_B) experiments were fitted to the following equation:

\[ I_E = \frac{-0.5\eta \tanh(\sqrt{\eta^2 + \delta^2 T})}{\sqrt{\eta^2 + \delta^2 - \delta \tanh(\sqrt{\eta^2 + \delta^2 T})}} \] (S5)

Here \( \eta \) is the cross-correlation rate, \( \delta \) depends on the \(^1\text{H}-^1\text{H}\) cross-relaxation between external protons, and \( T \) is the relaxation time. The \( \eta \) values were converted into order parameters \( S_{ax}^2 \) using:

\[ S_{ax}^2 = \frac{40}{9} \gamma_H^4 \tau_C \frac{r_{HH}^6}{\eta} \] (S6)

where \( r_{HH} = 1.813 \) Å is the intra-methyl \(^1\text{H}-^1\text{H}\) distance. All other terms have their usual meanings.

**Distance restraints from the measurement of paramagnetic relaxation enhancement**

Paramagnetic relaxation enhancement (PRE, \( \Gamma_2^{\text{net}} \)) rates for the methyl groups of Ile (\( \delta_1 \) only), Leu and Val methyl groups were measured using HMOC-based \(^1\text{H}\) relaxation measurements (19) and the following samples in D$_2$O-based NMR buffer: 115 \( \mu \)M ILV, U-[\(^{15}\text{N}, ^2\text{H}\)]-labeled Ets\Delta138$_{cysless}$ containing 173 \( \mu \)M MTSL-labeled inactive ERK2$_{cysless,Gly240Cys}$ (900 MHz; 25 PRE values) or 140 \( \mu \)M ILV, U-[\(^{15}\text{N}, ^2\text{H}\)]-labeled Ets\Delta138$_{cysless}$ containing 204 \( \mu \)M MTSL-labeled inactive ERK2$_{cysless,Leu252Cys}$ (800 MHz; 25 PRE values). PRE rates were obtained using a two-time point approach \( (T_A=0 \) ms, \( T_B=23 \) or \( 24 \) ms; see below) in the absence (paramagnetic) and presence (diamagnetic) of 1 mM ascorbic acid. PRE rates \( (\Gamma_2^{\text{mid}}) \) for backbone amides were measured using \(^{15}\text{N}, ^1\text{H}\) TROSY-based (20) experiments (600 MHz) with and without incubation with 1 mM ascorbic acid on the following samples: 150 \( \mu \)M ILV, U-[\(^{15}\text{N}, ^2\text{H}\)]-labeled Ets\Delta138$_{cysless}$ in the presence of 200 \( \mu \)M MTSL-labeled inactive ERK2$_{cysless,Gly240Cys}$ (82 PRE values) or 160 \( \mu \)M ILV, U-[\(^{15}\text{N}, ^2\text{H}\)]-labeled Ets\Delta138$_{cysless}$ in the presence of 160 \( \mu \)M MTSL-labeled inactive ERK2$_{cysless,Leu252Cys}$ (76 PRE values).

For the two point measurements the PREs and their corresponding errors were determined using the following equations (20):

\[ \Gamma_2^{\text{net}} = \frac{1}{T_B - T_A} \ln \left[ \frac{I_D(T_B)I_P(T_A)}{I_D(T_A)I_P(T_B)} \right] \]

\[ \sigma_2^{\text{net}} = \frac{1}{T_B - T_A} \sqrt{\left( \frac{\sigma_D(T_A)}{I_D(T_A)} \right)^2 + \left( \frac{\sigma_P(T_A)}{I_P(T_A)} \right)^2 + \left( \frac{\sigma_D(T_B)}{I_D(T_B)} \right)^2 + \left( \frac{\sigma_P(T_B)}{I_P(T_B)} \right)^2} \] (S7)
I_p and I_d are the peak intensities for the paramagnetic and diamagnetic datasets, respectively. \( \sigma_p \) and \( \sigma_d \) are standard-deviations of the noise measured in empty regions of the spectra for the paramagnetic and diamagnetic spectra, respectively.

For some resonances, the \( \Gamma^\text{met} \) values could not be determined precisely using the methodology describe above. In such cases, and for amide resonances, the PRE values were obtained utilizing ratios of HMQC/TROSY spectra (for methyl/amide groups) measured for the same samples described above processed without the use apodization functions and numerical solutions to the following equations:

\[
\begin{align*}
\left( \frac{I_p}{I_d} \right)_{\text{met}} &= \frac{R_{2,\text{direct},D}}{(R_{2,\text{direct},D} + \Gamma^\text{met}/2) (R_{2,\text{indirect},D} + \Gamma^\text{met}/2)} \exp(-\Gamma^\text{met} t) \\
\left( \frac{I_p}{I_d} \right)_{\text{amide}} &= \frac{R_{2,\text{direct},D}}{(R_{2,\text{direct},D} + \Gamma^\text{amide}/2)} \exp(-\Gamma^\text{amide} t)
\end{align*}
\]

(S8)

