Large-scale Discovery of ERK2 Substrates Identifies ETV3 as an ERK-Dependent Transcriptional Regulator

Supplemental Methods

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Recombinant ERK2 substrates and *in vitro* kinase reactions

Clones were from OpenBiosystems (clone IDs ETV3: 4918332, RIPK3: 3590770, STK10: 40111177, NID1: 40110859, RBMPS: 3586451) except CDC42EP1 (OriGene catalog #MC205344) and IRS2 (Addgene plasmid 11373). CDC42EP1, ETV3, NID1, RBPMS and RIPK3 were cloned into pET-16b (Stratagene), while IRS2 and STK10 were inserted into pET-100 (Invitrogen) using directional TOPO cloning. Vectors were transformed into BL21 Star (DE3) *E.coli* (Invitrogen) and induced using 1 mM IPTG for three hours during log-phase growth of a 2 mL culture. Bacterial pellets were lysed using B-Per bacterial lysis kit with DNAase, lysozyme, and Halt protease inhibitors (Thermo Scientific). Recombinant proteins were recovered from inclusion bodies by solubilization in 8M urea, except RIPK3, which was solubilized with 0.2% sodium dodecyl sulfate in 40 mM Tris-HCl pH 7.5, 100 mM NaCl. Recombinant proteins were stored at 10 ng/uL in 20 mM Tris-HCl pH 7.5, 50 mM NaCl, 50% glycerol.

Kinase reactions used 10-30 ng of substrate, 12.5-25 ng recombinant ERK2 and ATPγS at 50-100 µM in kinase buffer (25 mM Tris-HCl pH 7.5, 5 mM β–glycerolphosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$), and incubated 30 minutes at 30^{o}C. Reactions with RIPK3 were supplemented with 100 mM thiourea to increase solubility. Half of each reaction was silver stained as a loading control, while half was treated with PNBM at 2.5 mM for two hours prior to Western blotting for thiophosphate.

Phosphorylation mapping of recombinant proteins by HPLC-MS/MS

Kinase reactions were performed for 1 hour with approximately 100 ng of each substrate and ATP at 100 µM. Matched negative control reactions omitted ATP. Each reaction was run by SDS-PAGE and proteins visualized by Coomassie stain or mass-spectrometry compatible silver stain (Thermo Scientific). Matched bands were cut and destained, and processed as described for overnight digestion in trypsin (Promega, 12.5 ng/uL in 50 mM ammonium bicarbonate pH 8.9) or chymotrypsin (Sigma-Aldrich, 125 ng/uL in 100 mM Tris 10 mM $CaCl_2$ pH 8.0 at 30^{o} C).[1] Reactions were quenched with 5% formic acid in 50% acetonitrile and peptides were eluted by dehydrating the gel band twice with 100% acetonitrile. Peptide samples were dried to about 2 uL in a vacuum centrifuge and resuspended in 30 uL 0.1% acetic acid.

Immobilized metal affinity chromatography[2] was used to enrich phosphopeptides from digests of CDC42EP1 and IRS2, while ETV3 was loaded directly onto a C18 pre-column (10 cm, 100 µm I.D.). Pre-columns were placed in-line on an HPLC connected to a C18 column with electrospray tip (10 cm, 50 µm I.D., 1 µm tip flowing at approximately 20-40 nL/min). Peptides were eluted using a piece-wise linear gradient from 0% to 70% acetonitrile in 0.2M acetic acid (4 min: 9.1%, 50 min: 29.4%, 57 min: 42%, 60 min: 70%) and analyzed on an Orbitrap XL hybrid mass spectrometer. The mass spectrometer was running in data-dependent mode where each cycle included an Orbitrap MS scan with 100,000 target resolution followed by isolation and Collision-induced Dissociation of up to six ions (charge state 2-5) for analysis in the LTQ ion trap. MS/MS spectra were extracted using DTASuperCharge (version 1.19) and identified by MASCOT (Matrix Science, version 2.1). Phosphorylated peptides were confirmed by manual inspection of each spectrum.

