Synthetic Phosphorylation of p38α Recapitulates Protein Kinase Activity

Supplementary Information

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Contents

1 Supplementary Figures S4

2 General Measures S5
  2.1 Synthetic Chemistry .......................................................... S5
  2.2 Molecular Biology ............................................................. S5
  2.3 Protein Expression and Purification of p38α Variants ................. S6
  2.4 Gene Sequence Analysis ....................................................... S7
  2.5 Protein Modification Reactions ............................................ S7
  2.6 Enzymatic Reactions and Activity Assay .................................. S7

3 Synthesis of Protein Modification Reagents S8
  3.1 Preparation of 2,5-dibromoadipoyl dichloride (1) ...................... S8
  3.2 Preparation of 2,5-dibromo-adipic acid diamide (2) .................... S8

4 Protein Expression and Purification of p38α Variants for Single Modification S8
  4.1 Cloning of pGEX-2T-p38α-Cys172 Plasmid ............................... S8
  4.2 General Procedure for Mutagenesis of pGEX-2T-p38α-Cys172 Plasmid S9
  4.3 General Procedure for Protein Expression of p38α Mutants -Cys172 and -Cys180 . S11
  4.4 Protein Purification of p38α-Cys172 ........................................ S12
  4.5 Protein Purification of p38α-Cys180 ........................................ S15

5 Synthesis of Chemically Modified p38α Variants S18
  5.1 Synthesis of p38α-Dha172 ...................................................... S18
  5.2 Synthesis of p38α-pCys172 ..................................................... S19
  5.3 Synthesis of p38α-Dha180 ...................................................... S20
  5.4 Synthesis of p38α-pCys180 ..................................................... S21
  5.5 Comparison of Chemical Reaction Kinetics for Phosphocysteine Formation .... S22

6 Model Surface and Homology Calculations S22
  6.1 Electrostatic Calculations .................................................... S22
  6.2 Surface Accessibility Calculations ........................................ S22
  6.3 ATF2 Homology Modelling ................................................... S22

7 Methods for Characterising (Modified) Proteins S23
  7.1 General Procedure for Measurement of Circular Dichroism Spectra . . S23
  7.2 General Procedure for LC-MS/MS Analysis including Sample Preparation . . . S23
  7.3 LC-MS/MS Analysis for Detection of Phosphopeptides from p38α Variants . . S30

8 Activity Assay with Substrate Detection by Mass Spectrometry (MS) S33
  8.1 Measurement of the Activity of All Variants of p38α-X172 and -X180 .... S33
  8.2 Quantitative Measurement for Comparison of MS to Electrophoretic Radioassay . S36
  8.3 Quantitative Measurement of the Activity of Enzymatically Active Variants of p38α S37
  8.4 Control to Determine Phosphocysteine Stability against Assay Buffer Conditions . . S41
  8.5 Control to Determine Thiophosphate Stability against DTT Treatment ........ S41
  8.6 LC-MS/MS Analysis for Detection of Phosphopeptides after ATF2 Phosphorylation . . S42

9 Electrophoretic Radioassay S46
  9.1 Enzymatic Reaction ............................................................ S46
  9.2 SDS-PAGE analysis ............................................................ S46
  9.3 Gel Image Acquisition ....................................................... S47
9.4 Data Analysis and Quantification .................................................. S49

10 Determination of IC$_{50}$ ............................................................. S49
   10.1 Enzymatic Reaction ............................................................ S49
   10.2 Data Analysis and Quantification ............................................ S49

11 Protein Expression and Purification of MEK1 Variant ..................... S52
   11.1 Cloning of pET28a-MEK1-S222C/C277S/C376S plasmid ................. S52
   11.2 Mutagenesis of pET28a-MEK1-S222C/C277S/C376S Plasmid .............. S53
   11.3 Protein Expression of MEK1-Cys222 ....................................... S54
   11.4 Protein Purification of MEK1-Cys222 ..................................... S55

12 Chemical Modification of MEK1-Cys222 ..................................... S57
   12.1 Synthesis of MEK1-Dha222 .................................................. S57
   12.2 Synthesis of MEK1-Ethanolycysteine222 .................................... S59
   12.3 Synthesis of MEK1-pCys222 ............................................... S61
Fig. S1  Mass spectra of biologically and synthetically derived p38α, both before and after activation. (a) Enzymatically activated kinase exhibits three different phosphorylation states (observed masses 43481, 43562 and 43641 for the unglucosylated protein) while (b) the chemically modified one only displays one phosphoform. (c) Even prior to activation, the former displays partial phosphorylation due to phosphorylation during expression whilst (d) the synthetic precursor is pure.

Fig. S2  CD spectra of all variants of p38α-X180 and p38α-X172 (X = Cys, Dha or pCys), overall showing no significant changes between the unmodified variants (Cys variants, left) and phosphorylated variants (pCys variants, right). (a) p38α-Cys180 (b) p38α-Dha180 (c) p38α-pCys180 (d) p38α-Cys172 (e) p38α-Dha172 (f) p38α-pCys172
Electrostatic and surface accessibility calculations for p38α. (a) Notable residues with sites of chemical reaction (orange), surface accessible Cys (green) and hindered Cys (magenta) highlighted. (b) Surface accessibility calculations show that the two reaction sites have similar solvent accessibility. Cys→Ser mutations were designed according to this prediction, which was in agreement with the literature precedent. Residues that displayed a low level of accessibility were left untouched (39 and 211) while residues of similar accessibility to the reaction sites were mutated to Ser. Levels of accessibility: >100% (blue), 100–75% (green), 75–50% (orange), 50–25% (yellow), 25–0% (white), 0% (grey). (b) The region around Thr180 has net negative charge (red) while the region around Ala172 is positive (blue).

2 General Measures

2.1 Synthetic Chemistry

Melting points were determined under a light microscope at low power and are uncorrected. Infrared spectra were recorded using a Bruker TENSOR 27 spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded using a Bruker AV-400 spectrometer, proton NMR (1H NMR) at 400 MHz and carbon NMR (13C NMR) at 101 MHz. Chemical shifts (δ) are reported in parts per million (ppm) and are referenced relative to the residual proton-containing solvent (1H NMR: 2.52 ppm for DMSO; 13C NMR: 41.2 ppm for DMSO). Accurate mass spectra (ESI) were recorded using a Micromass Bruker MicroTOF instrument. TLC was performed using aluminium plates pre-coated with Merck Kieselgel 60 F254. The plates were visualised using either ultraviolet (UV) light or potassium permanganate staining as appropriate.

2.2 Molecular Biology

2.2.1 Media and Bacterial Strains

All bacterial handling was done in a sterile environment, either within close proximity to a Bunsen burner flame or inside a biological safety cabinet. Samples of p38α mutant plasmid construct p38α-C119S/C162S/A172C was kindly sent by Dr. Richard Bazin and co-workers, Pfizer, Sandwich, Kent. LB was bought as pre-formulated dry granules (Melford) and diluted according to the manufacturer’s specifications. SOC and NZY media were prepared from biological laboratory reagents. 2YT medium was bought as pre-formulated dry granules (Sigma Aldrich) and diluted according to the manufacturer’s specifications. All other biological laboratory buffer and media reagents were bought from common
suppliers and used as purchased. BL21(DE3) and NovaBlue *E. coli* chemically competent cells (Novagen) were handled according to the manufacturer’s instructions, thawing on ice before incubation with DNA. NovaBlue was used for cloning, while BL21(DE3) was used for protein expression. XL1-Blue *E. coli* chemically competent cells (Agilent) likewise were handled according to the manufacturer’s instructions. XL1-Blue was used for cloning.

### 2.2.2 Mutagenesis and Bioinformatics

Site-directed mutagenesis was done using PfuUltra DNA polymerase AD (Agilent), dNTPs (Sigma Aldrich) and DpnI (NEB). Primers for mutagenesis were designed using the MutaPrimer design function in SimVector 4 from Premier Biosoft, which follows the guidelines given by Stratagene. The sequences were then sent to Life Technologies (Invitrogen) for custom oligonucleotide synthesis. Plasmid purification was done by using the QIAprep® Miniprep Kit (Qiagen) according to the manufacturer’s recommendations. LyseBlue reagent had been added to buffer P1 from the Miniprep kit to aid identification of cell lysis. DNA and protein concentration determination was done using a NanoDrop 1000 spectrophotometer (Thermo Scientific), which allowed the calculation of yield. The wavelengths used were a ratio of 260/230 nm and 280–260 nm respectively. The extinction coefficients for proteins were calculated using ProtParam tool (ExPASy). Other bioinformatics tools from the ExPASy website for various applications were also used.

### 2.3 Protein Expression and Purification of p38α Variants

**Fast protein liquid chromatography (FPLC)** was done using a ÄKTA Purifier™ system (Amersham Biosciences, now part of GE Healthcare). Pre-packed columns for FPLC and G-25 desalting columns (SpinTrap™, MiniTrap™ and MidiTrap™) were purchased from GE Healthcare. Unless otherwise stated, the analytes were filtered before loading onto the column for each chromatographic step. Dialysis was performed using Slide-A-lyser® MWCO 10 000 dialysis cassettes (Thermo Scientific). Concentration of protein was performed using Vivaspin 500, 15 or 6, MWCO 10 000 spin columns (Sartorius Stedim Biotech) as appropriate. Autoclaving was performed at 121 °C for 10–15 min. Solutions which were used as media or buffers and which could not be autoclaved were filtered through either 0.2 µm cellulose acetate filters, 0.2 µm poliamide filters, or 0.2 µm Minisart® filter cartridges (Sartorius Stedim Biotech), as appropriate. In particular, all filtrations of buffers, solutions and lysates involved in protein purification or chemical reactions involving proteins were done through 0.2 µm filters. Filtration of volumes of <2 mL (for HPLC) was done using 0.2 µm spin filters (Corning). HPLC samples were otherwise clarified by centrifugation (13 200 rpm, 10–20 min, 4 °C). Microcentrifugation was performed using an Eppendorf Centrifuge 5415R. Shaking incubation was performed using either a New Brunswick Scientific incubator shaker or a New Brunswick Scientific Model G25 incubator shaker. Plate incubation was performed using a Heraeus B6030 incubator. Low speed centrifugation of large volumes (>2 mL) was performed using a Beckman Coulter Allegra™ X-12R centrifuge. High speed centrifugation of large volumes (>2 mL) was performed using a Beckman Coulter Avanti™ J-25 centrifuge. Sonication was performed using a Sanyo Soniprep 150 sonicator, equipped with a microtip.

**SDS-PAGE protein gel analysis** was done using NuPAGE® gels (Invitrogen). The samples (5 µL) were prepared by mixing with equal volumes of 2× SDS gel-loading buffer (100 mM Tris-Cl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol), with β-mercaptoethanol (200 mM) being added to the buffer just before use. The samples were mixed by vortexing and heated at 95 °C for 10 min. The samples were run on 10% bis-tris gels as appropriate. The gels were run in MOPS running buffer (50 mM MOPS pH 7.7, 50 mM Tris base, 0.1% SDS, 1 mM EDTA) at 200 V. Samples of 5–9 µL were loaded as appropriate. Staining of the gels was done with Instant Blue™ Coomassie® stain for 12–18 h and destained with MilliQ water for a minimum of 18 h.

**Solid LB agar medium** was prepared by suspending LB granules (6.25 g) and agar (3.75 g) in MilliQ water (250 mL). The suspension was autoclaved as previously described to give a solution which once
sufficiently cooled, was supplemented with the appropriate antibiotic (ampicillin: final concentration: 100 µg/mL) and poured into petri dishes (10–20 mL per plate).

**Glycerol Stocks:** For cloning strains (XL1-Blue), glycerol stocks were made by adding bacterial broth (750 µL) to glycerol solution (250 µL of a 60% stock solution in water) and stored at −80°C. For expression strains (BL21(DE3)), glucose (28 µL of a 2 M stock solution in water) was added as a supplement before adding the bacterial broth (722 µL) and stored as above.

### 2.4 Gene Sequence Analysis

DNA sequencing was done by Geneservice, Oxford using the primers pGEX forward (5′-GGGCTGGCAAGCCACGTTTGAGT-3′) and pGEX reverse (5′-CCGGGAGCTGCATGTGTCAGAGG-3′) for genes contained in pGEX vectors. The forward and reverse complimentary sequences returned from the gene sequencing service were analysed by sequence alignment of the two returned sequences, followed by string comparison to the desired reference sequence. Sequence data manipulation was done using scripts written in MATLAB. In the case of serious mismatching between observed and desired sequences, the raw data were visualised using FinchTV version 1.4.0.