Here \( R_{2,\text{direct},D} \) and \( R_{2,\text{indirect},D} \) are effective relaxation rates estimated by line fitting of the peaks in the direct and indirect dimensions for the diamagnetic sample, respectively, assuming Lorentzian lineshapes in both dimensions; \( t=8.0 \text{ ms} \) is the total polarization transfer time. The corresponding ratio in the case of the backbone amides (\( t=9.78 \text{ ms} \)) were determined using the following equation (21):

\[
\left( \frac{I_p}{I_d} \right)_{\text{amide}} = \frac{R_{2,\text{direct},D}}{(R_{2,\text{direct},D} + \Gamma^\text{amide}/2)} \exp(-\Gamma^\text{amide} t)
\]

(S9)

Overall errors in the PREs derived from peaks intensity ratios were estimated using a Monte-Carlo approach as follows: the error over the intensity ratios was estimated from the following equation assuming a 20% error for the \( R_{2,\text{direct/indirect}} \) in Equation S9:

\[
\sigma_{\text{ratio}} = \frac{I_p}{I_d} \sqrt{\left( \frac{\sigma_p}{I_p} \right)^2 + \left( \frac{\sigma_d}{I_d} \right)^2}
\]

(S10)

The errors determined using Equation (S10) were then used to generate 200 random values from a Gaussian distribution for each of \( I_p/I_d \) and \( R_{2,\text{direct/indirect}} \). The standard deviation was calculated over the solutions of resulting ensemble.

The \( \Gamma^\text{met/amide} \) rates for the fully-bound states were extrapolated using the concentrations involved together with the corresponding \( K_D \) values and converted into distances using the following equation:

\[
r^6 = \frac{K}{\Gamma^2} \left( 4\tau_c + \frac{3\tau_c}{1 + \omega_H^2 \tau_c^2} \right)
\]

(S11)

where the constant \( K \) for the nitroxide label is \( 1.23 \times 10^{-32} \text{ cm}^6 \text{ s}^{-2} \), \( \omega_H \) is the proton Larmor frequency expressed in radians s\(^{-1}\), and the \( \tau_c \) is the correlation time (22). The latter was estimated from the initial models of the EtsD138•ERK2 complex using HYDRONMR (23) and was
consistent with that calculated using the backbone relaxation data (extrapolated to the fully-bound state; this leads to some errors due to the exchange effects on the R₂ rates) and assuming isotropic overall diffusion. A τ_c value of 25 ns was used in Equation (S11) to convert the measured Γ₂ values into distances. It should be noted that calculated distances are largely insensitive, within limits, to the choice of the τ_c value used (24).

Distance restraints from NOESY experiments

LV, U-[¹⁵N, ²H]-labeled inactive ERK2 was prepared using a sodium α-ketoisobutyrate precursor and the expression and purification scheme described earlier (6). The peaks corresponding to the Leu and Val positions were assigned using a ¹³C, ¹H sof-HQMC spectrum (25) (14 and 12 ppm sweep-widths, with 512 and 128 complex points for the ¹H and ¹³C dimensions, respectively) in a D₂O-based NMR buffer at 600 MHz and the previously published resonance assignments by Xiao and coworkers (26). Using these assignments, the Leu, Val methyl resonances for a sample containing of 180 µM of inactive LV, U-[¹⁵N, ²H]-labeled ERK2 containing an equal concentration of IT, U-[¹⁵N, ²H]-labeled EtsΔ138 were assigned by proximity to corresponding Leu, Val resonances of free ERK2. Ambiguities were resolved and stereospecific assignments were obtained, where possible, through a structure-based analysis (using the structure of inactive ERK2; PDB: 3ERK) of the cross-peak pattern in a 3D ¹³C-NOESY-HMQC experiment (400 ms mixing time, sweep-widths of ¹H, ¹⁴N, ¹⁴C, and ¹³C dimensions: 10, 2, 16 ppm with 512, 32, 47 complex points, respectively). To further confirm the assignments, the sample was back-titrated towards the unbound form using progressive dilutions with buffer (see below). Peak intensities for the 6 intermolecular NOEs were converted into distances using a calibration constant obtained from NOEs involving a subset of stereoscopically assigned ERK2 Leu methyl groups not localized at the DRS and assuming an r⁻³ distance averaging. Before being converted into distances, intermolecular and EtsΔ138 intramolecular NOEs were rescaled respect the expected bound population (here we assumed that contribution towards the cross-peak intensity from the unstructured free form of EtsΔ138 N-terminus is negligible). Additional intramolecular NOEs were derived from a 3D ¹³C, ¹³C-HMQC-NOESY-HMQC spectrum optimized for methyl groups (800 MHz, 700 ms mixing time, ¹H, ¹³C, ¹⁴C sweep-widths of 15, 17.5, 17.5 ppm with 512, 42, 41 complex points, respectively) acquired on a sample containing 500 µM ILV, U-[¹⁵N, ²H]-labeled EtsΔ138_cysless bound to 780 µM unlabeled ERK2 in a D₂O-based NMR buffer. In the last case, intensities were classified either as “strong” (5 to 1 Å) or “weak” (9 to 2 Å). Cumulatively, 8 EtsΔ138 (to Ile or Thr) and 10 (to Leu or Val) ERK2 intramolecular NOEs were used.

