List of Supplemental Figures and Tables

**Regulatory Roles of Conserved Phosphorylation Sites in the Activation T-Loop of the MAP Kinase ERK1**

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Running Head: Inhibitory phosphosites in the activation loop of ERK1

**SUPPLEMENTAL FIGURE S1**: Kinase specificity of ERK1-WT, T207A and T207E on the Kinex™ Kinase Substrate Microarray.

**SUPPLEMENTAL FIGURE S2**: Phosphorylation of ERK T207/T188 in NIH-3T3 cells, A431 cells, A549 cells and HeLa cells under various treatment conditions.

**SUPPLEMENTAL FIGURE S3**: Amino acid residue interactions with activation T-loop phosphosite residues in ERK2.

**SUPPLEMENTAL TABLE S1**: Alignment of the catalytic domain sequences and known phosphosites in human protein kinases.

**SUPPLEMENTAL TABLE S2**: Frequency of mass spectrometry reports of phosphorylation sites in the activation T-loops of human protein-serine/threonine kinases.

**SUPPLEMENTAL TABLE S3A**: Summary of literature reports of phosphomimetic mutations in human proteins.

**SUPPLEMENTAL TABLE S3B**: List of reported engineered phosphorylation site mutations in human proteins.

**SUPPLEMENTAL TABLE S4A**: Amino acid interactions with ERK1 phosphoacceptor residues in the activation T-loop.

**SUPPLEMENTAL TABLE S4B**: Amino acid interactions with ERK2 phosphoacceptor residues in the activation T-loop.
SUPPLEMENTAL FIGURE S1: Kinase specificity of ERK1 Wild-type, T207A and T207E on the Kinex™ Kinase Substrate Microarray. Purified ERK1 and its mutants were activated by MEK1-ΔN3EE before incubation with the kinase substrate peptide microarray at 30°C for 2 h. MEK1 activity was suppressed by UO126. The control field was incubated with MEK1/UO126 only.

SUPPLEMENTAL FIGURE S2: Phosphorylation of ERK1 T207 and ERK2 T188 in NIH-3T3 cells (Lanes 1-4), A431 cells (lanes 5-7), A549 cells (Lane 8) and HeLa cells (Lanes 9-11) under various treatment conditions. Abbreviations: AsO₂-, arsenite; Ctrl, Untreated; EGF, epidermal growth factor; PMA, phorbol 12-myristate 13-acetate; Noco., nocodazole; TNFα, transforming growth factor-alpha. The samples were subsequently subjected to SDS-PAGE and Western blotting with antibodies that were specific for the ERK1 T202 and Y204 and ERK2 T183 and Y185 phosphosites (pTEpY), the ERK1 pT207 and ERK2 T188 phosphosite (pT207/pT188) or the pan-expression of ERK1 and ERK2 (ERK-CT). The migration positions of phospho-ERK1 (**), phospho-ERK2 (**), ERK1 (*) and ERK2 (*) on the SDS-PAGE gel are indicated.
SUPPLEMENTAL FIGURE S3: Interactions with T185, Y187, T190 and Y193 residues in the 3D structure of human ERK1. The x-ray crystallographic structures of the unphosphorylated human ERK2 (Panels A, C, and E; PDB Id 4XJ0; originally deduced by Yin J, Wang W. (2015)
Discovery of highly potent, selective, and efficacious small molecule inhibitors of ERK1/2. J Med Chem 58, 1976–1991) and T185/Y187 dual phosphorylated ERK2 (Panels B, D, and F; PDB 2ERK; originally deduced by Canagarajah BJ, Khokhlatchev A, Cobb MH, Goldsmith EJ (1997) Activation mechanism of the MAP kinase ERK2 by dual phosphorylation. Cell 90, 859-869.) were rendered with Jmol on the RCSB PDB website. The backbone atoms of ERK2 are shown with white bonds, and the side-chain amino acid residues with oxygen, nitrogen, carbon, phosphorus and sulphur atoms are colored red, blue, grey, orange and yellow, respectively. Atoms in the side chains of T185, Y187, T190 and Y193 that reside close to the atoms of other amino acid side chains are indicated with orange dashed lines and the distances are indicated in Angstroms. These analyses reveal profound changes associated with the interactions of T185 and Y187 with other side chains following their phosphorylation with little perturbation of T190 interactions with K151, N154 and D149, and Y193 interaction with E220. Supplemental Table S4B provides additional listing of the distances between the phosphoacceptor residues in ERK2 with neighbouring amino acid residues in other ERK2 x-ray crystallographic structures.

