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Supporting information for article:

Discovery of a novel allosteric inhibitor-binding site in ERK5: comparison with canonical kinase hinge ATP-binding site

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Table S1 List of reagents

| Reagent | Company | Catalogue Number |
|--|-----------------|----------------------|
| PEG 4000 | Sigma | 95904 |
| PEG 6000 | Fluka | 81255 |
| MES | Sigma | 69890 |
| Tris Base | Fluka | 93349 |
| DTT | Molekula | 42579915 |
| sodium formate | Sigma | 71539 |
| sodium chloride | ROTH | 9265.2 |
| glycerol | Sigma | 49767 |
| PBS | AppliChem | A0964,9010 |
| magnesium chloride | Fluka | 63064 |
| HEPES | AppliChem | A3714,1000 |
| DMSO | Fluka | 41639 |
| 2,3-butane-diol | Sigma | 300349 |
| XMD8-92 (compound 2) | Wuxi PharmaTech | Custom synthesized |
| Compound 3 | Astrazeneca | In-house synthesized |
| Compounds 4 to 6 | Pharmaron | Custom synthesized |
| ATP | Sigma | A7699 |
| eIF4E-binding protein 1 peptide | PerkinElmer | TRF0128 |
| MgCl ₂ | Sigma | M1028 |
| EGTA | Beyotime | ST068 |
| Tween 20 | Sigma | P9416 |
| EDTA | Invitrogen | AM9262 |
| Eu-anti-phospho-eIF4E-binding protein 1 antibody | PerkinElmer | TRF0216 |

Table S2 Translated sequence for human ERK5(46-402) after rTEV cleavage. Residues arising from the rTEV cleavage site are shown in italics.

GD⁴⁶VTFDVGDEYEIIETIGNGAYGVVSSARRRLTGQQVAIKKIPNAFDVVTKAKRTLRELKIL
 KHFHDNIIAIKDILRPTVPYGEFKSVYVVLDMESDLHQIIHSSQPLTLEHVRYFLYQLLRGL
 KYMHSAQVIHRDLKPSNLLVNENCELKIGDFGMARGLCTSPAEHQYFMTEYVATRWYRAPE
 LMLSLHEYTQAIDLWSVGCIFGEMLARRQLFPGKNVHQLQLIMMVLGTPSPAVIQAVGAER
 VRA^{YI}QSLPPRQPVPWETVYPGADRQALSLLGRMLRFEPSPARISAAAALRHPFLAKYHDPDD
 EPDCAPPFDFAFDREALTRERIKEAIVAEIEDFHARREGIRQQIRFQ⁴⁰²