### 2.5 Protein Modification Reactions

Sodium thiophosphate was bought from Sigma Aldrich and used as purchased. Shaking incubation of the reactions was performed using PHMT-PSC-18 from Grant Bio. Incubation without shaking was done in a waterbath at the appropriate temperature. LC-MS analysis was done on a Micromass LCT Premier instrument using either a ProSwift® RP-4H monolith, 1 × 50 mm column (Dionex) or a Chromolith® FastGradient RP-18 endcapped 50 × 2 mm monolithic HPLC column (Merck). A linear gradient was run from 5–95% of solvent mixture B (1% formic acid in acetonitrile) into solvent mixture A (1% formic acid in water). The gradient was run with a flow rate of 0.4 mL/min over 4 min for the ProSwift column and with a flow rate of 0.3 mL/min over 6 min for the Chromolith column. Modified proteins used in subsequent reactions had their yield determined by volume measurement using a Gilson-type pipette, and concentration measurement by UV absorbance as described previously, making the assumption that the functional group incorporated in the chemical reaction did not have a large effect on the extinction coefficient as predicted for the protein’s unmodified sequence. In determining the protein concentration in reaction mixtures during reaction monitoring, the samples of the reaction mixtures were first filtered and compared against a blank “reaction” (the reaction buffer with the other reagents, but no protein), which was also filtered. Analysis of the MS data collected was done using MassLynx version 4.1. Deconvolution of the protein ion series was done using the in-built maximum entropy algorithm with calibration of the MS done with equine myoglobin from heart.

### 2.6 Enzymatic Reactions and Activity Assay

Sequencing grade modified trypsin (Promega), ATF2 (Sigma Aldrich), ATP (Sigma Aldrich), active and unactive p38α (Merck Millipore) and [γ-32P]-ATP (Perkin Elmer) were all used as per manufacturer’s instructions. More specifically, active p38α purchased had been obtained by initial NiNTA purification of the non-phosphorylated p38α. The kinase was activated using MKK6-EE (constitutively active mutant) before subsequent re-purification by NiNTA agarose. Inhibitors VX-745 (Key Organics), TAK-715, JX401 (Tocris), BIRB-796 (Selleckchem) and SB202190 were made into DMSO stock solutions (2 mM) on arrival and stored at −20°C. Samples of the remaining 2 inhibitors were kindly sent by Dr. Lyn Jones and co-workers, Pfizer, Cambridge, MA, USA and similarly made into stock solutions. All other chemicals and buffer components were bought from common suppliers and used as purchased.

S7
3 Synthesis of Protein Modification Reagents

3.1 Preparation of 2,5-dibromoadipoyl dichloride (1)

![Chemical Structure](image)

Adipic acid (10.00 g, 68.4 mmol, 1.0 equiv.) was suspended in thionyl chloride (30 mL, 414 mmol, 6.0 equiv.) and the mixture heated under reflux at 80 °C under a drying tube of CaCl₂ in air. After 30 min heating, all the adipic acid had dissolved. Heating was continued for a further 1.5 h, after which the mixture was returned to room temperature and diluted with carbon tetrachloride (40 mL). N-bromosuccinimide (24.27 g, 136 mmol, 2.0 equiv.) and conc. HBr (4 drops of a 36 % solution in water) were subsequently added and the mixture returned to reflux at 80 °C for 2 h. As the reaction progressed, the reaction mixture first turned red and red vapour was observed and after 1 h, then transiently lost its colour before turning black. The mixture was then cooled to 0 °C and allowed to stand for 30 min before being filtered and washed with Et₂O (40 mL). The filtrate concentrated in vacuo to give 2,5-dibromoadipoyl dichloride (1) as a dark red oil. The product was not further purified or characterised and was directly subjected to the next reaction.

3.2 Preparation of 2,5-dibromo-adipic acid diamide (2)

![Chemical Structure](image)

To a stirred solution of NH₄OH (80 mL of a 25 % solution in water, 1180 mmol, 17.2 equiv.) at 0 °C was added dropwise the crude acid chloride 1 over 25 min. The mixture was allowed to stir at 0 °C for 1 h before being filtered to give a light olive precipitate and a dark filtrate. The precipitate was titurated by suspending in 1:1 methanol:H₂O (80 mL) and heating the mixture at 60 °C for 30 min before further filtration. The precipitate was further washed with methanol (80 mL) and dried to give 2,5-dibromo-adipic acid diamide (2, 11.281 g, 55 %) as a white powder: νmax (film) 3196, 1666 (C=O), 1606, 1418, 1321, 1220 cm⁻¹; ¹H NMR (400 MHz, DMSO) δ ppm 7.71 (2 H, s, NH₂), 7.30 (2 H, br s, NH₂), 4.29–4.36 (2 H, m, 2×CHBr), 1.76–2.10 (4 H, m, CH₂CH₂); ¹³C NMR (101 MHz, DMSO) δ ppm (both diastereomers reported) 170.84, 170.78 (C=O), 49.2, 48.9 (CH₂CHBr), 33.4, 33.3 (CH₂CHBr); m/z (LRMS: ESI+) 327, 325 ([M+Na]⁺); in agreement with previous data.⁶

4 Protein Expression and Purification of p38α Variants for Single Modification

4.1 Cloning of pGEX-2T-p38α-Cys172 Plasmid

Transformation: Plasmid DNA of pGEX-2T-p38α-Cys119S/C162S/A172C (referred to as p38α-Cys172) (1 µL) was transformed into NovaBlue E. coli chemical competent cells (25 µL) by heat shock. The DNA was incubated with the thawed cells on ice for 5 min before heat shock was performed at
42 °C for 30 s, returning immediately on ice. After incubating on ice for a further 2 min, the cells were fed with SOC medium (125 µL) before incubation at 37 °C, 250 rpm for 15 min.

**Bacterial Culturing:** The cells were plated onto LB agar medium supplemented with ampicillin (100 µg/mL) at different cell concentrations (80 µL, 50 µL and 20 µL + 20 µL SOC medium) and incubated at 37 °C for 14 h. All plates had bacterial lawns growing on them with small numbers of distinct colonies growing on plates of lowest cell concentration. 2 colonies were picked and grown up further in liquid LB medium (2 × 10 mL) supplemented again with ampicillin (100 µg/mL) in a shaking incubator at 37 °C, 250 rpm for 17 h.

**DNA Purification and Storage:** After glycerol stocks were made, the remaining cells were then pelleted by centrifugation (3000 rpm, 20 min, 4 °C). The resulting cell pellet was resuspended in P1 buffer and the plasmid DNA purified out using the QIAprep® Miniprep kit. The plasmid DNA was eluted in EB buffer and stored at −20 °C. DNA sequencing confirmed that the sequences of the plasmid samples received were as expected.

**Sequence of pGEX-2T-p38α-Cys119S/C162S/A172C:**

```
1 ATG AGC CAG GAA CGT CCG ACC TTT TAT CGT CAG GAA CTG AAT AAA
46 ACC ATT TGG GAA GTC CCG GAA CTG CAT CAG AAT CTG TCT CCG GTT
91 GGT AGC GGT GCA TAT GGT AGC GGT TGT GCA GCA TTT GAT ACC AAA
136 ACC GGT CTG CGT GTT GCA GTT AAA AAA CTG AGC CGT CCG TTT CAG
181 AGC ATT CAT GGC AAA GTC ACC TAT CGT CAG GGA CTG CTT CTG CAG
226 AAG CAT ATT AGA CAT GAA AAT GTC ATT GGT CTG CTG GAT TTT TTT
271 ACA CCG GCA GCT AGC CTG GAA CAG TTT AAT GAT GTG TAT CTG GTG
316 ACA CAT CTG ATG GGT GCA GAT CTG CTG CTG AAA AAG CAG
361 AAA CTG ACC GAT GAT CAT CTG TCC TTC TTA ATC TAT CAG ATT CTG
406 CTG GGC CTG AAA CAT ATT CAT AGC GCA GAT TTT ATT CAT CTG GAT
451 CTG AAA CCG AGT CAT GTC GCA AAT AAA AAT GAA CAT GAA GCTT GAA
496 ATT CTG GAT TTT GGT CTG CTG CTG CAT ACC GAT GAA ATG ACC
541 GGT TAT GTT GCA ACC GGT TGT GAT GCA GCA GAA ATT ATG CTG
586 AAT TGG ATG CAT TAT AAT CAG ACC GTG GAT ATT TGG AGC GTT GTG
631 TGT ATT ATG GCA GAA CTG CTG ACC GGT CTG ACC CTG TTT CCG GTT
676 ACA CAT GAT CAT ATT GAT CAG CTG AAA CTG ATT CTG CTG GAT TTT
721 ACA CCG GGT GCC GAA CTG CTG AAA AAA ATT AGC AGC GAA AGC GCA
766 CGC AAT ATT CAG AGC ATG CTG ACC AGC CCT AAA ATG AAT TTT
811 GCC AAT TGG TTT ATT GGT GCA AAT CCG CTG GCA GTT GAT CTG CTG
856 GAA AAA ATG CTG GTC TTT GAT GAC TTT AAT CAG AGC GAA AGC GCA
901 CAG GCA CTG CTG GCA TAT AAT TTT GCC GAT CAG CAT GAT CCG GAT
946 GAT GAA CCG GTT GCA GAT CCG TAT GAT CAG AGC TTT GAA AGC GTC
991 CTG GTC CTG ATT GAA TGG AAA AGC CTG ACC CAG GAT GAA GTG
1036 ATT AGC TTT GTT CCG CCTCCA CTG GAT CAA GAA GAA ATG GAA AGC
1081 TAA
```

4.2 **General Procedure for Mutagenesis of pGEX-2T-p38α-Cys172 Plasmid**

**Mutagenesis Primers:** The following primers were used to generate further p38α-Cys119S/C162S/T180C (referred to as p38α-Cys180):

- **C172A forward:** 5′-GGATTTTGTCTGGCCTGGCTGATACGGATGAAATGAGCCG-3′
- **C172A reverse:** 5′-CGGTCATTTCATCGTGCAGGATGACCTGCGGACCACAAAAATCC-3′
- **T180C forward:** 5′-CCGATGATGAAATGTCGCGGCCGTTAATGCGAACCAGTTGGG-3′
• T180C reverse: 5’-CCAACGGGTGCAACATAACCGCACATTTCATCATCGG-3’

**Mutagenesis Reaction:** The mutagenesis reactions were made up by adding the following components in the given order:

| Substance                   | Concentration | Quantity/µL |
|-----------------------------|---------------|-------------|
| dNTPs                       | 10 mM each    | 0.25        |
| forward primer              | 12.5 µM       | 0.25        |
| reverse primer              | 12.5 µM       | 0.25        |
| MilliQ water                | –             | 9.625 (10.25 if no DMSO was used) |
| Pfu DNA polymerase          | 2.5 U/µL      | 0.25        |
| 10× reaction buffer         | –             | 1.25        |
| DMSO                        | –             | 0.625       |
| plasmid                     | 10 or 50 ng/µL| 0.25        |
| **final volume**            |               | 12.5        |

In this way, 2 reactions with different DNA template concentrations were run for each mutation made. The reactions were then heated in a thermal cycler using the programme:

| Segment | No. of Cycles | Temp./°C | Time |
|---------|---------------|----------|------|
| 1       | 1             | 95       | 30 s |
| 2       | 16            | 95       | denaturation 30 s |
|         |               | 55       | annealing 1 min |
|         |               | 68       | extension 15 min |
| 3       | 1             | 4        | ∞     |

After this time, DpnI (0.25 µL of a 10 U/µL solution) was added and the mixture incubated for 1–2 h at 37 °C. Extent of DNA synthesis was determined by running samples (2.5 µL) of the reaction mixtures on a 0.8 % agarose gel supplemented with ethidium bromide (5.3 µL of a 1 % stock solution) in TAE buffer (50 mL) with the gel run at 150 V in TAE buffer. The samples were loaded with 6× DNA loading dye (0.5 µL). The gel was visualised under a UV lamp and only those reactions were plasmid DNA was observed were transformed into *E. coli*. Where there were 2 reactions containing the same template/primer combination, only the reaction with lower template concentration was subsequently used.

**Transformation:** The selected reaction mixtures were transformed into XL1-Blue *E. coli* supercompetent cells by heat shock. Reaction mixture (1 µL) was added to aliquots of freshly thawed cells (25 µL) and incubated on ice for 5 min. Heat shock was then performed at 42 °C for 40 s before returning the cells onto ice and incubating for a further 2 min. The cells were fed with NZY+ broth (250 µL) and incubated in a shaker incubator at 37 °C, 250 rpm for 1 h.

**Bacterial Culturing:** The cells were plated onto LB agar supplemented with ampicillin (100 µg/mL). The plates were incubated at 37 °C for 21–24 h. 3 colonies of each construct were then selected from the plates and cultured further in LB medium (3×10 mL), again supplemented with ampicillin (100 µg/mL) and the cultures incubated for a further 13–22 h in a shaker incubator at 37 °C, 250 rpm.