Structure determination using HADDOCK

For the docking protocols, active and passive residues for the AIR (ambiguous interaction restraints) were derived from CSP data as follows: first perturbed residues were defined from either amide or methyl perturbations as those with CSPs that were one standard deviation above the mean calculated over a subset of CSP values. This subset was chosen based on an iterative protocol described in detail previously (7). Briefly, resonances above the average plus 3 standard deviations of a given subset were progressively excluded, and the statistics were recalculated on the new subset until convergence (no residues above the threshold). Quenched residues were also considered to be perturbed. Active residues were taken from the set of perturbed residues having at least 20% solvent accessibility (RSA). Passive residues were defined as solvent exposed (RSA
values of 20% or above) and contiguous to active residues. Solvent exposure (% accessibility relative to that of the same residue X placed in an extended AXA tripeptide) was calculated using NACCESS (http://wolf.bms.umist.ac.uk/naccess/).

The structure of the EtsΔ138•ERK2 complex was determined using HADDOCK by separately obtaining the structural ensembles corresponding to the DRS/N-terminus and FRS/CTR interactions, the latter through a three-way docking protocol (27) between ERK2 and ATP, ERK2 and the V34-P39 (that includes the phosphorylation site) and S40-K138 fragments of EtsΔ138. The compatibility of the orientation of the two docking calculations was assessed by linking them through the intervening disordered loop using MODELLER 9.17 (28).

The N-terminal P9-I14 fragment from EtsΔ138 (EtsΔ1389-14; was selected based upon CSPs and TALOS+-based dihedral angle analysis; see the RESULTS section for details) was docked at the ERK2 DRS using HADDOCK 2.2 (29). For the EtsΔ1389-14 fragment, eight distinct starting conformations were chosen for the docking protocol. One of these conformations was fully extended, and the remaining seven were visually selected from an ensemble of structures generated using a standard torsion angle simulated annealing protocol using CNS 1.3 (1000 steps at 50000 K followed by 1000 cooling steps to 250 K, integration time 15 fs) and employing TALOS+-derived dihedral angles and intra-molecular methyl-methyl NOEs as the only sources of experimental restraints. These structures were used in combination with a set of six starting structures for ERK2 (PDB ID: 2FYS, 2GPH, 2Y9Q, 3ERK, 3O71, 4IZA) for the docking calculations. When necessary, mutations to the rat ERK2 sequence were introduced using PyMOL (Open-Source Version 1.8.2.1) and gaps in the structures were patched using MODELLER 9.17 (28). For the docking calculations, 600 final structures were generated starting from 10000 rigid docking models following a protocol for docking of flexible peptides described elsewhere (30). During the simulated annealing stages, backbone dihedral angles for the following segments Leu110-Leu128 and Leu153-Ile163 of ERK2 were restrained within the range of values observed among a set of 25 ERK2 crystal structures that included various inactive, active, DRS-free and DRS-bound forms of the kinase. TALOS+ (31) derived dihedral restraints were also imposed for EtsΔ1389-14, where available.

Ambiguous Interaction Restraints (AIR) were generated by considering all the peptide residues except P9 as active, while for ERK2, previously published CSPs (6) were used to define active and passive residues following previously published procedures (7). A total of 25 methyl-methyl distance restraints (including 6 inter-molecular restraints) derived from the methyl-methyl NOESY experiments described above were also introduced as unambiguous restraints starting at iteration 1. The results were clustered using a 2.0 Å RMSD threshold.

