Robust production of recombinant phosphoproteins using cell-free protein synthesis

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Understanding the functional and structural consequences of site-specific protein phosphorylation has remained limited by our inability to produce phosphoproteins at high yields. Here we address this limitation by developing a cell-free protein synthesis (CFPS) platform that employs crude extracts from a genomically recoded strain of Escherichia coli for site-specific, co-translational incorporation of phosphoserine into proteins. We apply this system to the robust production of up to milligram quantities of human MEK1 kinase. Then, we recapitulate a physiological signalling cascade in vitro to evaluate the contributions of site-specific phosphorylation of mono- and doubly phosphorylated forms on MEK1 activity. We discover that only one phosphorylation event is necessary and sufficient for MEK1 activity. Our work sets the stage for using CFPS as a rapid high-throughput technology platform for direct expression of programmable phosphoproteins containing multiple phosphorylated residues. This work will facilitate study of phosphorylation-dependent structure–function relationships, kinase signalling networks and kinase inhibitor drugs.

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phosphorylation, the reversible attachment of phosphate groups to proteins, is one of the most important post-translational modifications (PTMs) in nature, serving as a mechanism for enormous diversification in the function and molecular recognition of proteins. However, understanding the role of site-specific phosphorylation events remains a significant challenge due to technological limitations. Specifically, the inability to produce phosphoproteins with defined phosphorylation status at high purity and yield has restricted our capacity to elucidate the phosphorylation 'code' within the human phosphoproteome, probe complex signalling networks and develop novel phosphoprotein-based therapeutics. Moreover, most phosphoproteins isolated from cells and tissues are complex heterogeneous mixtures of different chemical structures owing to their mode of biosynthesis, subcellular distribution and diversity of PTMs. Furthermore, extensive crosstalk between kinase and phosphatase networks in vivo expands the spatio-temporal diversity of PTMs within the cell, confounding genetic phosphatase networks.

Notably, the inability to produce phosphoproteins with defined phosphorylation status at high purity and yield has restricted our capacity to elucidate the phosphorylation 'code' within the human phosphoproteome, probe complex signalling networks and develop novel phosphoprotein-based therapeutics. Moreover, most phosphoproteins isolated from cells and tissues are complex heterogeneous mixtures of different chemical structures owing to their mode of biosynthesis, subcellular distribution and diversity of PTMs. Furthermore, extensive crosstalk between kinase and phosphatase networks in vivo expands the spatio-temporal diversity of PTMs within the cell, confounding genetic phosphatase networks.

Various methods have been developed to manufacture and study phosphorylated proteins. State-of-the-art approaches that mimic phosphorylation by replacing a target site with an aspartate/glutamate residue or chemical analogue are powerful, yet are limited by their inability to match the charge density of the phosphate group. Alternatively, kinases known to phosphorylate a target site of interest have been employed in vitro. However, in vitro kinase phosphorylation often lacks site specificity. In a different strategy, semisynthetic approaches can use native chemical ligation, which has proven useful but is limited to sites near the protein termini. More recently, pioneering efforts have expanded the genetic code of Escherichia coli for site-specific incorporation of L-phosphoserine (Sep) into proteins using amber suppression via incorporation of amino acids Q, Y, K and G.

Results

Cell-free phosphoprotein synthesis of sfGFP. Our goal was to develop a CFPS platform capable of rapidly synthesizing an active human kinase. To accomplish this, we first optimized conditions that enabled site-specific incorporation of Sep into the superfolder GFP (sfGFP) reporter protein at a single in-frame amber codon at position 2 (sfGFP-S2TAG). Notably, the sfGFP gene sequence also encoded a C-terminal Strep-tag to enable purification and proteomics analysis. To assess phosphoprotein production in CFPS, 15 μl scale batch reactions were conducted for 20 h at 30 °C using the PANOx-SP system. Combined transcription and translation in the CFPS reaction were driven by either the wild-type sfGFP or the sfGFP-S2TAG DNA template. Negative controls lacking DNA templates were also conducted. Using this approach, we synthesized 685 ± 48 μg/ml−1 of wild-type sfGFP and 567 ± 37 μg/ml−1 of sfGFP-S2TAG in batch CFPS reactions that used extracts containing the overexpressed Sep-OTS (Fig. 1c, Table 1; mean ± s.d.; n = 4).