**DNA Purification and Storage:** Glycerol stocks of the cultures were made before cells were harvested by centrifugation (3750 rpm, 10 min, 4 °C) of the cultures and the supernatant decanted off. The cells were resuspended in P1 buffer for plasmid purification by Miniprep, where the manufacturer’s protocol was used. DNA sequencing confirmed the colonies containing the desired constructs, which were selected for either expression or further rounds of mutagenesis.
Sequence of pGEX-2T- p38α-Cys119S/C162S/T180C:

1 ATG AGC CAG GAA GGT CCG ACC TTT TAT CGT CAG GAA CTG AAT AAA
46 ACC ATT TGG GAA GTG CCG GAA CTG TAT CAG AAT CTG TCT CGT CTG
91 GGT AGC GGT GCA TAT GGT AGC GGT TGT GTC GCA GCA TTT GAT ACC AAA
136 ACC GGT CTT GGT CTT GTC TTT GCA GTT AAA AAA CTG AGC GTT CCG TTT CAG
181 AGC ATT ATT CAT GCC AAA AGT ACC TAT CGT GAA CTG GAG CTG CTG CTG
226 AAG CAT ATG AAA CAT GAA AAT GTG ATT GGT CTG CTG GAT GTT TTT
271 ACA CCG GCA CGT AGC CTT GAA GAG TTT ATT GAT TGT TAT CTG GTG
316 ACA CAT CTG ATG GGT GCA GAT CTG AAT AAT ATT GTG AAA AGC CAG
361 AAA CTG ACC GAT GAT CAT CTG CAG TTC TTA ATC TAT CAG ATT CTG
406 CGT GGC CTT AAA TAT CAT AGC GCA GAT ATT ATT CAT CTG GAT
451 CTG AAA CCG AGC AAT CTG GCA GCT ATT AAT GAA GAT AGC GAA CTG AAA
496 ATT CTG GAT TTT GGT CTT CCG GCT CGT CAT ACC GAT GAA ATG TGC
541 GGT TAT GTC GAA ACC CGT TGG TAT GGT GCA CCG GAA ATT ATG CTG
586 AAT TGG CAT TAT AAT CAG GCC GAA CTG GAT ATT TGG AGT GGT
631 TGT ATT ATG GCA GAA CTG CTG ACC GGT CTT GTC CTT CGT GGT
676 ACA GAT CAT ATT GAT CAG CTG AAA CTG GAT ATT CTG CTG CTG
721 ACA CCG GGT GCC CTT CGT CTG AAA TAT ATG AGC GAA GAT GCA
766 CGC AAT TAT ATT CAT GCG CTG ACC CAG ATG CCG AAA ATG AAT TTT
811 GCC AAT TGT GCA ACC AGC ATG GCC CTA CTT CCT GCA GAT
856 GAA AAA ATG AGC GCA GAT TTT GGT CTT GTC GAT ATT GGT GAT
901 CAG GCA CTG GCA CAT GCA TAT TTT GCC CAG TAT CAT GAT CTG GAT
946 GAT GAA CCG GTC GCA GAT CCG TAT GCA GAT CAT GCG TTT GAA AGC CTG
991 GAT CTG CTG ATT GAT GAA TGG AAA AGC CTG ACC TAT GAT GAA GAT
1036 ATT AGC TTT GGT CCG CCT CCA CTG GAT CAA GAA GAA ATG GAA AGC
1081 TAA

4.3 General Procedure for Protein Expression of p38α Mutants -Cys172 and -Cys180

Transformation: Plasmid DNA of pGEX-2T-p38α-Cys172 and -p38α-Cys180 (1 µL each) were transformed into BL21(DE3) E. coli chemical competent cells (20–25 µL) by heat shock. The heat shock conditions used were the same as that described for cloning. After feeding with SOC medium (100–125 µL), the cells were incubated in a shaker incubator at 37 °C, 250 rpm for 1 h.

Bacterial Culturing: The cells were plated on LB agar plates supplemented with ampicillin (100 µg/mL) at different cell concentrations (5 µL and 20 µL + 40 µL SOC medium, 50 µL) and incubated at 37 °C for 14–16 h. 2–3 colonies were selected and cultured further in liquid LB medium (2–3×10 mL), supplemented again with ampicillin (100 µg/mL) in a shaking incubator at 37 °C, 250 rpm for 15–15.5 h.

Protein Expression: After glycerol stocks of the cell starter cultures were made, one of the cultures (2×1–4 mL) was used to inoculate a larger culture in 2YT medium (2×800 mL) supplemented with ampicillin (100 µg/mL). These larger cultures were grown to OD₆₀₀ = 0.4 at 37 °C, 180 rpm. The temperature was reduced to 20 °C and the cultures grown further to OD₆₀₀ = 0.65–0.85 before protein expression was induced with IPTG (800 µL of a 1 M solution in water, final concentration: 1.0 mM). Expression was allowed to continue at 20 °C, 180 rpm for 14.5–16 h. The cells were harvested by pelleting with centrifugation (8000 rpm, JA10 rotor, 3×10 min, 4 °C). The pellets were flash frozen in liquid nitrogen and stored at −80 °C.
4.4 Protein Purification of p38α-Cys172

**Cell Lysis:** The frozen pellets (10 g) were combined and thawed on ice. Ice cold GST lysis buffer (PBS pH 7.3, 0.5 mM TCEP) (40 mL) was added and the mixture stirred until the pellets were completely resuspended. Lysosyme (50 mg) was added and the mixture stirred on ice for a further 1.5 h, after which the cells were sonicated using a sonicator equipped with a microtip (15×2 s blasts). DNase (1 mg) was then added and the mixture stirred on ice for 20 min before centrifugation (22 000 rpm, JA25.50 rotor, 20 min, 4°C) and the supernatant taken.

**GST Affinity Chromatography:** The supernatant was further clarified by sequential filtration through 0.8 µm, 0.45 µm and 0.2 µm filters before loading onto a pre-packed 5 mL GSTrap™ HP GST affinity column using an ÄTKA Purifier® system, chasing through (5 mL, 1 CV) with GST lysis buffer. The column was washed (40 mL, 8 CV) with GST wash buffer (PBS pH 7.3, 1 % Triton X-100) and further washed (50 mL, 10 CV) with GST lysis buffer. Elution was with a linear gradient (75 mL, 15 CV, 5 mL fractions) from lysis buffer to GST elution buffer (50 mM Tris pH 8.0, 20 mM L-glutathione). The fractions containing the p38α mutant fusion protein, as determined from the FPLC report file and by SDS-PAGE analysis, were combined.

**GST Tag Cleavage:** The buffer of the combined fractions was exchanged by dialysis (MWCO 10000) overnight against PBS pH 7.3. The protein solution was warmed to room temperature and thrombin (350 U) added. Incubation was continued at room temperature with gentle rocking for 3.5 h with samples being taken at regular intervals for timecourse monitoring. After this time, PMSF (50–110 µL of a 10 mg/mL solution in isopropanol) was added to a final concentration of 0.3 mM. Some precipitation was also observed after around 2.5 h.
**GST Tag Rebinding:** The digested protein was filtered before passing through a pre-packed 5 mL GSTrap™ HP GST affinity column. The fractions in the flow-through containing protein, as determined from the UV trace and from SDS-PAGE analysis were combined, while the column was regenerated by elution (75 mL, 15 CV) with GST elution buffer.

**Anion Exchange Chromatography:** The protein was diluted by $2 \times$ with anion exchange start buffer (25 mM HEPES pH 7.5, 5% glycerol, 0.5 mM TCEP) before being re-filtered and loaded onto a 5 mL HiTrap™ HP anion exchange column. The column was washed (50 mL, 10 CV) with anion exchange start buffer before the protein was eluted using a linear gradient (100 mL, 20 CV, 5 mL fractions) from start buffer to 50% anion exchange elution buffer (same as start buffer but with 1.0 M NaCl). The protein corresponding to the major peak in the UV trace from the FPLC report file and with the correct mass as determined from SDS-PAGE analysis was taken and these fractions were combined.
Size Exclusion Chromatography and Storage: The protein was concentrated by Vivaspin (MWCO 10 000) to a final volume ≈2.5 mL before filtering. The protein was loaded onto a HiLoad™ 16/60 Superdex 75 gel filtration column and filtered (180 mL, 1.5 CV, 1.2 mL fractions) into p38α storage buffer (50 mM HEPES pH 7.8, 50 mM NaCl, 5% glycerol, 0.5 mM TCEP). The fractions containing the protein were combined and stored either without further concentration in aliquots (100 µL) or with further concentration by Vivaspin (MWCO 10 000) in aliquots (50 µL). Aliquots were flash frozen in liquid nitrogen before being stored at −80 °C. Yield: 7 mL of a 1.8 mg/mL solution in p38α storage buffer, (calculated mass: 41 437, observed mass: 41 437).
Protein sequence obtained by translation of the corresponding gene sequence:

```
tag GS
1  MSQERPTFYR QELNKTIWEV PERYQLSPV GSGAYGSVCA AFDTKGRLRV
51 AVKKLSRPFQ SIIHAKRTYR ELRLDHMKH ENVIGLDVF TPARSLEEFN
101 DVYLVTLLMG ADLNNIVKSQ KLTDDHVQLFL YQILRGLKY IHSADIHRD
151 LKPSNLAUNE DSELKILDFG LCRRHDDDEM GYVATRWYRA PEIMLNWMYH
201 NQTVDIWSVG CIMAELLTGR TLFGDTHID QLKLILRLVG TPAGELKKI
251 SSESARNYIQ SLQMPKMNFL ANVFIGNPL AVDLEKMLV LDDSKRITAA
301 QALAHAYFAQ YHDPDEFPVA DPYDFSFR DLLIDEWKSL TYDEVISFVP
351 PPLDQEEMES
```

4.5 Protein Purification of p38α-Cys180

p38α-Cys180 was purified using a similar procedure as used for p38α-Cys172:

**Cell Lysis:** A pellet (14.0 g) containing p38α-Cys180 was thawed on ice and re-suspended (60 mL) in ice-cold GST lysis buffer (PBS pH 7.3, 0.5 mM TCEP). Lysozyme (70 mg) was then added and the mixture stirred at 4 °C for 2 h before being sonicated with a sonicator equipped with a microtip (15 × 2 s blasts, 60% amplitude, 1291 J total energy). The lysate was further treated with DNase (1 mg) at 4 °C for 1 h before pelleting by centrifugation (20 000 rpm, JA25.50 rotor, 40 min, 4 °C) and the supernatant taken.

**GST Affinity Chromatography:** The cleared lysate was further clarified by filtration (0.2 µm filter) and loaded in 2 batches onto a pre-packed GSTrap™ HP GST affinity column (GE Healthcare) on an ÄTKA Purifier system. On each run, the lysate was chased through (5 mL, 1 CV) with more lysis buffer before washing, first (40 mL, 8 CV) with GST wash buffer (PBS pH 7.3, 1% Triton X-100), then again (50 mL, 10 CV) with lysis buffer. The bound proteins were eluted (75 mL, 15 CV, 5 mL fractions) by a linear gradient from lysis buffer to GST elution buffer (50 mM Tris pH 8.0, 20 mM l-glutathione). The fractions containing the desired protein from both separations were determined from the UV trace of the FPLC report file and from SDS-PAGE analysis, and combined.
**GST Tag Cleavage:** The combined fractions were partially concentrated (23 mL final volume) by Vivaspin (10,000 MWCO) before dialysis against PBS at 4°C for 16 h. Thrombin (400 U of a 1 U/µL solution) in PBS was added and the protein incubated at room temperature with gentle rocking for 4 h before the thrombin was inhibited with PMSF (170 µL of a 10 mg/mL solution) in isopropanol and the protein returned on ice. Small amounts of precipitate had formed on this reaction.

**GST Tag Rebinding:** After filtering, the protein was re-loaded (2 mL fractions collected) onto the GSTrap™ HP GST affinity column, again in 2 batches and in each run, the column was washed (10 mL, 2 CV) with lysis buffer before elution (75 mL, 15 CV) with 100% elution buffer. The fractions from the flow-through containing the target protein of both batches were determined from the UV trace of the FPLC report file and from SDS-PAGE analysis, and combined.

**Anion Exchange Chromatography:** The combined fractions were diluted 2× with anion exchange start buffer (25 mM HEPES pH 7.5, 5% glycerol, 0.5 mM TCEP), re-filtered, and loaded as a single batch onto a 5 mL HiTrap™ Q HP anion exchange column (GE Healthcare). The column was further washed (50 mL, 10 CV) with start buffer before elution (100 mL, 20 CV, 5 mL fractions) with a linear gradient from start buffer to 50% anion exchange elution buffer (same as start buffer but with additional 1000 mM NaCl). SDS-PAGE analysis of the fractions determined that the desired protein corresponded
to 2 distinct peaks in the UV trace of the FPLC report file, with the first peak being the major one. Protein corresponding to this major peak was taken as the desired protein and further treated.

Fractions A2–B12: Batch 1, Fractions E2–F12: Batch 2. Cleavage timecourse on left of gel.