Supplemental Table Legends

SUPPLEMENTAL TABLE S1: Alignment of the catalytic domain sequences and known phosphosites in human protein kinases. The amino acid positions of 496 human protein kinase domains were aligned based on their primary and tertiary structures. The most evolutionarily related protein kinase domains are positioned next to each other to facilitate comparison. Known activatory phosphosites are highlighted in green, inhibitory phosphosites in red, and phosphosites of undefined function in yellow.

SUPPLEMENTAL TABLE S2: Frequency of mass spectrometry reports of phosphorylation sites in the activation T-loops of human protein-serine/threonine kinases. The amino acid positions of phosphorylation sites in the activation T loops (between kinase Subdomains VII (DFG) and VIII (APE) are presented for 174 human protein kinases that have been reported to feature phosphorylation at the aligned 155 or 158 positions by mass spectrometry (ms). Phosphorylation sites are highlighted in light purple. The data for the number of ms reports of phosphorylation were retrieved from the PhosphoSite Plus website (www.phosphosite.org). If a phosphosite was documented only by immunoblotting or Edman degradation, but not by ms, it was assigned a value of 1, even though there may have been multiple reports in the literature.

SUPPLEMENTAL TABLE S3A: Summary of literature reports of phosphomimetic mutations in human proteins. A "True" match corresponds to when a mutation successfully mimics the expected effect of phosphorylation of the target protein. For example, when a glutamic acid or aspartic acid residues substitution results in functional activation of the target protein as would its phosphorylation at the same site. Likewise, an alanine or phenylalanine mutations of an inhibitory phosphorylation site would leads to increased functional activity of the target protein. A "False" match corresponds to when the mutation has no effect or the opposite effect as would be expected on the functional activity of the target protein as would its phosphorylation.

SUPPLEMENTAL TABLE S3B: List of reported engineered phosphorylation site mutations in human proteins. Mutations: Loss-of-function (LOF); Gain-of-function (GoF); No identified
functional effect (NoE). This data was primarily derived from the UniProt website. The functional information was also retrieved from Phosphosite Plus, PhosphoNET and directly from original scientific reports.

**SUPPLEMENTAL TABLE S4A:** Amino acid interactions with ERK1 phosphoacceptor residues in the activation T-loop. Those amino acids residues and their atoms that were located 5 Angstroms or closer to T202, Y204, T207 and Y210 side chain atoms were identified from the 3D crystal structures represented in PDB files 4QTB and 2ZOQ. In addition to the locations of these amino acid residues in the full-length proteins, the corresponding positions in their aligned kinase catalytic domains are also shown along with their occurrence within any of the highly conserved subdomains. Note that many of the internal amino acid interactions between the phosphoacceptor amino acid residues T207 and Y210 are preserved whether the Y204 position is phosphorylated or not. Many of the amino acid residues that interact with unphosphorylated T207 and Y210 are located within kinase Subdomains VI and IX, and their interactions may serve to stabilize the active form of ERK1.

**SUPPLEMENTAL TABLE S4B:** Amino acid interactions with ERK2 phosphoacceptor residues in the activation T-loop. Those key amino acids residues and their atoms that were located 5 Angstroms or closer to T185, Y187, T190 and Y193 side chain atoms were identified from the 3D crystal structures represented in PDB files 4XJ0, 4G6N, 4ZZM and 2GPH for unphosphorylated and 2ERK and 4IZA for T185 and Y187 phosphorylated ERK2. In addition to the locations of these amino acid residues in the full-length proteins, the corresponding positions in their aligned kinase catalytic domains are also shown along with their occurrence within any of the highly conserved subdomains. Note that many of the internal amino acid interactions between the phosphoacceptor amino acid residues T190 and Y193, including those with amino acid residues in Subdomain VI, are preserved whether the T185 and Y187 sites are phosphorylated or not. By contrast, with phosphorylation the T185 and Y187 sites, there are many new interactions, which includes several with amino acid residues in Subdomains III, VI, VII and VIII depending on the crystal structure considered.