Affinity-purified sfGFP was analysed by SDS–polyacrylamide gel electrophoresis (PAGE) to confirm production of full-length protein (Supplementary Fig. 1). We then digested the affinity-purified sfGFP with trypsin and confirmed incorporation of Sep at sfGFP-S2TAG by mass spectrometry (MS) using label-free shotgun proteomics. A representative tandem MS spectrum from a N-terminal tryptic peptide of sfGFP reporting incorporation of Sep at position S2 of sfGFP-S2TAG is provided in Fig. 1d. To assess the purity of modified sfGFP, we performed label-free quantitation using two strategies: spectral counting and MS1 intensity-based quantitation (Supplementary Tables 1–3). Relative intensities of peptides were added/synthesized and maintained at precise concentrations, circumventing limitations in cellular uptake of non-standard amino acids (nsAAs). Furthermore, cell-free systems bypass limits imposed by the fitness of the organism. This enables the use of cytotoxic nsAAs, the production of cytotoxic proteins and removes constraints arising from toxic orthogonal translation system (OTS) components.

Recent works highlight the ability of CFPS to synthesize complex human proteins in high throughput, even some harbouring site-specifically incorporated nsAAs, which is the focus of this work.

We developed a CFPS system that can substantially improve throughput and yields of phosphoprotein synthesis (Fig. 1a). This approach comprised three important features. First, highly active S30 crude extracts were generated from a genomically recoded release factor 1-deficient E. coli strain (E. coli C321.DAA) that also lacks the Sep-specific phosphatase SerB. Second, an improved Sep-OTS was expressed during the growth of the recoded E. coli strain to enrich the lysate with the OTS components (that is, phosphoseryl-tRNA synthetase, tRNA-Sep and EF-Sep) necessary for phosphoprotein synthesis (Fig. 1b). The Sep-OTS was improved from previous efforts by combining the OTS components onto one vector, and by increasing the tRNA-Sep gene copy number from 1 to 5 (ref. 12). Third, these engineered components were integrated into a CFPS platform that has been previously shown to mimic the E. coli cytoplasmic environment. We used this platform to activate long-lived and efficient cell-free phosphoprotein synthesis.

Prior studies have suggested that the efficiency of amber codon suppression may be position dependent. In other words, the position in which the nsAA is incorporated affects protein synthesis yields and incorporation efficiency. To study this effect, we further examined Sep incorporation in sfGFP at position E17. While sfGFP-E17 TAG yields remained high at 516 ± 9 μg/ml−1 (Table 1), efficiency of Sep incorporation as determined by MS1 intensity-based quantitation was lower, ~20% for sfGFP-E17 TAG versus ~90% for sfGFP-S2TAG (Supplementary Table 3). Similar to our results with S2, near-cognate suppression via incorporation of amino acids Q, Y, K and G.
Figure 1 | CFPS platform with an expanded genetic code for the production of phosphoproteins. (a) Schematic of the production and utilization of an S30 crude extract containing the Sep-OTS for phosphoprotein biosynthesis. The plasmid-based Sep-OTS is induced during cell growth in the presence of Sep supplemented to the culture media. Cells expressing the Sep-OTS are then collected, lysed and processed to generate S30 extracts. CFPS reactions are supplemented with nucleoside triphosphates (NTPs), amino acids, T7 RNA polymerase and template plasmid DNA to direct the transcription and translation of a desired phosphoprotein product. (b) Schematic of the Sep-OTS. tRNA<sub>sep</sub> is aminoacylated with Sep by SepRS. EF-Sep then delivers Sep-tRNA<sub>sep</sub> to the ribosome. Site-specific incorporation of Sep at UAG (amber codon) is directed via the CUA anticodon of tRNASep. (c) Time-course and endpoint analysis of sfGFP-S2TAG with sfGFP-S2TAG DNA template added (dark grey) and as a control with no template DNA added (white). Expression of wild-type sfGFP-S2S (black) in the presence of the Sep-OTS. Error bars represent s.d. from three independent samples. (d) Annotated tandem mass spectrum from sfGFP-S2TAG confirming the site-specific incorporation of Sep at position S2. Doubly charged ions and fragments that have lost ammonia are marked by + + and * respectively.