**Size Exclusion Chromatography and Storage:** The protein was concentrated (∼3 mL) before being loaded onto a HiLoad™ 16/60 Superdex 75 gel filtration column and filtered (180 mL, 1.5 CV) into p38α storage buffer (50 mM HEPES pH 7.8, 50 mM NaCl, 5% glycerol, 0.5 mM TCEP). The fractions containing the target protein were determined from the UV trace of the FPLC report file and from SDS-PAGE analysis, pooled, and concentrated by Vivaspin. The protein was then divided into aliquots, flash frozen in liquid nitrogen and stored at −80°C; Yield: 2.8 mL of a 8.2 mg/mL solution in p38α storage buffer; (calculated mass: 41 407, observed mass: 41 404–41 409).
Protein sequence obtained by translation of the corresponding gene sequence:

```
tag
1  MSQERPTFYR QELNKTIWEV PERYQLSPV GSGAYGSVCA AFDTKTGLRV
51  AVKKLRSRPFQ SIIHAKRTYR ELRLKKHMKH ENVIGLDDVF TPARSLEEFN
101  DYLHVTLMG ADLNNIVKSQ KLTDDHVFQ FL IQILRLGYK IHSADIHRD
151  LKPSNLAVNE DSELKIDFG LARHTDDEM C GYVATRWYRA PEIMLNWMY
201  NQTVDIWSVG CIMAELHTGR TLFGTDHID QKLILRNVG TPQEAEKLI
251  SSESARNYIQ SRLQMFKMNF ANVFIGANPL AVDLLEKMLV LDSDKRITAA
301  QALAHAYFAQ YHDPDEPVA DPYDQSFRSL DLDIEWKSL TYDEVISFVP
351  PPLDQREMES
```

5 Synthesis of Chemically Modified p38α Variants

5.1 Synthesis of p38α-Dha172

p38α-Cys172 (500 µL) of a 1.8 mg/mL solution, 22 nmol, 1 equiv.) in p38α storage buffer (50 mM HEPES, pH 7.5, 50 mM NaCl, 5% glycerol, 0.5 mM TCEP) was thawed on ice and the buffer exchanged using a G-25 MiniTrap™ desalting column (GE Healthcare) to p38α reaction buffer (the same as storage buffer but without TCEP). A suspension of 2 (252 µL of a 3.9 mg/mL suspension, 3.3 µmol, 150 equiv.) in p38α reaction buffer was added and the mixture shaken at 37 °C, 550 rpm for 4 h. LC-MS after this
time showed >95% conversion to the Dha product. The reaction mixture was desalted using a G-25 MidiTrap column to give p38α-Dha172, which was either kept on ice for immediate use, or flash frozen in liquid nitrogen and stored at −80°C for short-term use: Yield: 755 µL of a 0.68 mg/mL solution in p38α reaction buffer, 57%, (calculated mass: 41 403, observed mass: 41 404).

5.2 Synthesis of p38α-pCys172

p38α-Dha172 (470 µL) of a 0.48 mg/mL solution, 5.4 nmol, 1 equiv.) in p38α reaction buffer (50 mM HEPES, pH 7.5, 50 mM NaCl, 5% glycerol) was thawed on ice. To the thawed protein was added batchwise (5 min intervals) sodium thiophosphate (≈ pH 8.0, 5×7.11 µL of a 690 mg/mL suspension, 5×27 µmol, 5×5000 equiv.) in H2O/HCl and the mixture shaken at 37°C, 550 rpm for 4 h. LC-MS after this time showed >95% conversion to the Dha product. The reaction mixture was desalted back to reaction buffer using a G-25 MiniTrap column, followed by repeated concentration/dilution by Vivaspin (MWCO 10 000) to give p38α-pCys172, which was kept on ice for immediate use: (calculated mass: 41 517, observed mass: 41 515).
5.3 Synthesis of p38α-Dha180

p38α-Cys180 was thawed on ice and the buffer exchanged using a G-25 MiniTrap™ desalting column (GE Healthcare) to p38α reaction buffer (50 mM HEPES pH 8.0, 50 mM NaCl, 5% glycerol). To the resulting protein (580 µL of a 2.51 mg/mL solution, 35 nmol, 1 equiv.) was added a suspension of dibromide 2 (410 µL of a 3.9 mg/mL suspension, 5.3 µmol, 150 equiv.) in the same buffer. The mixture was then shaken at 37 °C, 550 rpm for 2 h. LC-MS showed >95% consumption of the unmodified protein p38α-Cys180 with some residual bromide adduct intermediate. The reaction mixture was desalted again using a G-25 MidiTrap™ desalting column (GE Healthcare) and the protein incubated on ice for 18 h. LC-MS showed >95% conversion to the Dha product p38α-Dha172. The reaction mixture was divided into aliquots and kept on ice for immediate further use: Yield: 1250 µL of a 0.87 mg/mL solution in p38α reaction buffer, 75%, (calculated mass: 41 373, observed mass: 41 375).
5.4 Synthesis of p38α-pCys180

Sodium thiophosphate was added portion-wise (6 × 13.7 µL of a 690 mg/mL solution at pH 8, 6 × 53 µmol, 6 × 5000 equiv.) at 5 min intervals to p38α-Dha180 (500 µL of a 0.87 mg/mL solution, 10.5 nmol, 1 equiv.) in p38α reaction buffer (50 mM HEPES, pH 7.5, 50 mM NaCl, 5% glycerol). The sodium thiophosphate solution was made by dissolving sodium thiophosphate (68.2 mg) in water (27.6 µL) and neutralising with 5 M HCl (29.6 µL). The reaction mixture was then heated with shaking at 37 °C, 550 rpm for 8 h. LC-MS after this time showed >90% conversion to the thiophosphate adduct. The reaction mixture was desalted back to reaction buffer using a G-25 MiniTrap column, followed by repeated concentration/dilution by Vivaspin (MWCO 10 000) to give the thiophosphate product p38α-pCys180: (calculated mass: 41 487, observed masses: 41 489).
5.5 Comparison of Chemical Reaction Kinetics for Phosphocysteine Formation

Mass spectra from the timecourses of the phosphocysteine forming reactions with both p38α-Dha172 and p38α-Dha180 were quantitatively analysed (see Section 8.2.2 for more details) which revealed that the reaction with p38α-Dha172 was faster than with p38α-Dha180.

6 Model Surface and Homology Calculations

6.1 Electrostatic Calculations

The crystal structure file (1R3C.pdb) was prepared for electrostatic surface calculation using PDB2PQR\(^7,8\) (CHARMM forcefield) before use of the ABPS PyMOL plugin\(^9\) for calculation of the surface itself. The resultant images were manipulated in PyMOL.

6.2 Surface Accessibility Calculations

Surface accessibility was assessed using Naccess program\(^10,11\), calculated using 1R3C.pdb. The default probe size (1.4 Å) was used. Conversion of the percentage data from Naccess into colour information for the figures was done using scripts written in Python. The relative percentages of total side-chain accessibility were used for this conversion. The percentage data used is plotted below. Due to the methods used in the calculation, percentages of over 100% are possible.

6.3 ATF2 Homology Modelling

For the structure of ATF2, a homology model was made using sequence (amino acids 1–287 from Bos Taurus, sequence ID: AA133291.1) obtained by BLAST search\(^12\) of sequence from 1BHI.pdb. The homology model was then calculated using Phyre2\(^13\) to give the images as displayed in the figures.
7 Methods for Characterising (Modified) Proteins

7.1 General Procedure for Measurement of Circular Dichroism Spectra

Prior to measuring the spectra, samples not already in p38α reaction buffer (50 mM HEPES pH 7.5, 50 mM NaCl, 5% glycerol) were desalted into this using a combination of G-25 SpinTrap™ desalting column (GE Healthcare) and repeated concentration/dilution by Vivaspin (MWCO 10 000). Samples were then diluted (0.36–0.72 mg/mL) to an appropriate final volume (190–220 µL), loaded into a cuvette with thin pathlength (1.0 mm) before the spectra were collected using a Chirascan (Applied Photophysics). The spectra were collected as a temperature melts (Wavelength range: 180–260 nm, Temperature range: 10°C→90°C→10°C, Temperature step: 10°C, Temperature equilibration time: 450 s, Repeat scans: 5). Data collected were exported as raw data and reprocessed in MATLAB to give the final 3D plots (mean average of repeat scans taken, spectra smoothed using Savitzky-Golay filtering. Smoothing parameters: MATLAB function: sgolayfilt from Signal Processing toolbox, Polynomial order: 1, Window size: 5).

7.2 General Procedure for LC-MS/MS Analysis including Sample Preparation

The protocol is adapted from the one associated with the “in-solution tryptic digestion and guanidination kit” (Thermo Scientific), with modifications made to the reduction step.

Sample Preparation: A sample of the protein of interest (10.5 µL) was diluted with ammonium bicarbonate (15 µL of a 50 mM solution) in water. The volume was made up with water (1.5 µL) and the mixture heated at 60°C for 10 min. Iodoacetamide (3 µL of a 100 mM solution in water) was then added, the reaction mixture protected from light and incubated at room temperature for 30 min. The sample was digested by the addition of trypsin (1.0 µL of a 0.1 µg/µL solution), incubating at 37°C for 2 h. A second batch of trypsin (1.0 µL) was added and incubation continued at either 30°C for 18 h, or 37°C for 2 h. The digested sample was flash frozen in liquid nitrogen and stored at −80°C for further purification of the peptides.

Sample Purification: Peptides obtained from digestion were desalted using a C18 Sep-Pak cartridge (Waters). The cartridge was equilibrated by washing 5 mL with solution B (65% acetonitrile, 35% water, 0.1% formic acid), followed by washing (10 mL) with solution A (98% water, 2% acetonitrile, 0.1% formic acid). The sample was loaded onto the cartridge and the cartridge further washed (10 mL) with solution A. The peptides were then eluted (2×1 mL) with solution B, collecting the two fractions separately. The majority of the acetonitrile was removed in vacuo before the peptides were dried by lyophilisation. Finally, the dried peptides redissolved in solution A (20 µL).

Data Collection and Analysis: The sample of redissolved, purified peptides was given to the Mass Spectrometry service to analyse by LC-MS/MS. The data were collected using MassLynx (v. 4.1) and any necessary file reformatting (to .PKL file format) was done using ProteinLynx (v. 2.2.5). The data were then analysed using the MASCOT database search engine (MatrixScience, v. 2.4 CBRG Cluster): (Search parameters: Peptide tolerance: ±50 ppm, Number of 13C: 2, MS/MS tolerance: 0.2 Da, Peptide charge: 2+, 3+ and 4+, Variable modifications considered: Acetyl (Protein N-term), Carbamidomethyl (C), Cys→Dha (C), Deamidated (N), Deamidated (Q), Oxidation (C), Oxidation (M) and Phospho (C)). The data displayed below are: 1. Sequence coverage of each analysis with the peptides detected in red, 2. Highlighted peptides of interest that are present, 3. MS fragmentation spectra for the corresponding peptides.
p38α-Cys172:

1 GSMSQERPTF YRQELNKTIW EVPERYQNLSPGVGGAYGSVC AAFDTKGL
51 RVAVKLRLSRPFQSIIHAKRT YRELRLKHM KHEINVGLLD VFTPASLEE
101 FNDVVLVTHL MGADLNNIVK SQKLITDDHVQ FLIQIIRGL KYIHSADIIH
151 RDLKPSNLA V NEDSELKILD FGLCRHTDDE MTGYVATRQY VAEPEIMLNWML
201 HYNGTVDW VS HCSIIMAELL GRTLFLPTDID IQQLKLILRL VGPLGAEgLK
251 KISSESAR NY IQSLTOMPKM NFANVFIGAN PLAVDLEKLM LVLDSDKRIT
301 AAGALAHAYF AQYHDPDEP VADPYQSFE SRDLLIDEWK SLTYDEVISF
351 VPPPLQDEEM ES

Peptide: R.YQNLSPVGSGAYGSVC AAFDTK.T + Carbamidomethyl (C)

| Start | End  | Observed  Mr (expt) | Mr (calc) | ppm | Score | Expect |
|-------|------|--------------------|-----------|-----|-------|--------|
| 26    | 47   | 2292.0771          | 2291.0698 | 7.41| 90    | 3.9e-09|

Peptide: K.ILDFGLCRC.R + Carbamidomethyl (C)

| Start | End  | Observed  Mr (expt) | Mr (calc) | ppm | Score | Expect |
|-------|------|--------------------|-----------|-----|-------|--------|
| 168   | 175  | 994.5047           | 993.4974  | 992.5113 | 994  | 49    | 9e-05  |
p38α-Dha172:

1 GSMSQERPTF YRQELNKTIW EVPERYQNSL PVGSGAYGSV CAAFTKTLGL
51 RVAVKLSRP FQSIIHAKRT YRELRLKHM KHEVIGLLD VFPPARSLEE
101 FNDVYLVTHL MGADLNNIVK SQKLTDDHVQ FLIYQILRGL KYIHSAADIH
151 RDLPFSNALV NEDSELKILD FGLCRHTDDE MTGYVATR Wy RAEI MLNWM
201 HYNQTDVWSVGÇIAMELLT GRTLFPGTDH IDQLKLILRL VGTGPAELLK
251 KISSESARNY IQSLTOMPKM NFANVFIGAN PLAVDLLEKML VLVDSKRIT
301 AAQLAHAYF AQYHDPDDEEP VADPYDQSFSE SRDLDIEMK SLTYDEVISF
351 VPPPLDQEEM ES

Peptide: R.YQNLS PVGSGAYGSVCAAFTKTL + Carbamidomethyl (C)

| Start | End  | Observed | Mr (expt) | Mr (calc) | Δ ppm | Score | Expect |
|-------|------|----------|----------|----------|-------|-------|--------|
| 26    | 47   | 2292.0630| 2291.0557| 2291.0529| 1.25  | 165   | 6.6e-16|

Peptide: K.ILDFGLCR.H + Cys–Dha (C)

| Start | End  | Observed | Mr (expt) | Mr (calc) | Δ ppm | Score | Expect |
|-------|------|----------|----------|----------|-------|-------|--------|
| 168   | 175  | 902.5085 | 901.5012 | 901.5022 | -1.05 | 36    | 0.0074 |
p38α-pCys172:

1 GSMSQERPTF YRQELNKTIW EVPERYQNLSPVGSGAYGSVCAAFTDKITGL 51 RAVVKKLSRP FQSIIHAKRT YRELRLKHM KHENVIGLLDVFTPARSLEE 101 FNDVYLVTHL MGADLNIVKV SQKLTDHHVQ FLIYQILRLGL KYIHSADIIH 151 RDLKPSNLAV NEDSELKILD FGLCRHGDDE MTGYVATR WyRAPEIMLNWM 201 HYMQTVGILWS GCQIAEMLLT GRTLFPGTDDH IDQLKLILRL VGTPGAEILK 251 KISSESARNY IQSLTOMPKM NFAVFGIGAN PLAVDLEKLMVLVSDKRIT 301 AQQALAHAYF AQYHDSEPDDP VADPYGQSFE SRDGiDEWKS LTYDEVISF 351 VPPPLDQEBEM ES

Peptide: R.YQNLSPVGSGAYGSVCAAFTDK.T + Carbamidomethyl (C)

| Start | End | Observed Mr(expt) | Mr(calc) | ∆ppm | Score | Expect |
|-------|-----|--------------------|----------|------|-------|--------|
| 26    | 47  | 2292.0637          | 2291.0564| 1.56 | 75    | 1e-07  |

Peptide: K.ILDFGLC.R.H + Carbamidomethyl (C)

| Start | End | Observed Mr(expt) | Mr(calc) | ∆ppm | Score | Expect |
|-------|-----|--------------------|----------|------|-------|--------|
| 168   | 175 | 994.5047           | 993.4974 | 992.5113 | 994  | 49   | 9e-05  |
p38α-Cys180:

1 GSMSQERPTFYRQELNKTIW EVPERYQNLSPVGSAGYSVCAAFTDKTG
51 RAVAVKLSRPFQSIHIAKRTYRELRLKHM KHEVIGLDVFTPARSLEE
101 FNDVYLVTHL MAGDLNNIVK SQKLTDHVQFLQILRGLKYIHSADI
151 RDLKPSNLAVNEDSELKILDFGLARHTDEMCGYVATRWRPAPEIMLNWM
201 HYNQTVDIWSVGCIYAAELETRTLFPQTDHDIQQLKLILRLVGTGPAELLLK
251 KHSSESARNYYIQSLTOMPKMNFANVFIGANPLAVLLEKMLVLDSDKRIT
301 AAQALAHAYFAQYHDPPDEPVAPPYQSFESRDLLIDEWKSLTYDEVISF
351 VPPPLDQEEMES

Peptide: R.YQNLSPVGSAGYSVCAAFTDK.T + Carboxyamidomethyl (C)

| Start | End  | Observed Mr (expt) | Mr (calc) | ∆ppm | Score | Expect |
|-------|------|--------------------|----------|------|-------|--------|
| 26    | 47   | 2292.0615          | 2291.0542| 0.60 | 83    | 1.6e-08 |

Peptide: R.HTDDEMCGYVATR.W + Carboxyamidomethyl (C)

| Start | End  | Observed Mr (expt) | Mr (calc) | ∆ppm | Score | Expect |
|-------|------|--------------------|----------|------|-------|--------|
| 176   | 188  | 1554.6293          | 1553.6220| -1.20| 73    | 4.2e-07 |
p38α-Dha180:

1 GSMSQE\textsuperscript{ERPTF YRQLNLKTIW EVPERYQNSLS PVGSGAYGSV \textsuperscript{CAA}AFDKTGL
51 RVALVKLSRP FQ\textsuperscript{S}IHAKRT YRELRLKHM KHENVIGLLD VFTP\textsuperscript{S}LEEE
101 FNDVLYVTHL MG\textsuperscript{ADL}NIVK SQ\textsuperscript{K}LTDH\textsuperscript{VQ FLIYQILRGL KYI\textsuperscript{H}SADIIH
151 RDLKPSNLAV NED\textsuperscript{SEL}KILD FGL\textsuperscript{AR}HTDE M\textsuperscript{CG}GYVATRWY RAPEIM\textsuperscript{L}NMW
201 HYNQTV\textsuperscript{D}IWS VGC\textsuperscript{\textsc{\textbf{I}}M}AELLTT GRL\textsuperscript{F}P\textsuperscript{G}TDH \textsuperscript{IDQ}\textsuperscript{L}KL\textsuperscript{L}ILRL V\textsuperscript{GT}P\textsuperscript{G}A\textsuperscript{EL}LK
251 KISS\textsuperscript{E}S\textsuperscript{AR}NY IQSL\textsuperscript{T}OMPKM \textsuperscript{N}FAN\textsuperscript{V}FIGAN \PLAV\textsuperscript{D}LLEK\textsuperscript{M}LV\textsuperscript{L}DS\textsuperscript{D}KRIT
301 A\textsuperscript{AQ}Q\textsuperscript{A}LA\textsuperscript{H}AYF \textsc{\textbf{A}}\textsuperscript{C}YHD\textsuperscript{D}P\textsuperscript{DEP} V\textsuperscript{A}D\textsuperscript{P}D\textsuperscript{Q}\textsuperscript{S}FE SD\textsuperscript{D}L\textsuperscript{L}I\textsuperscript{D}E\textsuperscript{W}K SL\textsuperscript{T}YDEV\textsuperscript{IS}F
351 V\textsuperscript{PP}P\textsuperscript{L}DQE\textsuperscript{E}EM ES

Peptide: R.YQNLSPVGSGAYGSV\textsubscript{\textsc{Caa}}AFDKT.T + Carbamidomethyl (C)

| Start | End  | Observed Mr (expt) | Mr (calc) | ∆ppm  | Score | Expect |
|-------|------|--------------------|-----------|-------|-------|--------|
| 26    | 47   | 2292.0593          | 2291.0520 | -0.36 | 159   | 3.2e-15 |

Peptide: R.HTDDE\textsubscript{MC}GYVATR.W + Cys\textrightarrow{}Dha (C)

| Start | End  | Observed Mr (expt) | Mr (calc) | ∆ppm  | Score | Expect |
|-------|------|--------------------|-----------|-------|-------|--------|
| 176   | 188  | 1463.6230          | 1462.6157 | 0.69  | 75    | 1.8e-07 |
p38α-PCys180:

1 GSMSQERPTF YRQELNKT IW EVPERYQNL S PVGSGAYGSV ÇCAAFTKG TL GL
51 RVAVKKLSRP FQSIHAKRT YRELRLKHM KHEVIGLLD VFTPAS LEE
101 FNDVYLVTHL MGADLNJNI V SQKLTDH TVQ FLIIQILRGL KYIHSADIIH
151 RDLKPSNLAV NEDSELKILD FGLARHTDDE MÇGYVATRWHY RAPEIMLNWM
201 HYNQTDVIWS VÇÇÍMAELLT GRTLFPGTDH IDQLKLILLRL VGFPGAELLK
251 KISSESARNY IQSLOTMKPM NFANVFIGAN PLAVDLLEK LMVLDSKRIT
301 AQAQLAHYF AQYHDPDDEP VADPYQSFSE SRDLIDEWK SLYDEVISF
351 VPPPLDQEM ES

Peptide: R. YQNLSPVGSGAYGVS ÇCAAFTKT. T + Carbamidomethyl (C)

| Start | End | Observed Mr (expt) | Mr (calc) | ∆ppm | Score | Expect |
|-------|-----|-------------------|-----------|------|-------|--------|
| 26    | 47  | 2292.0618         | 2291.0545 | 0.73 | 173   | 9.6e-17|

Peptide: R. HTDDEMÇGYVATR.W + Phospho (C)

| Start | End | Observed Mr (expt) | Mr (calc) | ∆ppm | Score | Expect |
|-------|-----|-------------------|-----------|------|-------|--------|
| 176   | 188 | 1577.5786         | 1576.5713 | 1.64 | 6     | 0.28   |
7.3 LC-MS/MS Analysis for Detection of Phosphopeptides from p38α Variants

7.3.1 Protein Digestion

p38α-pCys180 (40 µg) was buffer exchanged using Vivaspin™ (MWCO 10 000 Da) against ammonium bicarbonate buffer (50 mM solution in water) to a final protein concentration of 0.4 mg/mL. Urea (48 mg, 8 M final concentration) was then added and the sample incubated at room temperature for 10 min with occasional vortexing. The protein was reduced with DTT (200 mM solution in water) at 56 °C for 25 min and alkylated with iodoacetamide (400 mM solution in water) at room temperature for 30 min in the dark. The sample was diluted 4× with ammonium bicarbonate buffer and trypsin added (final enzyme:protein 1:50 (w/w) ratio). The sample was incubated overnight at 37 °C. The digested protein was diluted (final concentration: 100 fmol/µL) with water. Variant p38α-pCys172 was treated similarly.

7.3.2 LC-MS/MS Analysis

The samples were analysed on an Orbitrap Elite (Thermo Fisher Scientific, DE) connected to a UHPLC Proxeon EASY-nLC 1000 and an EASY-Spray nano-electrospray ion source with EASY-Spray column (Thermo Fischer Scientific, DK). Peptides were trapped on an Acclaim PepMap® trapping column (100 µm i.d. × 20 mm, 5 µm C18) and separated on an EASY-spray Acclaim PepMap® analytical column (75 µm i.d. × 500 mm, RSLC C18, 2 µm, 100 Å). Samples were loaded at a pressure of 500 bar with 100% solvent A (0.1% formic acid in water) and the peptides separated by a linear gradient (length: 15 min, 7% to 30% solvent B (0.1% formic acid in acetonitrile), flow rate: 200 nL/min). Full scan MS spectra were acquired in the Orbitrap (350-1500 m/z, resolution 120 000, AGC target 1 × 10^6, maximum injection time 250 ms). CID and ETD spectra were acquired in Ion Trap (resolution 7500, AGC cation target 3 × 10^4, AGC Anion target 2 × 10^5, maximum injection time 100 ms). After the MS scans, the 5 most intense peaks were selected for fragmentation based on data-dependent decision tree (DDDT). The signal threshold for fragmentation of parent ion was set to 500 ion counts. For CID fragmentation, the normalized collision energy and default charge state was set to 35% and 2 respectively. For ETD fragmentation, reaction time was set to 50 ms and supplemental activation was enabled. Charge state screening was enabled; parent ion with unassigned charge state and charge state 1 was rejected. Dynamic exclusion was enabled (exclusion size list 75, exclusion duration 5 s). The setting for DDDT is as such: ETD fragmentation was followed for charge states 3, 4 and 5 with m/z less than 750. For charge states greater than 5, ETD fragmentation was always performed. In all other cases, CID activation was performed. In a separate experiment, HCD was used. All the settings for the instrument were kept the same, except HCD spectra were acquired in Orbitrap and the 3 most intense peaks were selected for fragmentation and normalized collision energy was set to 32%.

7.3.3 Data Analysis

The raw data files generated were processed using MaxQuant software (Version 1.4.1.2), integrated with Andromeda search engine as described elsewhere.14,15 For identification of phosphocysteine peptides, Andromeda searched peak lists against the sequence of p38α as well as against a list of common contaminants. Trypsin was selected as specific digestion mode with maximum number of missed cleavages set to be 4. In case of p38α-pCys180 protein; acetylation (N-term), carbamidomethylation (C), cysteine to serine, oxidation (M), phosphorylation (STY), threonine to carbamidomethylation, threonine to cysteine, threonine to dehydroalanine, and threonine to phosphocysteine were used as variable modifications. For p38α-pCys172, similar rational variable modifications were used. All spectra were manually validated.
P38α-pCys172
8 Activity Assay with Substrate Detection by Mass Spectrometry (MS)

8.1 Measurement of the Activity of All Variants of p38α-X172 and -X180

Kinase activity buffer stock (5.00 µL of a 10× stock containing 200 mM Tris pH 7.5, 20 mM DTT, 50 mM β-glycerophosphate, 2.0 mM Na3VO4, 100 mM MgCl2, 50 mM NaF) was diluted with water (13.4 µL). A sample of (un)modified p38α kinase (10.0 µL of a 20 µM solution) in p38α reaction buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 5% glycerol) was added to the buffer, followed by ATP solution (5.00 µL of a 5.0 mM solution in water, pH 7.0). A sample (3.34 µL) corresponding to t = 0 was taken before the substrate ATF2 (7.16 µL of a 18.9 µM solution) in ATF2 storage buffer (20 mM HEPES pH 7.5, 0.5 mM EGTA, 1 mM DTT, 5 µg/mL aprotinin, 10 mg/mL leupeptin, 0.25 mg/mL AEBSF, 0.03% Brij-35, 150 mM NaCl, trehalose 10%) pre-diluted with water (7.75 µL) was added. Samples (5 µL) were taken at given timepoints (1, 5, 10, 30 and 60 min, then 3 h and 16–18 h) and the samples quenched with urea (10 µL of a 10 M solution in water). The samples were then analysed by LC-MS for the mass of the substrate. For all the reactions with p38α-X180 species, samples taken after 150 min were also analysed by SDS-PAGE. MS displayed are for the timepoint >16 h: (Unmodified substrate: expected mass: 53 kDa (value provided from manufacturer’s product specification sheet), observed range of masses: 53 242–53 248, Monophosphorylated substrate: expected mass: +80 from unmodified substrate, observed range of masses: 53 326–53 327, Diphosphorylated substrate: expected mass: +160 from unmodified substrate, observed mass: 53 405).