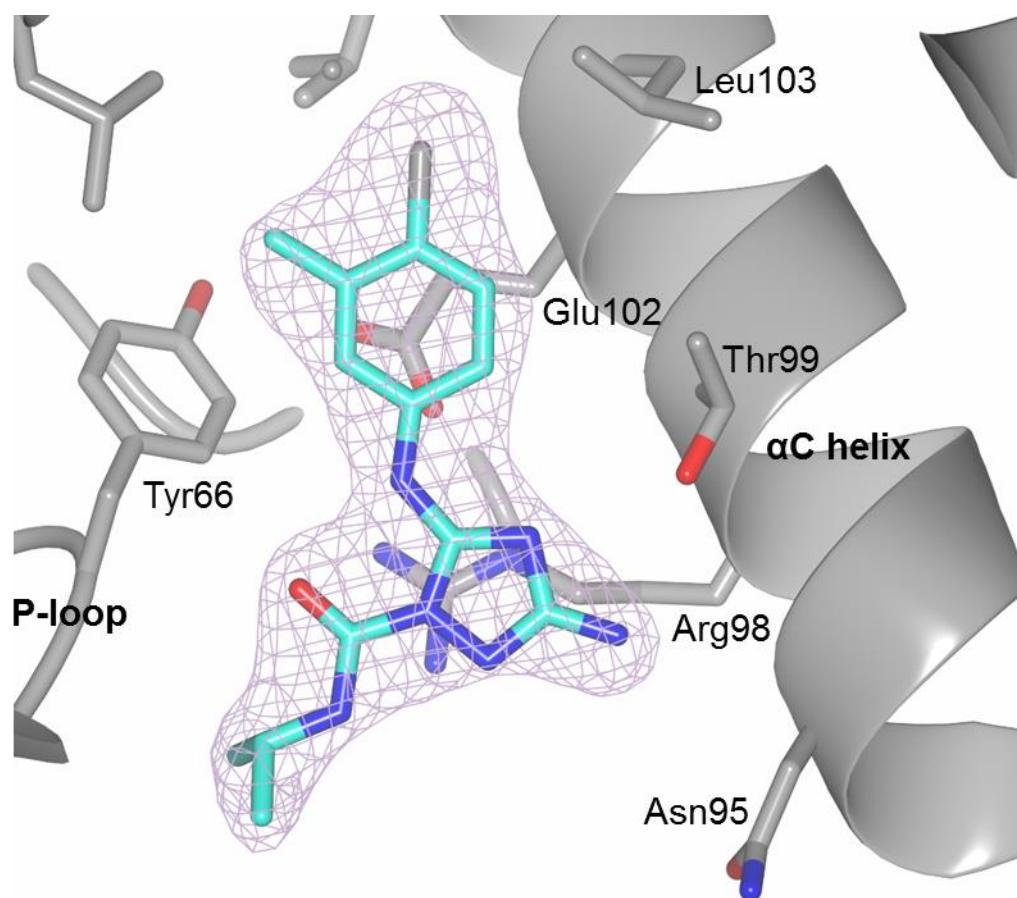


Figure S1 $2mF_o - DF_c$ OMIT electron-density map (scaled to 1.0σ , and represented as a lilac mesh) surrounding compound **5** (carbon atoms in cyan). The compound could be unambiguously fitted in the electron density.

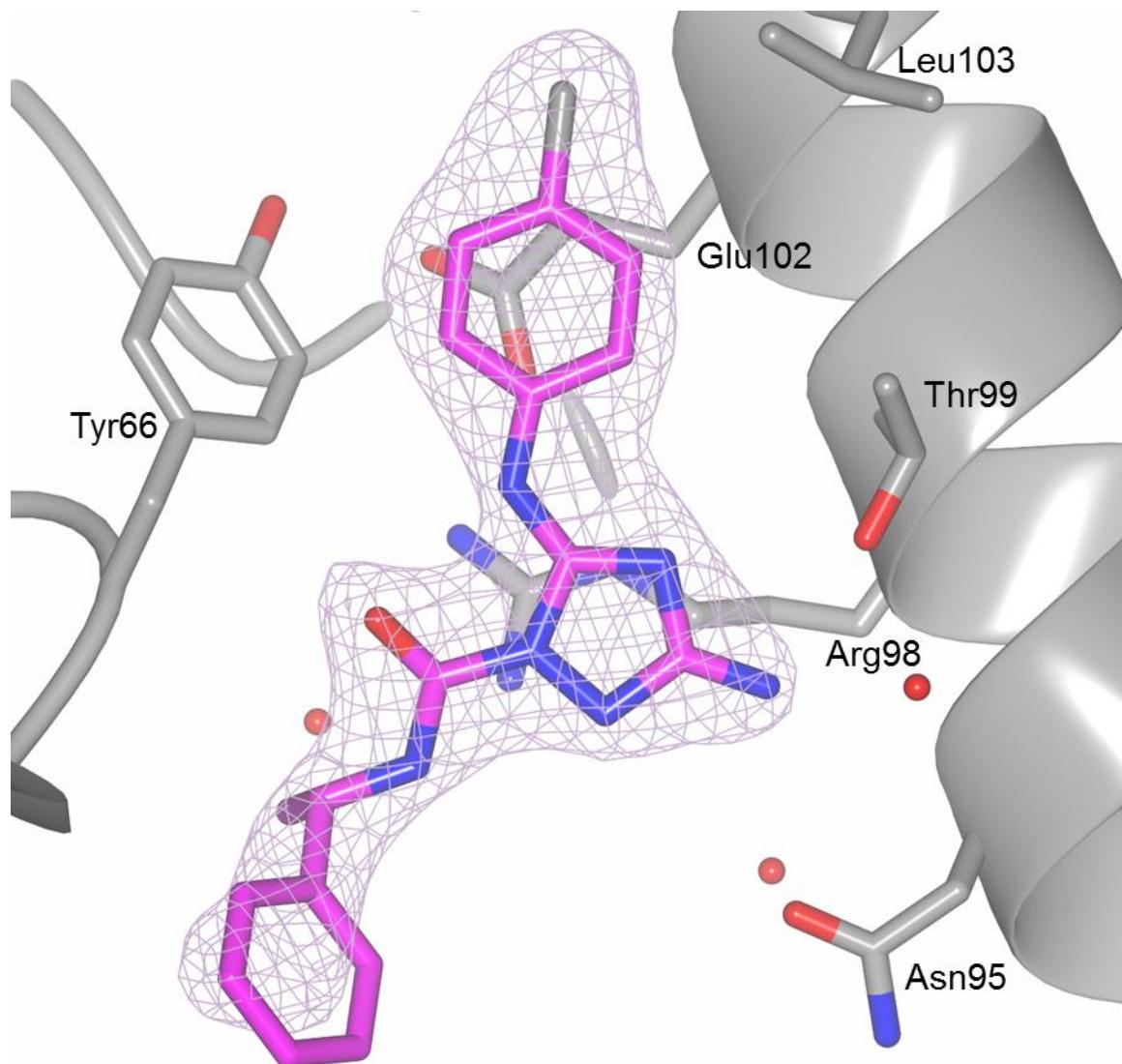


Figure S2 $2mF_o - DF_c$ OMIT electron-density map (scaled to 1.0σ , and represented as a lilac mesh) surrounding compound **6** (carbon atoms in magenta). The compound could be unambiguously fitted in the electron density.