Table 1 | Comparison of CFPS titres in this study relative to previous works.

| Protein | Yield (µg ml<sup>-1</sup>) | No. of TAG | Position | % Yield of WT | Strain | Reference |
|---------|------------------|-----------|----------|--------------|--------|-----------|
| sfGFP   | 686 ± 48         | 0         | 0        | WT           | E. coli C321ΔA | This work |
| sfGFP   | 567 ± 37         | 1         | S2       | 83% of WT    | E. coli C321ΔA | This work |
| sfGFP   | 516 ± 9          | 1         | E17      | 75% of WT    | E. coli C321ΔA | This work |
| sfGFP   | 289 ± 21         | 2         | S2, E17  | 42% of WT    | E. coli C321ΔA | This work |
| MEK1    | 308 ± 20         | 0         | 0        | 100% of WT   | E. coli C321ΔA | This work |
| MEK1    | 343 ± 9          | 1         | S218     | 83% of WT    | E. coli C321ΔA | This work |
| MEK1    | 328 ± 36         | 1         | S222     | 100% of WT   | E. coli C321ΔA | This work |
| MEK1    | 269 ± 28         | 2         | S218, S222 | 3.5% of WT | EcAR7   | This work |
| GFP     | 1.1              | NA        | E17      | <4% of WT    | BL21   |           |
| MEK1    | 0.001            | NA        | S218, S222 |           |        |           |

NA, not applicable; WT, wild type.

was also observed at position 17 (Supplementary Table 1, Supplementary Fig. 4). Our results provide evidence for the positional dependence of nonsense suppression efficiency.

Since we intended to study a human kinase with two Sep residues (see below), we also studied site-specific phosphorylation into sfGFP at positions S2 and E17. As compared with wild-type sfGFP expression, yields were lower when synthesizing sfGFP-S2TAG/E17TAG (289 ± 21 µg ml<sup>-1</sup>; Table 1). Unfortunately, efforts to detect the doubly phosphorylated sfGFP by MS failed due to technical limitations. Specifically, addition of multiple phosphorylated residues into peptides reduces their ionization efficiency. In addition, we speculate that the addition of two negative charges into the reporter peptide shifts the charge state of the peptide to a singly charged peptide with an m/z ratio outside of our detection window. To verify Sep incorporation in doubly phosphorylated sfGFP and validate both mono-phosphorylated forms, we used a Phos-tag-mediated mobility shift assay that has been previously described<sup>31,32</sup> (Supplementary Fig. 5). The Phos-tag reagent binds phosphorylated proteins and causes slower migration of phosphorylated proteins during SDS–PAGE. Using the Phos-tag method, we observed distinct signature shifts for phosphoserine-containing fractions of S2TAG, E17TAG and S2TAG/E17TAG sfGFP preparations compared with the unmodified, wild-type sfGFP (Supplementary Fig. 5). Overall, Phos-tag and MS data validated a novel CFPS platform that permits biosynthesis of proteins harbouring site-specifically incorporated Sep residues.

Cell-free phosphoprotein synthesis of MEK1 kinase. We next demonstrated the utility of our method by applying the platform...
for the in vitro synthesis of highly active doubly phosphorylated human MEK1 kinase (mitogen-activated ERK activating kinase 1). In previous works, bacterial expression of soluble MEK1 in vivo has required fusion partners, such as the maltose binding protein or glutathione S-transferase23 fusion tags. Since CFPS systems have shown benefits for expressing mammalian proteins in soluble form as compared with in vivo expression platforms34, we attempted to express MEK1 without fusion partners. Wild-type MEK1 with serines at positions S218 and S222 (MEK1-SS) and doubly phosphorylated MEK1 with phosphoserines at the same positions (MEK1-S^SSP^) were produced in 20-h CFPS batch reactions at 30 °C (Fig. 2a). A unique feature of our technology for co-translational incorporation of Sep is the ability to produce monophosphorylated forms of kinases without having to add alanine mutations to suppress second site phosphorylation events. We therefore also synthesized MEK1 variants phosphorylated at either S218 (MEK1-S^SS^) or S222 (MEK1-S^SSP^) having the native activation loop amino-acid sequence to determine if each site individually could activate the kinase alone. The total MEK1 expression yields for each variant were quantified by 14C-leucine incorporation. We observed synthesis of 308 ± 19 µg ml⁻¹ MEK1-SS, 343 ± 9 µg ml⁻¹ MEK1-S^SS^, 328 ± 36 µg ml⁻¹ MEK1-SSP^ and 269 ± 28 µg ml⁻¹ of MEK1-S^SSP^ (Table 1, Fig. 2a). These volumetric yields (g l⁻¹) exceeded previously reported in cell produced MEK1 by >1,000-fold (Table 1).