As mentioned above, for the docking of the PNT domain to the FRS site, a multi-domain docking approach was employed (27). An ATP molecule was initially docked into the nucleotide binding site of active ERK2 (PDB: 2ERK) using MODELLER (28) and using the nucleotide orientation within the crystal structure of CDK2 bound to cyclin A3 and an optimal substrate peptide (PDB: 1QMZ) (32) as template. During the docking protocol the ATP molecule was considered to be fully flexible in order to allow rotation of the dihedral angles. However, specific distance restraints between ERK2 and ATP were used in order to constrain the latter to the nucleotide binding pocket. Similarly, the ERK2 activation loop was also treated as fully flexible. For EtsΔ138, residues encompassing H0 and a region that included H0 and the CTR (S40-K138) were taken from the lowest energy structure in the NMR ensemble (PDB: 2JV3) describing unbound Δ29EtsΔ138 and docked against ERK2 using ambiguous (AIR) and unambiguous distance restraints. The first 10 N-terminal residues were treated as fully flexible during the docking, but backbone dihedral restraints derived from the NMR structure were introduced using energy constants that increased
from 0.1 to 1.0 kcal mole\(^{-1}\) rad\(^{2}\) in the N- to C-terminal direction. AIR restraints were derived from the CSP perturbations measured in this work and from previous NMR studies (6, 33). Distance restraints derived from the PRE-measurements, as described above, were introduced among between EtsA138 amide and methyl groups, and the oxygen atoms from two MTSL moieties were introduced \textit{in-silico} at positions 240 and 252 (a cysteine residue was placed at position 240 using the pymol mutagenesis module) on ERK2. This was achieved using a protocol within the HADDOCK package that allows the generation of an ensemble of four distinct, independent MTSL groups for each spin-labeled position (34) from which the intermolecular distances are calculated using \(r^{6}\) averaging. Simultaneously, the EtsA138 fragment encompassing V34-P39 (that includes the phospho-acceptor T38) was also included in the calculation, and a distance restraint between the oxygen on T38 and the ATP \(\gamma\)-phosphate was imposed based on the analogous distance observed in the CDK2 crystal structure mentioned earlier. Distance restraints were also imposed between the carbonyl of P39 and the amide of S40 as described by Karaca and coworkers (27). Clustering was done using a 3.0 Å RMSD threshold. The automodel module from MODELLER 9.17 was used to stitch the various EtsA138 fragments obtained from this procedure (EtsA138\(_{34-39}\), EtsA138\(_{40-138}\)) and from the DRS (EtsA138\(_{9-14}\)) docking protocol. This last step was used to confirm that the interactions at the ERK2 DRS and FRS were compatible with each other.

**Measurement of steady-state kinetic parameters**

Kinase assays were performed at 28 °C in assay buffer (25 mM HEPES, pH 7.6, 50 mM KCl, 2 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA, 0.5% glycerol, 2.5 mg/mL BSA), containing 2 nM of ppERK2, 5 mM MgCl\(_{2}\) and various concentrations of wild-type EtsA138 (or \(\Delta 24\)EtsA138) and several variants thereof (Tables 1, 2) as substrates. Rates were measured under conditions where total product formation represented less than 10% of the initial substrate concentrations. The reaction mixture was incubated for 10 min before the reaction was initiated by the addition of \([\gamma-^{32}\text{P}]-\text{ATP}\). Aliquots (5–10 \(\mu\)L) were taken at set time points and applied to 2 \(\times\) 2-cm\(^{2}\) P81 cellulose paper. The papers were washed for 3 \(\times\) 10 min in 50 mM phosphoric acid (\(\text{H}_3\text{PO}_4\)), then in acetone and dried and the amount of labeled protein determined by counting the associated counts/min on a Packard 1500 scintillation counter at a sigma value of 2 (5). Initial rates were determined by linear least squares fitting to plots of product formed against time using

\[
\frac{k_{\text{obs}}}{k_{\text{cat}}} = \frac{[S]}{K_M + [S]} \tag{S12}
\]

The parameters used in deriving equations are defined as follows; \(k_{\text{obs}}\) observed rate constant; \(k_{\text{cat}}\), apparent catalytic constant; \([S]\), concentration of substrate \(S\); \(K_M\), apparent Michaelis constant for substrate \(S\).

**Measurement of the rate of release of EtsA138 from the EtsA138•ppERK2 complex**

The construct EtsA138\(_{\text{C31}}\) (see above) was labeled with fluorescein using 5-iodoacetomido-fluorescein as described before to generate the fluorescent EtsA138\(_{\text{C31}^*}\) (35). Stopped flow experiments were conducted at 27 °C on a Kintek SF 2001 Airforce 1 stopped flow apparatus fitted with 530 nm bandpass filters with a 25 nm bandwidth (Corion). An excitation wavelength of 492
nm was used. For competition experiments in which Ets\(\Delta\)138 competes for binding with Ets\(\Delta\)138\(_{C31}\)*, one syringe contained a 100 nM solution of Ets\(\Delta\)138\(_{C31}\)* and 40 \(\mu\)M ERK2 in 25 mM HEPES pH 7.5, 50 mM KCl, 2 mM DTT, 40 \(\mu\)g/mL BSA, 20 mM MgCl\(_2\), 0.1 mM EDTA, and 0.1 mM EGTA while the other syringe contained Ets\(\Delta\)138 (12.5-1000 \(\mu\)M) in the same buffer. The solutions were incubated at 27 °C for 3 minutes before being mixed to give a final concentration of 50 nM Ets\(\Delta\)138\(_{C31}\)*, 20 \(\mu\)M ERK2, and 6.25-500 \(\mu\)M Ets\(\Delta\)138. The reaction was monitored for a total of 20 msec and an average of 5 traces was used for data analysis. The observed fluorescence \(F\) was fitted to Equation (S13) to obtain \(k_{obs}\).

\[
F = A + Be^{-k_{obs}t}
\]  
(S13)