Immunofluorescence

Approximately 10^5 per cm^2 of DLD1 or HEK 293T cells were thoroughly dispersed and plated overnight on coverslips. 293T cells were then transfected with HA-tagged mouse ETV3 in the pBabe-IRES-EGFP vector (AddGene vector 14430) using FuGene HD (Roche) according to the manufacturer's instructions and grown for two more days. Cells were treated as described with U0126 (10 µM) and/or PMA (100 nM), fixed in 4% formaldehyde for 15 minutes and washed 3 times with PBS. Cells were blocked for one hour with 5% goat serum (DLD1) or 5% mouse serum (293T) in PBS with 0.1% Triton-X. DLD1 cells were incubated overnight at 4^{o}C with anti-ETV3 at 8 µg/mL in PBS with 1% BSA 0.1% Triton-X, then washed three times with PBS and incubated for 1 hour with anti-rabbit Alexa488 secondary antibody at 1:1000 dilution with goat anti-rabbit Alexa488 conjugate. Following antibody incubations slides were washed twice with PBS, treated with DAPI in PBS for 15 minutes, washed once with PBS, treated with phalloidin conjugated to Alexa647 (Invitrogen), washed twice with PBS, and mounted on slides using

ProLong Gold (Invitrogen). Images were captured on a DeltaVision Spectris microscope from Applied Precision and processed by software deconvolution.

ChIP-qPCR

8 x 10^6 293T cells were prepared and processed as described for ChIP-Seq experiments, except that only 10^6 cells and 5 ug of antibody were used. Samples were analyzed by real-time PCR on a Roche Lightcycler 480 system using Roche SYBR Green I PCR master mix. Primers for *ddx20* were 5' GAGGCGGAGATACGAACT TG 3' and 5' TACCACATTGGCTGGTGTGT 3', for *dusp6* 5' GCTGGAACAGGTTGTGTTGA 3' and 5' AAGTGCCCTGGT TTATGTGC 3', and for *myc* 5' CCAACAAATGCAATGGGAGT 3' and 5' CCAGAGTCCCAGGGAGAGTG 3'. Mutations in the ETV3-pBabe-IRES-EGFP vector were introduced using Stratagene QuikChange.

ERK binding site motif analysis

ScanSite 2.0 (http://scansite.mit.edu) was used to search SwissProt protein sequences for high-stringency ERK1/2 binding sequences (ScanSite motifs "Erk D-domain" and "Erk1 Binding" for D-domain and DEF-domain respectively){Obenauer, 2003}. Where an appropriate protein sequence was not available in SwissProt, protein sequences were uploaded directly from the National Center for Biotechnology Information Entrez protein database. The frequency of each motif in AS-ERK2 substrates was compared to SwissProt mouse proteome (Sept 22 2009 release) using the X^2-test.

ETV3 binding motif analysis

To identify an optimal ETV3 binding motif, the most enriched unrefined ETS family motif (ELK-1) from TRANSFAC found under the U0126-treated ChIP-Seq peaks was used as the initial hypothesis. The expected motif strength score for ELK-1 was calculated by scanning a randomly sampled set of background sequences that match the length, GC content, and distance from TSS distributions of the U0126 peaks. In this dataset, peak strength as determined by p-value positively correlates with motif strength, and the *p*-value cutoff of 1e-25 was identified as the threshold below which the U0126 peaks contain stronger motifs than the expected background. The ELK-1 motif was refined by THEME using the peaks with p-value < 1e-25 to arrive at an ETV3 motif. See THEME citation for more information on the motif refinement procedure.