While phosphoprotein production in 15 µl batch reactions provides throughput, we next set out to demonstrate the potential for scale-up, noting that several previous works have demonstrated the ability to scale CFPS to the litre scale35,36. For example, Yin et al.36 used the open reaction environment of CFPS to produce 300 µg ml⁻¹ aglycosylated trastuzumab in reactions ranging from 60 µl to 4 l. By increasing the batch reaction scale more than 10-fold to 300 µl reactions in a 24-well flat-bottom plate, we observed production of 467 ± 15 µg ml⁻¹ MEK1-S^SSP^ (Fig. 2b). The increase in productivity from our 15 µl reactions is consistent with previous reports37,38, which have noted that increasing the surface area-to-volume ratio can increase CFPS yields. We pooled together eight CFPS reactions to make 1.05 ± 0.12 mg of total MEK1-S^SSP^ as determined by radioactive incorporation (Fig. 2b). The capacity to manufacture milligram quantities of site-specifically phosphorylated proteins demonstrates that our approach is not restricted to microgram quantities for analytical purposes.

Having successfully expressed different forms of MEK1, we next characterized the protein products in several ways. First, we validated the production of full-length MEK1 by western blot analysis using an antibody specific for the C-terminal His tag (Fig. 2c; full gels available in Supplementary Fig. 6). As expected, full-length MEK1, MEK1-S^SS^, MEK1-SSP^ and MEK1-S^SSP^ were produced when the DNA template contained site-specific in-frame amber codons. Second, we confirmed MEK1-S^SSP^ production by western blot using a phosphospecific antibody for MEK1 (Supplementary Fig. 7). Third, we validated the presence of Sep using the Phos-Tag gel-shift assay, because similarly to doubly phosphorylated GFP, we were unable to detect the MEK1-S^SSP^ peptide by MS for technical reasons. The gel shifts for MEK1-S^SS^, MEK1-SSP^ and MEK1-S^SSP^ were distinct, and as noted by others39, the doubly phosphorylated MEK1 protein counter-intuitively produced a characteristic shift that was intermediate when compared with the mono-phosphorylated MEK1 variants (Fig. 2c), an observation that is also consistent with the phosphorylated sGFP (Supplementary Fig. 5). Our Phos-tag data provided clear demonstration of single- and two-site phosphorylation into MEK1. While our data suggest that the product is not completely pure (that is, there are some non-phosphorylated species), we believe that continued developments to build a more efficient Sep-OTS40,41 or removal of phosphatases from the extract will provide notable improvements in purity as seen for other OTSs. We then used our platform to elucidate sequence-function relationships for MEK1.

**Figure 2 | In vitro synthesis of phosphorylated MEK1 variants.** (a) Volumetric yields of phosphorylated MEK1 variants MEK1-5, MEK1-S^SS^, MEK1-S^SSP^ and MEK1-S^SSP^ produced in 15 µl batch reactions. (b) Time-course plot showing the biosynthesis of MEK1-S^SSP^ in 300 µl batch reactions. The inset shows a representative autoradiogram of MEK1-S^SSP^ being synthesized over time. (c) Quantitation of total MEK1 production and phosphoprotein production by western blot analysis on SDS-PAGE and Phos-tag gels. The Phos-tag western blot shows characteristic shifted bands for the phosphorylated MEK1 variants MEK1-S^SS^, MEK1-SSP^ and MEK1-S^SSP^. These bands are absent in the wild-type MEK1-SS control sample. Error bars represent s.d. from three independent samples. N = 3 for all western blot and gel analysis.

**Functional effects of site-specific phosphorylation in MEK1.** MEK1 plays an important role in cellular signal transduction through the human MAP kinase cascade responsible for driving cellular proliferation and differentiation42. Within the cascade, the doubly phosphorylated MEK1 phosphorylates the
MEK1-SS, MEK1-S PS, MEK1-SS P or MEK1-S PSP with a kinase activities, we incubated the CFPS reaction products towards ERK2 with an

We therefore examined the enzymatic activity of cell-free extracellular-signal-regulated kinases ERK1 and ERK2 (Fig. 3a).

assayed by western blot analysis with an anti-His antibody. Total ERK was left lane). Representative western blots of phosphorylated ERK (Phos-ERK) ERK2 phosphorylation was determined in the absence of MEK1 protein (far left lane). Representative western blots of phosphorylated ERK (Phos-ERK) confirm the increased activity of phosphorylated MEK1 kinase variants in reactions carried out with equal amounts of ERK substrate. Total ERK was assayed by western blot analysis with an anti-His antibody. N = 3 for all kinase assays and western blot analysis.

extracellular-signal-regulated kinases ERK1 and ERK2 (Fig. 3a).