ATF2 after Treatment with p38α-Cys172:

ATF2 after Treatment with p38α-Dha172:
ATF2 after Treatment with p38α-pCys172:

ATF2 after Treatment with p38α-Cys180:
ATF2 after Treatment with p38α-Dha180:

ATF2 after Treatment with p38α-pCys180:
8.2 Quantitative Measurement for Comparison of MS to Electrophoretic Radioassay

8.2.1 Enzymatic Reactions

10× kinase assay buffer (7.5 µL) was diluted with water (20.16 µL and ATP (7.5 µL of a 5 mM solution in water) was added. ATF2 (12 µL) in ATF2 storage buffer was diluted with water (13 µL) and this solution was added (24.84 µL) to the buffer/ATP master mix. After taking a sample (4.0 µL) for t = 0, kinase (14.0 µL of a 20 µM solution) in p38α reaction buffer was added to initiate the reaction. Samples were taken at further timepoints (2, 5, 10, 18, 30, 45, 60, 75, 90, 105, 120, 150 and 180 min, times corresponding to the radioassay) and quenched in urea solution (10 M in p38α reaction buffer). Samples were then analysed by LC-MS using a Chromolith® FastGradient RP-18 endcapped 50×2 mm monolithic HPLC column (Merck).

8.2.2 Data Analysis and Quantification

LC-MS datasets were processed using MassLynx as previously described (Section 2.5). Data from the deconvoluted spectra were output from MassLynx as a spectrum list for quantitative analysis using MATLAB R2012b.

In MATLAB, quantities of the three substrate species (unphosphorylated, monophosphorylated and diphosphorylated) were estimated from relative peak intensity, setting the baseline at the median of all the values in the spectrum list. The peaks were picked by taking the maximum intensities from the three ranges where the peaks of these species were expected.

To convert the MS data so that it could be compared to the data from electrophoretic radioassay (ERA), the MS signals from the two phosphorylated species were added according to the equation

\[
\gamma^{32}\text{P on ATF2}_{\text{ERA}} = [\text{ATF2-P}]_{\text{MS}} + 2[\text{ATF2-PP}]_{\text{MS}}.
\]

Both the curves from MS and ERA were normalised for the comparison.
### 8.3 Quantitative Measurement of the Activity of Enzymatically Active Variants of p38α

#### 8.3.1 Enzymatic Reactions

A modified procedure to the one above was used. 10× kinase assay buffer (60 µL), water (4.8 µL) and ATP (60 µL of a 5 mM solution in water) were combined to give a master mix. Aliquots (20 µL) were taken and ATF2 (74.2 µL, final concentrations: 14, 8, 4, 2, 1 and 0.5 µM) in ATF2 storage buffer added. After taking a sample (4.75 µL) for t = 0, kinase (4.75 µL of a 1.6 µM solution of either p38α-pCys180 or p38α-pThr Tyr, final concentration: 80 nM) in p38α reaction buffer was added to initiate the reaction. Samples were taken at further timepoints (0.5 h and every h for 10 h, then every 3–4 h for 36 h total time for p38α-pCys180, 2, 5, 10, 18, 30, 45, 60, 75, 90, 105, 120, then every 180 min for 5 h total time for p38α-pThr Tyr) and quenched in urea solution (10 M in p38α reaction buffer). Samples were then analysed by LC-MS.

#### 8.3.2 Data Analysis and Quantification

The MS data was analysed and quantified using the procedure described in Section 8.2.2. The peak proportions were then normalised to the starting ATF2 concentration. Determination of $k_{cat}/K_M$ was done by taking the absolute value initial rates for each of the ATF2 species. This was plotted against substrate concentration and from the gradient, $k_{cat}/K_M$ could then be calculated. Finally, the standard error in the value for the gradient was estimated by inputting the [S] vs. rate data into Graphpad PRISM v.5.01.

**Example timecourses:**

![Timecourse of p38α\textsuperscript{T180pC} on ATF2](image1)

![Timecourse of Active p38α\textsuperscript{pT180pY182} on ATF2](image2)

ATF2:

![Determination of $K_M$ and $k_{cat}$ for p38α\textsuperscript{T180pC} wrt ATF2](image3)

![Determination of $K_M$ and $k_{cat}$ for p38α\textsuperscript{pT180pY182} wrt ATF2](image4)
ATF2-P:

\[
\frac{k_{\text{cat}}}{K_M} = 0.59 \, \mu\text{M}^{-1}\text{h}^{-1}
\]

ATF2-PP:

\[
\frac{k_{\text{cat}}}{K_M} = 0.666 \, \mu\text{M}^{-1}\text{h}^{-1}
\]

**MS of a representative timecourse (for p38α-pCys180, [ATF2] = 14 µM):**

Start:

\[
\text{TOF MS ES+ 110}
\]
After 5 h:

After 10 h:

After 18.5 h:
8.3.3 Data Reanalysis for Intermediate ATF2 Species Adducts

Close observation of the mass spectra revealed an adduct mass from the parent peak of +37–40 Da, which could be explained by cyanate adduct formation from the urea quench.\textsuperscript{16,17} Reanalysis of the data were done to take this adduct into account, adding the mass of the adduct onto the corresponding parent peak and repeating the subsequent timecourse and kinetic analysis as described above. The two analyses revealed the same trends as observed in the previous analysis.

Without adduct:

|                          | $p38\alpha$-pThr$p$Tyr | $p38\alpha$-pCys180 |
|--------------------------|------------------------|---------------------|
| $(k_{cat}/K_M \text{ global})/\mu\text{M}^{-1} \text{ h}^{-1}$ | $8.14 \pm 0.08$       | $0.65 \pm 0.03$     |
| $(k_{cat}/K_M \text{ ATF2-P})/\mu\text{M}^{-1} \text{ h}^{-1}$ | $5.30 \pm 0.33$       | $0.59 \pm 0.03$     |
| $(k_{cat}/K_M \text{ ATF2-PP})/\mu\text{M}^{-1} \text{ h}^{-1}$ | $2.84 \pm 0.29$       | $0.07 \pm 0.01$     |

With adduct:

|                          | $p38\alpha$-pThr$p$Tyr | $p38\alpha$-pCys180 |
|--------------------------|------------------------|---------------------|
| $(k_{cat}/K_M \text{ global})/\mu\text{M}^{-1} \text{ h}^{-1}$ | $7.43 \pm 0.21$       | $0.60 \pm 0.03$     |
| $(k_{cat}/K_M \text{ ATF2-P})/\mu\text{M}^{-1} \text{ h}^{-1}$ | $5.13 \pm 0.32$       | $0.51 \pm 0.02$     |
| $(k_{cat}/K_M \text{ ATF2-PP})/\mu\text{M}^{-1} \text{ h}^{-1}$ | $2.30 \pm 0.24$       | $0.09 \pm 0.02$     |
8.4 Control to Determine Phosphocysteine Stability against Assay Buffer Conditions

Due to the potential for phosphocysteine to be unstable towards reducing buffer conditions\textsuperscript{18}, the stability of the chemically modified p38\textsubscript{α}-pCys\textsubscript{180} was verified. The same buffer conditions as described in Section 8.3 were used. 10× kinase assay buffer (0.5 mL), ATF2 storage buffer (3.7 mL), water (40 µL), ATP (0.5 mL of a 5 mM solution, pH 7.0) and p38\textsubscript{α} reaction buffer (217 µL) were combined. An aliquot (993 µL) was taken and p38\textsubscript{α}-pCys\textsubscript{180} added (6.64 µL of a 1.0 mg/mL solution in p38\textsubscript{α} reaction buffer, final concentration: 80 nM). The protein was left to incubate at room temperature for 18 h. After this time, the protein was concentrated using Vivaspin 500 (MWCO 10 000) and the concentrated sample analysed by LC-MS. Analysis showed no detectable amounts of dephosphorylation product (p38\textsubscript{α}-pCys\textsubscript{180}: calculated mass: 41 487, observed mass: 41 487, p38\textsubscript{α}-Cys\textsubscript{180}: calculated mass: 41 407).

8.5 Control to Determine Thiophosphate Stability against DTT Treatment

To p38\textsubscript{α}-pCys\textsubscript{180} (10 µL of a 0.5 mg/mL solution) in p38\textsubscript{α} reaction buffer was added DTT (10 µL of a 414 mM solution, final DTT concentration: 207 mM) in reaction buffer. The mixture was incubated at room temperature for 18 h. LC-MS analysis after this time showed \(\approx 15\%\) conversion to the dephosphorylated product (p38\textsubscript{α}-pCys\textsubscript{180}: calculated mass: 41 487, observed mass: 41 487, p38\textsubscript{α}-Cys\textsubscript{180}: calculated mass: 41 407, observed mass: 41 406).
In a similar experiment, a lower DTT concentration (final concentration: 2.75 mM) was used. LC-MS after 18 h showed no detectable dephosphorylation.

8.6 LC-MS/MS Analysis for Detection of Phosphopeptides after ATF2 Phosphorylation

8.6.1 Protein Digestion

Proteolytic digestion of phosphorylated ATF2 was done using the FASP protocol\textsuperscript{19} with 10 kDa Microcon filtration devices (Millipore). Protein sample (1–2 µg) was mixed with urea solution (200 µL of an 8 M solution in 50 mM ammonium bicarbonate buffer), loaded into the filtration devices and centrifuged (14 000 \( \times \) g, 15 min). The concentrate was reduced in the filtration device by adding DTT (100 µL of a 50 mM solution in 50 mM ammonium bicarbonate buffer) at 50 °C for 20 min, followed by centrifugation (14 000 \( \times \) g, 15 min). The sample was alkylated with iodoacetamide (100 µL of a 50 mM solution in 50 mM ammonium bicarbonate buffer) for 25 min in the dark. Excess alkylating agent was removed by centrifugation (14 000 \( \times \) g, 15 min), followed by washes of the centrifugal unit with ammonium bicarbonate buffer (3 \( \times \) 100 µL). The resulting concentrate was diluted with ammonium bicarbonate buffer (50 µL of a 50 mM solution) containing trypsin (final enzyme:protein ratio: 1:50 (w/w)). The sample was incubated overnight at 37 °C. Following overnight digestion, peptides were eluted to a fresh Eppendorf tube by centrifugation. Ammonium bicarbonate buffer (50 µL) was added to the centrifugal unit and centrifuged again to elute the remaining peptides. The digested protein was diluted (final concentration: 100 fmol/µL) with 1% formic acid for analysis. LC-MS/MS analysis of the samples was then performed according to the conditions in Section 7.3.
8.6.2 Data Analysis

The data were processed similarly as for phosphopeptide data from the p38α variants (Section 7.3). The raw data files generated were processed using MaxQuant software (Version 1.4.1.2), integrated with Andromeda search engine as described elsewhere.\textsuperscript{14,15} For identification of phosphorylated peptides, Andromeda searched peak lists against the human database (UniProt) as well as against a list of common contaminants. Trypsin was selected as specific digestion mode with maximum number of missed cleavages set at 2. Acetylation (N-term), oxidation (M) and phosphorylation (STY) were used as variable modifications and carbamidomethylation (C) as fixed modification. All spectra were manually validated.

The analysis showed that only two of the three possible phosphopeptides corresponding to Thr69 and Thr71 phosphorylation were detected: 1. mono-phosphorylation at Thr69 and 2. bis-phosphorylation at both sites. As was consistent with observations from whole protein ESI-MS, MS intensity for the mono-phosphorylated peptide was stronger than for the bis-phosphorylated one.
ORE01_RR_131124_ATF2Phospho_FASP_02 3925 ITMS; CID 218.88 1692.77 ATF2

0 50 100
0 0.2 0.4 0.6 0.8 1
300 400 500 600 700 800 900 1000 1100 1200 1300 1400 1500
m/z

% Relative Abundance

Intensity [10^6]

y₁₄²⁺ y₁₂ y₁₁ y₁₀ y₉ y₈ y₇ y₆ y₅ y₄ y₃
b₄ b₅
b₆ b₇ b₈ b₉ b₁₀ b₁₁ b₁₂ b₁₃ b₁₄

ATF2-pThr69Thr71:
| Raw file Scan Method Score Mass Gene names |
|-------------------------------------------|
| ORE01_RR_131124_ATF2Phospho_FASP_02 5300 ITMS; CID 176.22 2160.98 ATF2 |