Where \(A\) and \(B\) are constants. \(k_{off}\) is obtained as the limiting value of \(k_{obs}\) with increasing Ets\(\Delta\)138 concentration (assuming a simple 1-site binding, \(k_{obs}\) at lower concentrations of Ets\(\Delta\)138 is increased by the fact that Ets\(\Delta\)138\(_{C31}\)* dissociated from ppERK2 can rebind thus affecting the observed fluorescence).
Fig. S1. The canonical docking sites on ERK2. The D-recruitment site (DRS) is targeted by D-site sequences with the basic and hydrophobic residues docking into negatively charged ($\phi_{\text{chg}}$) and hydrophobic ($\phi_{\text{hyd}}$) regions on the ERK2 surface, respectively. The DRS is present in both active and inactive forms of ERK2. The F-recruitment site (FRS) is targeted by F-site sequences, where only the two Phe residues participate in significant interactions with ERK2 while the Pro appears to be dispensable. The FRS is fully formed in active ERK2 (ppERK2) upon dual phosphorylation on Thr183 and Tyr185. Selected residues discussed in the text are shown in stick format and labeled.
Fig. S2. Ets-1 contains a pointed (PNT) domain, a transactivation domain (TAD), and a DNA-binding ETS domain. The first 138 residues are sufficient for the interaction with ERK2 and phosphorylation on T38 that is a part of a canonical TP motif targeted by MAP kinases. The sequence of the construct used in this study (EtsΔ138, Mus musculus, NP_001033731.1) that comprises the first 138 residues of Ets-1 is shown. Also shown is the structure of EtsΔ138 that was generated by computationally introducing the disordered residues, 1-28, into the solution structure comprising residues 29-138 (PDB: 2JV3). Helices H2-H5 comprise the PNT domain. Sidechains of key residues discussed in the text are shown in stick representation, labeled and colored on the structure (also underlined and colored on the sequence) as follows: key N-terminal hydrophobic residues (pink), the phospho-acceptor (T38, blue), helix H0 (light grey), helix H1 (dark grey) and the PNT domain (coral green). The construct used carries a S26A mutation (indicated by a ‘*’ on the sequence). Helix H1 and the PNT domain (H2-H5) are collectively termed the C-terminal region (CTR) in the text.
**Fig. S3.** (A) Isothermal calorimetry (ITC) traces for the interactions of EtsΔ138 with of ERK2 (left) and inactive ppERK2 (right). (B) Chemical shift perturbations for EtsΔ138 (200 μM) in the presence of increasing concentrations of either ERK2 (left) or ppERK2 (right) were fitted to quadratic binding isotherms. Fits using a single global K_{D} as opposed to individual K_{D} values for each trace were preferred for both ERK2 and ppERK2 (determined using the Akaike Information Criterion; >99.9% probability). The circles represent experimental data and the solid lines represent the theoretical curves from the global fit. Data and fits for only a selected number of residues are shown for optimal visualization. The affinities obtained in each case are consistent (within error) to those determined using ITC. (C) Correlation between the CSPs for the inactive (76%-bound) and active (71%-bound) complexes using the data shown in Fig. 1. The CSPs are well correlated overall (R=0.86). The scatter indicates local differences in specific regions between the inactive and active complexes. These differences are discussed in the main text.
Fig. S4. EtsΔ138 is phosphorylated by ppERK2. The large chemical shift change of the γ2 methyl resonance of T38 indicates phosphorylation by ppERK2. The resonance that is significantly upfield shifted in the $^{13}$C dimension corresponds to the phosphorylated species.
Fig. S5. The N-terminus of EtsΔ138 is dynamic in both the free and the ERK2-bound states. \(^1\)H-\(^{15}\)N NOE data (600 MHz) plotted against residue number for EtsΔ138 in the free state (A) and in the EtsΔ138•ERK2 complex (65%-bound) (B) suggests that while its N-terminus undergoes some degree of ordering upon interaction with ERK2, it remains largely disordered in the complex. The C-terminal region (CTR) that includes helix H1 and the PNT domain is rigid in both cases. An interesting observation is that certain parts of H0 that are also dynamic in the free state, are further destabilized upon interaction with ERK2. The dashed cyan line indicates a \(^1\)H-\(^{15}\)N NOE value of 0.6. Values larger than 0.6 indicate a relatively high-degree of order in solution. Gaps in the plot correspond to either prolines, or residues for which data could not be analyzed in a reliable fashion due to extensive line-broadening, low sensitivity or spectral overlap.
**Fig. S6.** EtsΔ138 engages the DRS and FRS of ERK2. Backbone amide chemical shift perturbations on ERK2 in the presence of EtsΔ138 obtained from $^{15}$N, $^1$H TROSY-based titrations plotted against residue number are shown on the extreme left panel. The largest perturbations are localized on the $\phi_{hyd}$ part of the DRS and at certain parts of the FRS. The CSP corresponding to Asp316, the only $\phi_{chg}$ residue that is perturbed in the presence of EtsΔ138 (see main text) is labeled. Shown in the middle panel are the backbone amide perturbations seen for ppERK2 in the presence of the canonical F-site peptide from Elk-1 (Elk387-399). In this case, no significant perturbations are seen at the DRS contrasting the large perturbations seen at the FRS. In addition, several resonances corresponding to a part of the FRS and the activation loop that are absent in the apo state reappear in presence of the Elk387-399 peptide. In contrast, significant amide perturbations are seen in the ppERK2 DRS (extreme right panel), at the both the $\phi_{hyd}$ and $\phi_{chg}$ sub-sites in the presence of the canonical D-site peptide, KIM15-31 from HePTP (36). The dashed lines on the plots indicate the 1.0σ, 1.5σ, 2.0σ and 3.0σ thresholds. The regions of ERK2 that are perturbed in the presence of EtsΔ138 are depicted on a ribbon representation of ERK2 on the bottom panel.
Fig S7. Positions of $^{13}$Cα (blue, left panels) and $^{13}$Cβ (red, right panels) resonances of T10, L11, T12, I13 and I14 methyl resonances in the free and 76% ERK2-bound states of EtsΔ138. T10 and L11 show substantial downfield and upfield shifts (indicated by the arrows) of $^{13}$Cα and $^{13}$Cβ resonances, respectively. Resonances for T12 and I13 are broadened out in the bound state (indicated by the circles). Bound-state data for these resonances have been plotted at close to the noise level to illustrate the absence of expected resonances. Data for I14 that shows no significant perturbations for the $^{13}$Cα and $^{13}$Cβ resonances are also shown.
Fig. S8. Comparison of the binding of the N-terminal region of EtsΔ138 at the ERK2 DRS with other DRS ligands. The top panel depicts interactions of EtsΔ138 at the ERK2 DRS for the lowest energy conformer of the best HADDOCK cluster (ERK2 represented as a surface; residues 9-14 from EtsΔ138 represented as a green ribbon; key sidechains are shown as sticks). Also shown are the binding modes of the canonical D-site peptides from MKP3 (yellow, PDB: 2FYS), HePTP (brown, PDB: 2GPH), DCC (purple, PDB: 3O71), RSK1 (orange, PDB: 4H3P), MEK2 (red, PDB: 4H3Q) and MNK1 (olive, PDB: 2Y9Q). The non-canonical interaction involving the C-terminus of PEA-15 (grey, PDB: 4IZ5) is also depicted. For all the crystal structures, only the interacting sidechains are shown as sticks. For MEK2 only, the backbone is shown as ribbon. The hydrophobic pockets $\phi_A$, $\phi_B$ and $\phi_L$ are colored violet, pink and blue, respectively. The bottom panel compares the conformation of the backbone and key interacting sidechains for the EtsΔ138 conformer from above (green) with the canonical D-site sequence from HePTP (brown) and the non-canonical interaction involving the PEA-15 C-terminus (grey).