We therefore examined the enzymatic activity of cell-free synthesized mono- and doubly phosphorylated MEK1 variants towards ERK2 with an in vitro kinase cascade assay. To evaluate kinase activities, we incubated the CFPS reaction products MEK1-SS, MEK1-S\textsuperscript{PS}, MEK1-SS\textsuperscript{P} or MEK1-S\textsuperscript{PS}P with a full-length ERK2 K54R variant with greatly reduced autophosphorylation activity. Western blot analysis with an anti-phos-ERK antibody showed that the doubly phosphorylated MEK1 could robustly phosphorylate ERK2 in vitro as expected (Fig. 3b; full gels available in Supplementary Fig. 7). Uniquely, our platform also enabled us to test the functional role of mono-phosphorylated forms on MEK1 activity. We discovered that phosphorylation at either S218 or S222 is necessary and sufficient for MEK1 kinase activity (Fig. 3b). To our knowledge, this is the first demonstration of active MEK1 synthesis by bacterial CFPS without the need for fusion proteins to enable soluble protein expression. Obitivating fusion proteins during the production of human proteins in CFPS systems as demonstrated here will provide new opportunities to study the kinase without confounding effects of the solubility protein partner. The flexibility and utility of the CFPS system is shown by the rapid, robust and scalable production of the active phosphorylated MEK1-S\textsuperscript{PS}P for biochemical and biophysical characterization.

**Discussion**

By expanding the genetic code in the CFPS environment our approach has shown the ability to rapidly produce (<24 h) and test an active human phosphoprotein in high yields. The capacity to utilize chemically synthesized linear DNA libraries\textsuperscript{14} in this platform will enable high-throughput synthesis of phosphoproteins, markedly increasing the speed and resolution at which we can define, manipulate and study phosphorylation-induced effects on protein structure and function. Indeed, our cell-free approach can measure and study the contributions of site-specific phosphorylation to understand how phosphoproteins perform their critical and versatile roles in cellular regulation. Looking forward, the CFPS technology described herein for the direct synthesis and interrogation of phosphoproteins sets the stage for new technological paradigms to (i) understand the human phosphoproteome, (ii) develop novel arrays of disease-related phosphorylated proteins and (iii) identify novel small molecule inhibitors with therapeutic potential. Improvements to the Sep-OTS for enhanced incorporation efficiency will open the way to even broader applications.

**Methods**

**Cell-free protein synthesis extract preparation**. E. coli C221 AA cells\textsuperscript{25} harbouring the Sep-OTS system were grown in 2 × YTPG media\textsuperscript{28} supplemented with 2 mM L-phosphoserine at 30°C. The SepOTS system was induced at an OD\textsubscript{600} of 0.6 with 1 mM isopropyl-β-D-thiogalactoside (IPTG) and cells were grown to a final OD\textsubscript{600} of 3.0 that represents the middle of the exponential growth phase. Cultures were then collected on ice and cells were pelleted by centrifugation for 15 min at 5,000 relative centrifugal force (RCF) and 4°C. Cell pellets were then washed three times with cold S30 buffer (10 mM Tris-acetate pH 8.2, 14 mM magnesium acetate, 60 mM potassium acetate and 1 mM diethanol). After final wash and centrifugation, the pellet were weighed, flash frozen in liquid nitrogen and stored at −80°C. To make cell extract, the thawed cells were suspended in 0.8 ml of S30 buffer per 1 g of wet cell mass and processed as reported by Kwon and Jee\textsuperscript{41}.

**Cell-free protein synthesis reactions**. CFPS reactions were performed under conditions similar to the PANOx-SP system\textsuperscript{27,43}. Batch reactions were conducted with a final reaction volume of 15 μl in 600-μl microfuge tubes. Reactions were performed either in duplicate or triplicate, with duplicate measurements for each condition tested. Reactions in 96-well plates (Corning Incorporated, Corning, NY) were performed with a final reaction volume of 30 μl. All reactions contained 30% v/v cell extract supplemented with 1.2 mM ATP, 0.85 mM each of GTP, UTP and CTP; 34.0 μg ml\textsuperscript{−1} folic acid; 170.0 μg ml\textsuperscript{−1} of E. coli tRNA mixture; 13.5 μg ml\textsuperscript{−1} plasmid; 100 μg ml\textsuperscript{−1} T7 RNA polymerase; 2 mM each of 20 standard amino acids; 0.33 mM NAD; 0.27 mM coenzyme-A (CoA); 1.5 mM spermidine; 1 mM putrescine; 4 mM sodium oxalate; 130 mM potassium glutamate; 10 mM ammonium glutamate; 12 mM magnesium glutamate; and 33 mM phosphoethanolpyruvate. Reactions were conducted for 20 h at 30°C in an incubator without shaking.