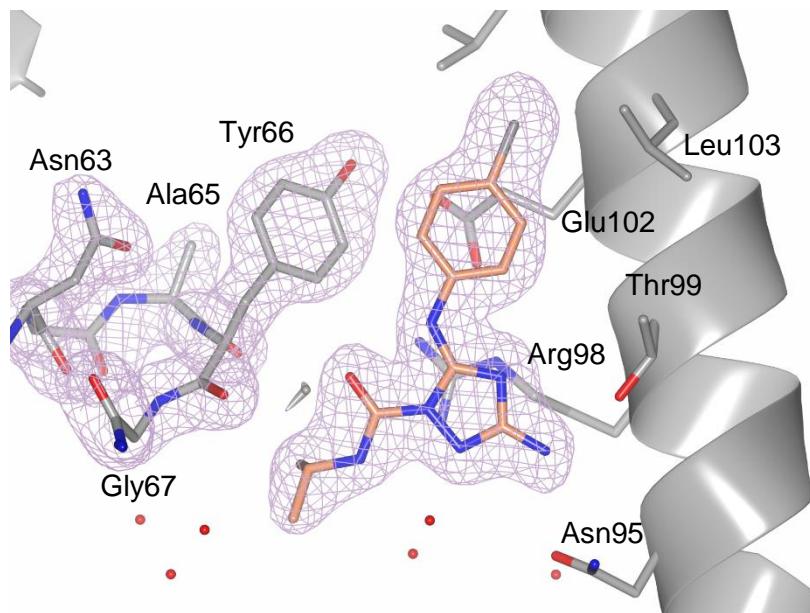


Figure S3 $2mF_o - DF_c$ OMIT electron-density map (scaled to 1.0σ , and represented as a lilac mesh) surrounding the P-loop (Asn63, Gly64, Ala65, Tyr66, Gly67 and Val68, carbon atoms in grey) and compound 4 (carbon atoms in coral). The P-loop residues and the compound could be unambiguously fitted in the electron density.

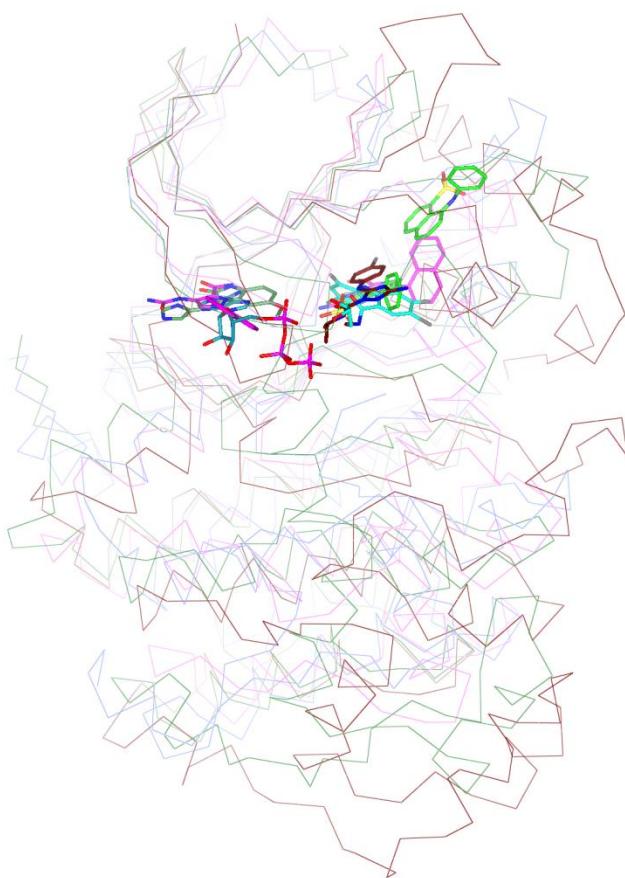


Figure S4 Overlay of the structures of ERK5 in complex with compound 4, MEK in complex with MgATP and PD318088 (PDB 1S9J), ITK in complex with an allosteric inhibitor (PDB 4M0Y), and CDK2 in complex with SU9516 and ANS (PDB 3PY1). Proteins are rendered as backbone C α traces in dark brown (ERK5), pale blue (MEK), pink (ITK), dark green (CDK2). Compounds are shown as sticks with carbon atoms coloured tan (compound 4), dark blue (ATP), cyan (MEK inhibitor, PD318088), magenta (pyrazole carboxamide inhibitor of ITK), dark green (SU9516, a type I inhibitor of CDK2) and green (ANS). Structures were superposed by matching residues corresponding to the β -sheet and hinge region of the N-terminal lobe of ERK5 kinase (amino acids 55-61, 70-88, 108-123, and 131-140).

S1. Z'-LYTE® Assay Protocol

Assay protocol details can be found at:

<https://www.thermofisher.com/content/dam/LifeTech/migration/files/drug-discovery/pdfs.par.60256.file.dat/20130430%20ssbk%20customer%20protocol%20and%20assay%20conditions.pdf>