Fig. S9. Spectral perturbations for Leu and Val methyl groups of ERK2 (obtained from $^{13}$C, $^1$H HMQC based titrations) confirm occupancy of parts of the DRS and FRS by EtsΔ138. Residues for which the corresponding Leu or Val resonances are broadened out are depicted by the cyan bars while the values of the CSPs are indicated by the black bars. Key residues discussed in the text are labeled. Leu232 and Leu235 that form part of the $\phi_2$ pocket of the canonical FRS show significant spectral changes; Leu198 (labeled with smaller font) that also forms part of this pocket does not display any appreciable spectral perturbation. The diamonds represent Leu and Val residues for which resonance assignments are not available.
Fig. S10. Comparison of the dynamics of EtsΔ138 residues I13 and I14 bound to ERK2. A comparison of the buildup of “forbidden” coherences due to intra-methyl $^1$H-$^1$H cross-correlation using a 3Q relaxation experiment of Sun et. al. (37) on the Ile δ1 positions of EtsΔ138 (77%-bound). Given the extensive broadening of the I13 δ1 resonance in the presence of ERK2, data for only 4 relaxation delays (15 ms maximum) compared to the I14 δ1 resonance for which 11 relaxation delays (up to 70 ms) could be analyzed. The ratio of the $\eta$ values for the two residues yields $S^2_{axis}(I13)/S^2_{axis}(I14) = 1.85\pm0.22$ compared to 1.37\pm0.07 in free EtsΔ138, suggesting a greater spatial restriction of I13 compared to I14 when bound to ERK2. Additionally, the value of $\delta$ (that relates to the density of external protons that lead to homonuclear $^1$H-$^1$H relaxation, in addition to the overall order) is much larger in magnitude for I13 (-74.3\pm5.9 s$^{-1}$) compared to I14 (-9.3\pm0.99 s$^{-1}$) suggesting the presence of a larger pool of external protons around I13, indicating a more significant burial. Though the sampling of the I13 build-up curve is limited and the actual precision of the analysis is lower than that for I14, these results qualitatively indicate a greater degree of order for I13 and a more substantial interaction with ERK2.
Fig. S11. (A) Amide CSPs induced by ERK2 binding are plotted on the ribbon structure of H0+CTR fragment of EtsΔ138 using a blue-white-red gradient ranging from 0.0 to > 0.2 ppm. Residues for which the resonances are quenched are shown in magenta. Perturbations for Ile, Leu, Val and Thr methyl groups shown as spheres are also depicted using the same coloring scheme as the amides. Methyl CSPs have been scaled respect to the amide CSPs to generate the same maxima in the two cases for representation using a common scheme. Perturbed amides or methyl resonances belonging to residues with 0% solvent accessibility (as in (B) below) have been labelled. (B) The same representation as in (A) colored according to the residue solvent accessibility (RSA) using a green (0%) to yellow (>50% or above) gradient. Perturbed methyl groups are represented as spheres as in (A). The solvent accessibility (relative to that of a residue X placed in an extended AXA tripeptide) has been calculated using NACCESS.
**Fig. S12.** Distances (straight lines; 152 total restraints) derived from the PREs are color coded using a blue-red gradient. Amides or methyls are shown as van der Waals spheres. EtsΔ138 is colored as in Fig. S2. The ERK2 FRS is shown in maroon, and attached the MTSL groups are shown in gold. For clarity, only one conformation for each MTSL moiety is shown. Partially ambiguous interatomic restraints (due to lack of stereospecific assignments) or restraints from residues showing no measurable PREs (49 amides, 7 methyls for which a minimal lower bound of 26 Å was used) are excluded from this figure. Note that the Cys at position 252 is native for wild-type ERK2; it was mutated to Leu to generate ERK2cysless.
Fig. S13. The NMR-determined N-terminal and C-terminal docking interactions of EtsΔ138 with ERK2 are mutually compatible. Model of EtsΔ138 (dark green) docked onto ERK2 (grey) obtained by combining the results of the HADDOCK calculations for the docking of the 9-14 segment of EtsΔ138 at the ERK2 DRS, and for the three-way docking involving the 34-39, 40-138 fragments of EtsΔ138 and ATP onto ERK2. The model highlights the proximity between the flexible N-terminal end of H0 and the catalytic site hosting ATP (shown as sticks). Thus, moving the phospho-acceptor T38 closer to the PNT domain would necessitate greater structural distortions at the C-terminal region to allow efficient phosphorylation. Note that the spatial proximity between T38 and ATP has been imposed computationally and does not reflect any experimentally derived NMR restraints. Some of the key EtsΔ138 sidechains involved in contacting ERK2 and discussed in the text are shown in stick representation. Relevant secondary structure elements for both ERK2 and EtsΔ138 are also labelled.
Fig. S14. Order parameters ($S^2_{\text{axis}}$) for Ile (δ1) and Thr methyl positions of free EtsΔ138 measured using the intra-methyl $^1$H-$^1$H dipolar cross-correlation rate ($\eta$) plotted against residue number. The phospho-acceptor T38 position is indicated.
**Fig. S15.** Comparison of the interactions of EtsΔ138 at the ERK2 FRS with other FRS ligands. Interactions involving the H0-H1-PNT region of EtsΔ138 (residues 40-138, shown in ribbon representation, green) for the lowest energy conformer within the best HADDOCK cluster superimposed on the structures of the complexes of ppERK2 with the canonical F-site sequence from Elk-1 (A, Elk387-399; gold ribbon representation) (7) or PEA-15 (B, gold ribbon representation, PDB: 4IZ5) (38). ERK2 is shown in surface representation with light blue coloring. Key sidechains of the PNT domain of EtsΔ138, the F-site sequence from Elk387-399 and the DED domain of PEA-15 are labelled and shown in stick representation. P396 of Elk387-399 clashes with F120 of EtsΔ138 in this orientation. For PEA-15, a part of the backbone comprising residues 14-59 has been made partially transparent for visual clarity. The hydrophobic pockets (ϕ₁ and ϕ₂) that accommodate the two phenylalanine residues (F395 and F397 in Elk387-399) of the canonical F-X-F F-site sequence, are colored blue and pink in both panels.
Fig. S16. Determination of the rate of release of EtsΔ138 from its complex with ppERK2. The competition assay was performed by adding various concentrations of unlabeled EtsΔ138 to a complex of ppERK2 and EtsΔ138C31* (all the native Cys except C31 in EtsΔ138 were mutated to Ala; C31 was fluorescently labeled using 5-iodoacetomido-fluorescein). The increase in fluorescence was monitored over time and the $k_{obs}$ value at each concentration was obtained by fitting the resultant traces (top panel) to Eq. S13. The lower panel shows the variation in the $k_{obs}$ values with increasing EtsΔ138 concentration. The $k_{off}$ is obtained as the limiting value of $k_{obs}$. Curves for EtsΔ138C31* concentrations of 6.25 and 12.5 µM yielded $k_{obs}$ values (>500 s$^{-1}$) faster than the dead-time of the instrument.
Table S1 – Parameters used in the NMR experiments