CFPS reactions were scaled to reaction volumes of 300 μl in untreated flat-bottom 24-well plates (Model 353226; BD Biosciences, San Jose, CA, USA). Eight 300-μl reactions were run in parallel for the production of milligram quantities of MEK1-S\textsuperscript{PS}P resulting in a total reaction volume of 2.4 ml. Remaining wells around the perimeter of the plate were filled with water for internal humidification, which resulted in reduced sample evaporation. Reactions in 24-well plates were conducted at 30°C while shaking at 300 r.p.m. in a ThermoMixer (Eppendorf, Mississauga, Ontario). Full details for cell growth, cell harvest, and CFPS extract preparation are provided in the Supplementary Information.

**sfGFP western blot analysis**. For western analysis of sfGFP, 100 ng of total sfGFP samples were loaded on either 4–15% acrylamide gels (Bio-Rad) or on a hardcast 10-well 12% acrylamide gel containing 25 μM Phos-tag acrylamide. Transferred PVDF membranes were blotted with 1:545 Anti-GFP (Invitrogen, Mouse Anti-GFP, Mouse Anti-GFP 33-2,600) followed by 1:10,000 DAM-HRP. Signal was detected by enhanced chemiluminescence (Bio-Rad) imaged on a ChemiDoc XRS + CCD camera. Densitometry was performed using the Bio-Rad Image Lab software.

**MEK1 western blot analysis**. Purified MEK1 protein was separated by SDS–PAGE on a 4–15% gradient polyacrylamide gel (Bio-Rad) or on a hardcast 10-well 12% acrylamide gel containing 25 μM Phos-tag acrylamide. Transferred PVDF membranes were blotted with either 1:2500 Anti-His (6x-His Epoxypeptide-TAG, PA1–983R, Thermo Fisher Scientific) for total MEK1 or 1:1,000 Anti-MEK1-S\textsuperscript{PS}P (Phospho-MEK1 1/2 (Ser217/221), 9154, Cell Signaling Technology), followed by 1:10,000 DAR-HRP.
Signal was detected by enhanced chemiluminescence (Bio-Rad) imaging on a ChemiDoc XRS + CCD camera. Densitometry was performed using the Bio-Rad Image Lab software.

**MEK1 kinase activity assay.** Kinase activity of purified full-length MEK1 (S218/222, S218/S222, S218/S222 or S218/222) was evaluated by measuring ERK2 phosphorylation. Approximately, 1.0 μM MEK1 variants were pre-incubated in kinase activity buffer (50 mM Tris-HCl (pH = 7.4), 150 mM NaCl, 1 mM diethiothreitol, 20% glycerol, 10 mM MgCl2, and 1 mM ATP) at 30 °C for 5 min, then 2.5 μM ERK2 substrate was added and the reaction was allowed to proceed at 30 °C. Aliquots of 7.5 μl were removed at 0, 0.5, 1, 2, 5 and 30 min and quenched with 7.5 μl of 2 × Laemmli sample buffer, then heated to 55 °C for 5 min. A negative control was run with only ERK2 substrate for 30 min and quenched in the same manner as kinase-containing samples. The quenched reactions were run on 4–15% acrylamide TBE-PAGE gels and transferred to a PVDF membrane. Membranes were blotted with either 1:1,000 Anti-Phospho antibody (Phospho Ser/Thr 32, 4, 7, 9, 20, 22, 23, 32, 34, 37, 44, 46, 68, 92) and/or 1:1,000 Anti-Phospho antibody (Phospho Thr 202, 204, 210). Densitometry was performed using the Bio-Rad Molecular Imaging, phosphorelay apparatus.

**Mass spectrometry and bioinformatics.** MS procedures and sample preparation details are given in the Supplementary Methods.

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**Author contributions**

M.C.J. conceived the project, supervised the research, assisted in analysing data, preparing the figures and writing the manuscript; J.O.P. and H.R.A. designed and performed the experiments, analysed the results, prepared the figures and wrote the manuscript; N.L.P., S.R., C.M.I.H., M.B.A. and K.W.B. assisted in cloning, expression, and preparation of MEK1 kinase and K54R ERK2.
performing experiments and analysing data; F.J.I. supervised the construction of the recoded strains.

Additional information
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