![Peptide sequence and mass spectrum](image-url)
9 Electrophoretic Radioassay

9.1 Enzymatic Reaction

Kinase assay buffer stock (5.00 µL of a 10× stock containing 200 mM Tris pH 7.5, 20 mM DTT, 50 mM β-glycerophosphate, 2.0 mM Na$_3$VO$_4$, 100 mM MgCl$_2$, 50 mM NaF) was diluted with water (appropriate quantity for a final reaction volume of 75 µL) and ATP (7.5 µL of a 5 mM solution in water, pH 7.0) added. Kinase (modified or unmodified mutant, 15.0 µL of a 20 µM solution) in p38α reaction buffer and subsequently [$\gamma$-$^{32}$P]-ATP (8–13 µCi in 0.75–3 µL of solution) were added. A sample (3.34 µL) corresponding to t = 0 was taken before initiating the enzymatic reaction with the addition of ATF2 (12.06 µL of a 18.9 µM solution) in ATF2 storage buffer (20 mM HEPES pH 7.5, 0.5 mM EGTA, 1 mM DTT, 5 µg/mL aprotinin, 10 mg/mL leupeptin, 0.25 mg/mL AEBSF, 0.03% Brij-35, 150 mM NaCl, trehalose 10%), pre-diluted with water (11.13 µL). Samples of further timepoints (2, 5, 10, 18, 30, 45, 60, 75, 90, 105, 120 and 150 min) were taken and the samples quenched with 1.5× SDS/EDTA loading dye (10 µL) for SDS-PAGE analysis. A similar reaction was run with p38α-pThrTyr but with a different final kinase concentration (80 nM). The final concentrations of all the reaction components:

| Component                      | Final Concentration |
|--------------------------------|---------------------|
| p38α kinase                    | 4.0 µM              |
| ATP                            | 500 µM              |
| [$\gamma$-$^{32}$P]-ATP        | 120–170 µCi/mL      |
| kinase assay buffer:           |                     |
| Tris                           | 20 mM               |
| DTT                            | 2 mM                |
| β-glycerophosphate             | 5 mM                |
| Na$_3$VO$_4$                   | 0.2 mM              |
| MgCl$_2$                       | 10 mM               |
| NaF                            | 5 mM                |
| ATF2                           | 3.0 µM              |

9.2 SDS-PAGE analysis

Gels were hand-cast using an appropriate casing block for the PROTEAN II xi Multi-Cell gel tank (Biorad). 12% gels were made from pouring the solution (for 6 gels):

| Stock Solution                                                                 | Quantity |
|-------------------------------------------------------------------------------|----------|
| 30% acrylamide (37.5:1 acrylamide:bisacrylamide)                              | 160 mL   |
| 1.0 M Tris pH 8.8                                                              | 150 mL   |
| water                                                                         | 82 mL    |
| 10% SDS                                                                       | 4 mL     |
| 10% APS                                                                       | 4 mL     |
| TEMED                                                                         | 160 µL   |

The gels were topped with sat. butanol after pouring. Once the gels were set, 5% stacking gels were poured on top from the solution.
### Stock Solution

| Stock Solution       | Quantity |
|----------------------|----------|
| 30% acrylamide       | 17 mL    |
| 1.0 M Tris pH 6.8    | 12.5 mL  |
| water                | 68 mL    |
| 10% SDS              | 1 mL     |
| 10% APS              | 1 mL     |
| TEMED                | 100 µL   |
| bromophenol blue     |          |

Samples (10 µL) were loaded in alternate lanes on the gel with 1× blank SDS/EDTA loading dye (10 µL) (12.5 mM Tris pH 6.8, 30 mM EDTA, 12.5% glycerol 2.5% SDS, 0.1% bromophenol blue) in the otherwise unloaded lanes. The gels were run in Tris/glycine running buffer (25 mM Tris base, 25 mM glycine, 0.1% SDS) for 1 h at 600 V, 1000 mA, 250 W.

### 9.3 Gel Image Acquisition

The dye front of the gels were cut off and the gels washed in EDTA solution (≈100 mM) before mounting between acetates and exposing to a phosphoscreen for 1.5 h.

To allow for quantification, a dilution series of the ATP (both ATP and [γ-32P]-ATP) as used in the reaction was also made. The dilution series was spotted on Whatman paper No. 1 and exposed to the phosphoscreen at the same time as the gels. Finally, the screens were scanned using a Storm Phospho-imager. The gels from all the variants showed timecourses of no conversion, except for the one with p38α-pCys180 in (f). The gels are displayed as triplicates. The remaining bright spots in the other gels are positive controls (except for in (e) where the positive control was (f)).

(a) Kinetic assay with p38α-Cys172. The only spot visible is a positive control where only one positive sample was run per triplicate.

(b) Kinetic assay with p38α-Dha172. The only spot visible is a positive control where only one positive sample was run per triplicate.
(c) Kinetic assay with p38α-pCys172. The only spot visible is a positive control where only one positive sample was run per triplicate.

(d) Kinetic assay with p38α-Cys180. The only spot visible is a positive control where only one positive sample was run per triplicate.

(e) Kinetic assay with p38α-Dha180. Gel (f) was run concurrently with this one and acts as the positive control.

(f) Kinetic assay with p38α-pCys180.
p38α-pThrpTyr Control:

9.4 Data Analysis and Quantification

Gel densitometry was done using ImageQuant (GE Healthcare). The data were output into the appropriate programs (Excel or MATLAB) for further mathematical and statistical analysis.

10 Determination of IC_{50}

10.1 Enzymatic Reaction

Stock solutions (2.0 mM) of all inhibitors were made in DMSO. The stock was serially diluted with water (twice 10× dilution) to give a solution in 1% DMSO (20 µM), diluted to the first working concentration (8 µM) with 1% DMSO before a 2× serial dilution series was made with 1% DMSO (11–13 dilutions made).

For enough samples corresponding to 10 IC_{50} curves, a master mix of 10× kinase assay buffer (75.0 µL), water (28.5 µL) and ATF2 (kinase substrate) (59.6 µL of a 1 mg/mL in ATF2 storage buffer) and kinase (either p38α-Cys180 and p38α-pThrTyr, 45 µL of a 400 nM solution) kinase in p38α reaction buffer was made. An ATP mixture of cold ATP (75.0 µL of a 2.5 mM solution in water) and [γ-32P]-ATP (5.63 µL of a 11.6 mCi/mL solution) was made separately.

Test samples were made by adding master mix (4.01 µL) to inhibitor solution (1.875 µL) and incubated for 30 min at room temperature before initiation of the reaction by addition of ATP mixture (1.50 µL). Samples were then incubated at 19 °C for 30 min for p38α-pThrTyr, or for 24 h for p38α-Cys180. After this time, the reactions were quenched using SDS/EDTA loading dye, the samples loaded onto 12% gels for SDS-PAGE analysis and the gels imaged by autoradiography as previously described (Section 9). Final reaction concentrations were also the same as previously described, except for kinase concentration (80 nM final concentration).

10.2 Data Analysis and Quantification

Gel densitometry was performed using ImageQuant (GE Healthcare) as described above. The data from the gel as determined from ImageQuant was input into MATLAB for fitting to the 4 parameter logistic function.
IC₅₀:

| Inhibitor | p₃⁸α-pThrPyr/nM | p₃⁸α-pCys180/nM |
|-----------|-----------------|-----------------|
| VX745     | 136 ± 5         | 99 ± 8          |
| TAK715    | 1264 ± 24       | 1091 ± 64       |
| BIRB796   | 1309 ± 40       | 1128 ± 68       |
| JX401     | 5 ± 2           | 11 ± 2          |
| SB202190  | 13 ± 1          | 14 ± 1          |
| 1         | 78 ± 2          | 68 ± 6          |
| 2         | 586 ± 9         | 558 ± 24        |

Hill Coefficient:

| Inhibitor | p₃⁸α-pThrPyr | p₃⁸α-pCys180 |
|-----------|--------------|--------------|
| VX745     | 2.6 ± 0.1    | 1.8 ± 0.1    |
| TAK715    | 4.8 ± 0.4    | 3.8 ± 0.7    |
| BIRB796   | 9.9 ± 1.1    | 14.0 ± 5.6   |
| JX401     | 0.9 ± 0.04   | 2.0 ± 0.1    |
| SB202190  | 1.4 ± 0.1    | 1.5 ± 0.3    |
| 1         | 2.1 ± 0.2    | 2.0 ± 0.1    |
| 2         | 9.3 ± 0.5    | 12.6 ± 6.2   |
11 Protein Expression and Purification of MEK1 Variant

11.1 Cloning of pET28a-MEK1-S222C/C277S/C376S plasmid

**Transformation:** Synthetic pET28a-MEK1-S222C/C277S/C376S plasmid (Genscript, 1.00 µL, 200 ng/µL) was transformed into NovaBlue *E. coli* chemically competent cells (50 µL) by heat shock. The DNA was incubated with the thawed cells on ice for 5 min before heat shock was performed at 42 °C for 30 s. After incubating on ice for a further 2 min, the cells were fed with SOC medium and incubated in a shaker at 37 °C, 250 rpm for 30 min.

**Bacterial Culturing:** The cells were plated onto solid LB agar medium containing kanamycin (50 µg/mL) at three different concentrations (200 µL, 50 µL and 20 µL + 20 µL SOC medium) and the plates were incubated at 37 °C for 17 h. The plate with the lowest cell concentration gave distinct colonies while the other plates giving bacterial lawns. 3 colonies were selected and cultured separately in liquid LB medium (10 mL) containing kanamycin (50 µg/mL) in a shaking incubator at 37 °C, 250 rpm for 16 h.

**DNA Purification and Storage:** After glycerol stocks were made, the remaining cells were then pelleted by centrifugation (3000 rpm, 20 min, 4 °C). The resulting cell pellet was resuspended in P1 buffer and the plasmid DNA purified out using the QIAprep® Miniprep kit. The plasmid DNA was eluted in EB buffer and stored at −20 °C. DNA sequencing confirmed that the sequence of the gene was intact: plasmid concentration 118 – 127 ng/µL.
Sequence of pET28a-MEK1-S222C/C277S/C376S:

1 ATG GCC AGC AGC CAT CAT CAT CAT CAT CAC AGC AGC GGC CTG GTG
46 CCG CGC GGC AGC CAT ATG CCG AAA AAA AAA CCG ACC CCG ATT CAA
91 CTG AAC CGC GCT CGT GAT GGC TCC GTT GGT AAT GGC ACC TCA AGC
136 GCA GAA AGC AAC CGC GAA CTG GAC AAA AAA CTT GAA GAA CTG
181 GAA CGT GAT GAA CAG CAA AAA GCG CTC GAA GCC CTT CTG ACC
226 CAG AAA CAA AAA CTT GGG GAA CTG AAA GAT GAC TAT TCT GAA AAA
271 ATC AGT GAA CGT GGC GCC GGT AAC GGC GTG GTT GTT TTT AAA GTC
316 AGC CAT AAA CCG TCT GGT CTG GTG ATG GCA CGT AAA CTG ATT CAC
361 CTG GAA ATC AAA CCG GCT ATT CAG ATT ATC GCG GAA CTG
406 CAA GTG CTG CAT GAA TGC AAT TCT CCG TAT ATT GTT GGG TTT TAT
451 GGT GCG TTT TAC AGT GAC GCC GAA ATT TCC ATC TGT ATG GAA CAC
496 ATG GAC GCC GTT AGC CTG GAT CAG GGT CTG AAA AAA GCA GGC GTG
541 ATC CCG GAA CAA ATT CTG GGT AAA GTC TCT ATT GCT GTG ATC AAA
586 GGC CTG AGC ATC CTG CAT AAA AAA CAT CTG ATC CAC CGC GAT
631 GTG AAA CCG TCA AAC ATC CTG GGT AAA AAA AAA GCA GGC CGT
676 CGT TGC GCC TTT GGC GCC GGT ACC CGT AGT TAT ATG TCC CCG GAA CGC CTG
721 AAC TCT TTT GTC GGC ACC CGT AGT ATG TAT ATG CCG GCC GAA CGC CTG
766 CAG GGT AGC CAT TAC TCA GTG CAA TCG GAT ATC TGG TCA ATG GGC
811 CTG TCG CTT GAT GAA ATG GCC GTT GGT GTC ATT CCG ACC CGC GAT
856 CGG GCC CGC AAA GAA CGG CAT GAA CTG AGT ATT TTT GCC TCT CAG GGT GAA
901 GGT GAT GCG GCC GAA ACC CGG CCG GTC ACC CGG GTG CTG
946 CCG CTG AGC TCT GGC GAT ATG AGC GTG CGT CCG ATG ATC GCA ATC
991 TTC GAA CTG CTG GAT TAC ATT GTG AAT GAA CCG CCG CCA AAA CTG
1036 CCG AGC GCC GTG GTC TTT AGC CTG GAA TTT CAG GAC TCC GTG AAC AAA
1081 TGT CTG ATC AAA AAT CCG GCA GAA CGT GCT CAT CTG AAA AAA CTG
1126 ATG GTC CAC GCT TTT ATT AAA CGC TCC GAC GCG GAA GGA GTG GAT
1171 TTC GCC GGT TGC CTG CTG TCT TCG ACC ATG GGC CTG AAT CAA CCG TCA
1216 ACG CCG ACC CAC GCT GCT GGT GTG

11.2 Mutagenesis of pET28a-MEK1-S222C/C277S/C376S Plasmid

Due to N-terminal gluconoylation\(^1\) of MEK1-S222C/C277S/C376S, a G(-19)F mutation in the His\(_6\)-tag was made. A procedure as described for the mutation of p38\(\alpha\) (Section 4.2) was used, along with the primers:

- G(-19)F forward: 5'-GAAGGAGATATACCATGTTTACCCATCATCATC-3'
- G(-19)F reverse: 5'-GATGATGATGATGGCTGAACATGGTATCTC-3'
Sequence of pET28a-MEK1-G(-19)F/S222C/C277S/C376S:

```
1  ATG  TTC  AGC  AGC  CAT  CAT  CAT  CAT  CAT  CAT  CAT  CAT  CAT  AGC  AGC  GGC  CTG  GTG
46 CCG  CGC  GGC  AGC  CAT  ATG  CCG  GAT  GGC  TCC  GCT  GTC  GAT  GAC  GGT  ACC  TCA  AGC
91  CGA  GAA  AGC  AAC  ATC  GAA  GGC  CTG  GAG  AAA  AAA  CTT  GAA  GAA  CTG
136 GAA  CTG  GAT  GAA  CAG  CAA  CTG  AAA  CGC  CAG  GAA  ACC  TTT  CTG  ACC
181  CAG  AAA  CAA  AAA  GTG  GAC  GGC  GGT  AGC  CTG  AAA  AAA  AAA  CCG  ACC  TCT  GAA
226  CCG  CGA  GCA  AGC  TGG  GAA  CAG  GAC  GGC  GGT  ACC  AAA  GCT  TTT  TTA  ATT  CAC
271  GAA  ATT  TGC  ATC  AAA  CCG  TCT  GGT  CTG  GTG  ATG  GCA  CGT  AAA  CTG  ATT  CAC
316  CTT  GAA  GAT  GAA  CAG  CAA  AAA  CTG  GAA  GAA  CTG  GAA  CTG  GAT  GAA  CAG  CAC
361  GCA  GAA  ACG  AAC  CTG  GAA  GCG  CTG  CAG  AAA  AAA  CTG  GAA  GAA  CTG  GAT  GAC
406  CAG  AAA  CAA  AAA  GTG  GGC  GAA  CTG  AAA  GAT  GAC  GAT  TTC  GAA  AAA  GCT  TTT
451  ATT  GAC  GGC  GGT  AGC  CTG  GAT  CAG  GTC  ATG  GCA  CGT  AAA  CTG  ATT  CAC
541  ATC  CCG  GAA  CAA  ATT  CTG  GGT  AAA  ATT  GTC  ATT  CGT  AGT  AAC  TTT  GGC  GCA
586  CTT  GGC  AGC  TTT  GCC  AGC  GGT  CTG  GAG  AAA  ATT  CTG  GGT  ATT  TCA  ATT  GGC
631  GTC  TGC  ATC  AAC  ATC  GAA  GGT  GAT  GAC  GGC  GGT  AGC  CTG  AAA  AAA  GCA  GGC
676  CTG  TGG  ATC  AAA  CCG  TCT  GGT  CTG  ATG  GCA  CGT  AAA  ATC  ATG  CAC  CGC  GAT
721  CTG  AAA  CAA  ATT  CTG  GGT  AAA  GTC  TCT  ATT  GCT  GAA  CTG  GAA  ATC  AAA  ATC
766  CTG  TGC  GAC  TTT  GGC  GTT  AGC  GGT  CAG  CTG  ATT  GAT  AGT  ATG  GCG  GGT  GCG
811  GGT  GAT  GCG  GCC  AAA  GAA  CTG  GAA  GAA  CTG  GAA  CTG  GAT  GAA  CAG  TCT  AGC
856  CAG  GGT  GCC  AAA  GAA  CTG  GAA  GAA  CTG  GAA  GAA  CTG  GAA  CTG  GAT  GAA  CAG
901  GTT  GGT  CGT  TCT  ATC  ATT  TCT  ATT  GTC  ATT  TCG  CGC  GGT  GAA  ATT  AAA  GAT
946  CTG  TTT  GCC  AAG  TCA  ATT  TCA  ATT  GGC  GTT  AAA  GAT  GGC  CAG  TCC  GCC  TTA
991  ATC  ATT  ATC  AAC  ATC  TCA  ATT  TAT  TCC  ATT  TCC  ATT  TCC  ATT  TCC  ATT  TCC
1036  TCC  AAG  ATT  TAT  TCC  ATT  TCC  ATT  TCC  ATT  TCC  ATT  TCC  ATT  TCC  ATT  TCC
1081  ATT  TCC  ATT  TCC  ATT  TCC  ATT  TCC  ATT  TCC  ATT  TCC  ATT  TCC  ATT  TCC  ATT
1126  TCC  ATT  TCC  ATT  TCC  ATT  TCC  ATT  TCC  ATT  TCC  ATT  TCC  ATT  TCC  ATT  TCC
1171  TCC  ATT  TCC  ATT  TCC  ATT  TCC  ATT  TCC  ATT  TCC  ATT  TCC  ATT  TCC  ATT  TCC
1216  TCC  ATT  TCC  ATT  TCC  ATT  TCC  ATT  TCC  ATT  TCC  ATT  TCC  ATT  TCC  ATT  TCC
```

11.3  Protein Expression of MEK1-Cys222

**Transformation:** Plasmid encoding the mutant MEK1-G(-19)F/S222C/C277S/C376S (referred to as MEK1-Cys222) were transformed into BL21(DE3) *E. coli* chemically competent cells by heat shock. BL21(DE3) *E. coli* competent cells (25 µL) were thawed on ice and plasmid DNA (1 µL) added. The mixture was incubated on ice for 5 min before heat shock was performed at 42 °C for 30 s and immediately returning on ice. After a further incubation period of 2 min, the cells were fed with SOC medium (125 µL) before incubation with shaking at 37 °C, 250 rpm for 1 h.

**Bacterial Culturing:** The freshly transformed cells (20 µL) were streaked onto LB/agar plates supplemented with kanamycin (50 µg/mL final concentration). 3 colonies were selected and cultured further in liquid LB medium (3×10 mL), again supplemented with kanamycin to give starter cultures.

**Protein Expression:** One of the starter cultures was further cultured. 2YT medium (5×800 mL), again supplemented with kanamycin was inoculated with the starter culture (5×1.6 mL). These new cultures were grown to OD<sub>600</sub> ≈ 0.1 in an incubator with shaking at 37 °C, 180 rpm before the temperature was turned to 20 °C. The cultures were grown further to OD<sub>600</sub> = 0.6–0.7. Protein expression was then induced by the addition of IPTG (1 mM final concentration) and incubation continued at the same conditions for 17 h. The cells were finally harvested by centrifugation (8000 rpm, JA10 rotor, 5×10 min, 4 °C), the pellets placed into bags, flash frozen in liquid nitrogen and stored at −80°C.
11.4 Protein Purification of MEK1-Cys222

**Cell Lysis:** The cell pellets (30 g) were combined, thawed on ice and re-suspended (100 mL) in ice cold Ni²⁺ affinity lysis buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 20 mM imidazole, 5 % glycerol, 0.5 mM TCEP) with stirring at 4 °C. Lysozyme (120 mg) was added and the mixture stirred for a further 3–4 h before sonication using a sonicator equipped with a microtip (20 × 3 s blasts, 60 % amplitude, 2770 J total energy). DNase (1 mg) was added and the mixture stirred for a further 1 h before the lysate was clarified by centrifugation (20 000 rpm, JA25.50 rotor, 40 min, 4 °C). The lysate was further clarified by filtration using 0.2 µm syringe filters.

**Ni²⁺ Affinity Chromatography:** Using an ÄKTA Purifier FPLC system, the clarified lysate was loaded (110 mL) onto a pre-packed 5 mL HisTrap™ HP Ni²⁺ affinity column, chasing through (17.5 mL, 3.5 CV) with more lysis buffer. The column was washed (50 mL, 10 CV) with Ni²⁺ affinity wash buffer (same as lysis buffer but with 50 mM imidazole) and the bound protein eluted using a linear gradient (100 mL, 20 CV, 5 mL fractions) from wash buffer to Ni²⁺ affinity elution buffer (same as lysis buffer but with 500 mM imidazole). The fractions containing the desired protein were determined from the UV trace of the FPLC report file and from SDS-PAGE analysis, and collected.

**Size Exclusion Chromatography and Storage:** The collected fractions were concentrated by Vivaspin (MWCO 10 000) to a final volume ≈2 mL before filtering. The concentrated proteins were loaded onto a HiLoad™ 16/60 Superdex 200 gel filtration column and filtered (180 mL, 1.5 CV, 1.2 mL fractions) into MEK1 storage buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 5 % glycerol, 0.5 mM TCEP). The fractions containing the desired protein were determined from the UV trace of the FPLC report file and their purity was determined from SDS-PAGE analysis. The pure and impure fractions of the desired protein were collected separately, re-concentrated by Vivaspin (MWCO 10 000, 3.1 mg/mL final concentration), divided into aliquots, flash frozen in liquid nitrogen and stored at −80 °C: Yield: 12 mg in MEK1 storage buffer.
Protein sequence obtained by translation of the corresponding gene sequence:

tag MFSSHHHHHH SSGLVPRGSH
  1 MPKKKPTPIQ LNAPADGSAV NGTSSAETNL EALQKKEEL ELDEQQRKRL
  51 EALTIQKQKV GELKDDEFEK ISELGANGG VVFVSHKPS GLVMARKLIH
  101 LEIKPAIRNQ IIRELQVLHE CNSPYIVGFY GAFYSDEGIS ICMEHMDGGS
  151 LDQVLKAKGR IPEQILGKVS IAVIKGLTYL REKHIMHRD VKPSNILVNS
  201 RGEIKLCDFG VSGQLIDSMAS NGFGTRSYM SPERLGTHY SVQSDIWSMG
  251 LSLVEAVAGR YPIPPPDAKE LEILFGSQVE GDAETPPRP RTPGRPLSSY
  301 GMDSRPMAH FELLYIVNE PPPKLPSGVF SLEFDQDFVNK CLIKNPAERA
  351 DLKQLMVHAF IKRSDAEVEVD FAGWLSSTIG LNQPSTPHTA AGV
12 Chemical Modification of MEK1-Cys222

12.1 Synthesis of MEK1-Dha222

MEK1-Cys222 in MEK1 storage buffer (50 mM HEPES pH 8.0, 150 mM NaCl, 5 % glycerol, 0.5 mM TCEP) was thawed on ice and the buffer exchanged to MEK1 reaction buffer (same as MEK1 storage buffer but with no TCEP) using a G-25 SpinTrap™ desalting column (GE Healthcare). The concentration of the protein was adjusted with reaction buffer. To the diluted, desalted MEK1-Cys222 (310 µL of a 1.83 mg/mL solution, 12.4 nmol, 1 equiv.) was added glycerol (56.8 µL of a 60% solution in water) and ATP (56.8 µL of a 100 mM solution in water, pH 7.0) and the mixture incubated at room temperature for 30 min. Dibromide (144 µL of a 3.9 mg/mL suspension in reaction buffer, 1.9 µmol, 150 equiv.) was then added and the mixture incubated with shaking at 37 °C, 600 rpm for 8 h. LC-MS after this time showed >85% conversion to the Dha product. The reaction mixture was desalted again using G-25 MiniTrap™ desalting column (GE Healthcare), followed by repeated concentration/dilution by Vivaspin (MWCO 10 000) to give MEK1-Dha222 which was either kept on ice for immediate use or flash frozen in aliquots and stored at −80 °C. LC-MS/MS analysis of a sample prepared using the method as described in Section 8.6 showed that Dha had been installed at the desired site: (calculated mass: 45 642, observed mass: 45 646).
ORE01_RR_131220_MEK1_Dha_FASP_02

Scan Method Score Mass Gene names
5763 ITMS; CID 97.15 2355.09 MAP2K1

C* denotes Carbamidomethyl modification; C† denotes Dehydroalanine (Dha) modification
To demonstrate that the installed Dha was reactive, MEK1-Dha222 was treated with β-mercaptoethanol. To an aliquot of MEK1-Dha222 (50 µL of a 1.0 mg/mL solution, 1.1 nmol, 1 equiv.) in MEK1 reaction buffer was added β-mercaptoethanol (1 µL of a 10% v/v solution in MEK1 reaction buffer, 1.4 µmol, 1260 equiv.). The mixture was incubated at room temperature for 1 h. LC-MS after this time showed complete conversion to MEK1-ethanolycysteine222. LC-MS/MS analysis of a sample prepared using the method as described in Section 7.3 showed that the adduct was installed in the desired position: (calculated mass: 45 720, observed mass: 45 722)
C* denotes Carbamidomethyl modification; C† denotes β-Mercaptoethanol adduct with Dehydroalanine (Dha) modification.

| Raw file          | Scan  | Method    | Score | Mass     | Gene names |
|-------------------|-------|-----------|-------|----------|------------|
| ORE01_RR_131218_MEK_bME_01 | 3181  | FTMS; HCD | 57.48 | 2433.11  | MAP2K1     |
12.3 Synthesis of MEK1-pCys222

To MEK1-Dha222 (105 µL of a 1.04 mg/mL solution, 2.4 nmol, 1 equiv.) in MEK1 reaction buffer was added ATP (3 µL of a 500 mM solution in water, pH 7.0). Separately, sodium thiophosphate (15.67 mg) was dissolved in water (12.97 µL) and HCl (6.81 µL of a 5 M solution) added to adjust the pH to 8.0. The thiophosphate solution (4 × 4.06 µL of a 530 mg/mL suspension, 4 × 12 µmol, 4 × 5000 equiv.) was added batchwise (5 min intervals) to the protein and the resultant mixture incubated at room temperature for 18 h. LC-MS after this time showed >85 % conversion to the thiophosphate adduct. The reaction mixture was desalted by dialysis against MEK1 reaction buffer to give MEK1-pCys222: (calculated mass: 45 756, observed mass: 45 759).

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