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<td>\textsuperscript{1}H</td>
<td>40</td>
<td>66.0</td>
<td>\textsuperscript{13}C</td>
<td>500</td>
</tr>
</tbody>
</table>

C. U-[\textsuperscript{15}N, \textsuperscript{2}H, \textsuperscript{13}C]-labeled Ets\textsuperscript{138 (300 \textmu M)} + Inactive ERK2 (250 \textmu M)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Points</th>
<th>SW</th>
<th>Nucleus</th>
<th>Points</th>
<th>SW</th>
<th>Nucleus</th>
<th>Points</th>
<th>SW</th>
<th>Nucleus</th>
<th>Field</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNCO</td>
<td>1024</td>
<td>15.5</td>
<td>H</td>
<td>24</td>
<td>8.9</td>
<td>\textsuperscript{13}C</td>
<td>32</td>
<td>29</td>
<td>\textsuperscript{15}N</td>
<td>600</td>
</tr>
<tr>
<td>HNCA/CB</td>
<td>1024</td>
<td>15.5</td>
<td>H</td>
<td>46</td>
<td>62</td>
<td>\textsuperscript{13}C</td>
<td>32</td>
<td>29</td>
<td>\textsuperscript{15}N</td>
<td>600</td>
</tr>
</tbody>
</table>

D. U-[\textsuperscript{15}N, \textsuperscript{2}H, \textsuperscript{13}C]-labeled Ets\textsuperscript{138 (200 \textmu M)} + U-[\textsuperscript{2}H]-labeled Inactive ERK2 (250 \textmu M)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Points</th>
<th>SW</th>
<th>Nucleus</th>
<th>Points</th>
<th>SW</th>
<th>Nucleus</th>
<th>Points</th>
<th>SW</th>
<th>Nucleus</th>
<th>Field</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNCO</td>
<td>1024</td>
<td>15.5</td>
<td>H</td>
<td>36</td>
<td>8.9</td>
<td>\textsuperscript{13}C</td>
<td>40</td>
<td>29</td>
<td>\textsuperscript{15}N</td>
<td>600</td>
</tr>
<tr>
<td>HNCA/CB</td>
<td>1024</td>
<td>15.5</td>
<td>H</td>
<td>52</td>
<td>62</td>
<td>\textsuperscript{13}C</td>
<td>32</td>
<td>29</td>
<td>\textsuperscript{15}N</td>
<td>600</td>
</tr>
</tbody>
</table>

E. U-[\textsuperscript{15}N, \textsuperscript{2}H, \textsuperscript{13}C]-labeled Ets\textsuperscript{138 (200 \textmu M)} + ppERK2 (200 \textmu M)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Points</th>
<th>SW</th>
<th>Nucleus</th>
<th>Points</th>
<th>SW</th>
<th>Nucleus</th>
<th>Points</th>
<th>SW</th>
<th>Nucleus</th>
<th>Field</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNCO</td>
<td>1024</td>
<td>15.5</td>
<td>H</td>
<td>24</td>
<td>8.9</td>
<td>\textsuperscript{13}C</td>
<td>32</td>
<td>29</td>
<td>\textsuperscript{15}N</td>
<td>600</td>
</tr>
</tbody>
</table>

F. ILV U-[\textsuperscript{15}N, \textsuperscript{2}H]-labeled Ets\textsuperscript{138cytless (500 \textmu M)} + Inactive ERK2 (780 \textmu M)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Points</th>
<th>SW</th>
<th>Nucleus</th>
<th>Points</th>
<th>SW</th>
<th>Nucleus</th>
<th>Points</th>
<th>SW</th>
<th>Nucleus</th>
<th>Field</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMQC-NOESY-HMQC (HCC)</td>
<td>512</td>
<td>15.0</td>
<td>H</td>
<td>40</td>
<td>17.5</td>
<td>\textsuperscript{13}C</td>
<td>42</td>
<td>17.5</td>
<td>\textsuperscript{13}C</td>
<td>800</td>
</tr>
<tr>
<td>HMQC-NOESY-HMQC (HCH)</td>
<td>512</td>
<td>15.0</td>
<td>H</td>
<td>40</td>
<td>17.5</td>
<td>\textsuperscript{13}C</td>
<td>42</td>
<td>2.5</td>
<td>\textsuperscript{1}H</td>
<td>800</td>
</tr>
</tbody>
</table>

G. IT, U-[\textsuperscript{15}N, \textsuperscript{2}H]-labeled Ets\textsuperscript{138 (180 \textmu M)} + Inactive LV, U-[\textsuperscript{2}H]-labeled ERK2 (180 \textmu M)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Points</th>
<th>SW</th>
<th>Nucleus</th>
<th>Points</th>
<th>SW</th>
<th>Nucleus</th>
<th>Points</th>
<th>SW</th>
<th>Nucleus</th>
<th>Field</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOESY-HMQC</td>
<td>512</td>
<td>10.0</td>
<td>H</td>
<td>46</td>
<td>2.0</td>
<td>\textsuperscript{1}H</td>
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<td>16.8</td>
<td>\textsuperscript{13}C</td>
<td>800</td>
</tr>
</tbody>
</table>

The SW is in ppm, the number of points indicate complex points and the field is \textsuperscript{1}H frequency in MHz, in all cases.
References