Detailed Protocol for ERK2 substrate labeling and identification

In vitro labeling of AS-ERK2 substrates

1. Plate 100,000 NIH 3T3-L1 fibroblasts expressing WT-ERK2 or AS-ERK2 each in a 10-cm plate with light or heavy SILAC media respectively (Pierce/Thermo, DMEM for SILAC + 10% dialyzed FBS + L-proline 230mg/L + light/heavy L-Arg/L-Lys).
2. 72 hours later split each 10-cm plate into to 15-cm plates using the same media.
3. 72 hours later serum-starve each plate in appropriate SILAC media for 3 hours.
4. Treat the cells with EGF (100ng/mL) for 5 minutes, remove the media, wash once with 1mL cold PBS (aspirate very thoroughly), lyse each plate in 400uL kinase buffer (scrape aggressively because the detergent concentration is low, final volume should be about 600µL).
5. Use a protein assay to adjust each lysate to the same concentration and total volume.
6. Add N6-PhEt-ATPγS (50µM), GTP (1mM), and TCEP (2mM) to each lysate and rotate at 30^{o}C for 30 to 90 minutes (Western blot with the thiophosphate ester antibody to determine the kinetics of particular reaction conditions).

Sample processing

1. Split each sample into 200µL aliquots in 1.5mL Eppendorf tubes.
2. Use methanol/chloroform precipitation to remove detergents
    a. Add 640uL MeOH and 180uL $CHCl_3$ to each 200µL aliquot and vortex briefly.
    b. Add 480uL ddH_2O to each tube and vortex.
    c. Spin the tubes at 4^{o}C in a mini-centrifuge at maximum speed for 5 minutes.
    d. Carefully remove the top liquid layer.
    e. Add 300uL MeOH, vortex briefly and spin for 15 minutes at 4^{o}C.
    f. Remove the supernatant (use a gel-loading tip to get all the liquid) and let the pellet air-dry for 10-15 minutes.
    g. Add 100uL digest buffer *without disturbing the pellet*.
    h. Sonicate the pellet for 15 seconds in a water-bath sonicator.
    i. Combine all the protein from every tube (light and heavy SILAC together).
3. Add $CaCl_2$ to 1mM, TCEP to 2mM, and sequencing grade trypsin at >1:100 w/w to protein.
4. Rotate overnight and room temperature on a rotor (rotation is critical).
5. Acidify the digest with glacial acetic acid 1:6 v/v.
6. Desalt the digest on a C18 Sep-Pak (Waters) (no more than 3mg peptide per Sep-Pak)
    a. Equilibrate the Sep-Pak with 10mL 0.1% acetic acid, 10mL 90% MeCN 0.1% acetic acid, and 0.1% acetic acid. Push each wash through at 2-3mL/min.
    b. Load the digest at a flow rate *less than 1mL/min*.
    c. Wash the Sep-Pak with 10mL 0.1% acetic acid at 1ml/min.
    d. Elute peptides with 5mL 40% MeCN + 0.1% acetic acid.
7. Reduce the elution to <1mL in a vacuum centrifuge.
8. Freeze the samples at -80^{o}C, then immerse in liquid nitrogen for 10 minutes.
9. Remove the solvent by lyophilizing. Refreeze the sample if it melts during this time.

Thiophosphate capture

1. Resuspend the lyophilized peptides in binding buffer + 2mM TCEP + BSA 250µg/mL (Bio-Rad Albumin Protein Standard). Final pH must be 5.5.

2. Transfer SulfoLink bead slurry to a tube (25µL for each mg of protein in the starting lysate), centrifuge briefly at 1000 rpm, and remove the supernatant.
3. Wash SulfoLink beads in 500µL binding buffer with 5 minutes rotation *wrapped in foil*. Remove the buffer by spinning the beads 10 seconds at 1000 rpm.
   (Note: SulfoLink beads degrade rapidly upon exposure to air. Their performance in this procedure degrades noticeably within 24 hours)
4. Repeat the wash twice.
5. Combine the sample with the beads and rotate overnight at room temperature wrapped in foil.
6. Pellet the beads and remove the supernatant.
7. Wash the beads sequentially in 500µL of binding buffer (2x), 5mM DTT in binding buffer pH 8.5, binding buffer, 5% formic acid, binding buffer. Each wash except formic acid should be on a rotor. Additional washes may be added.
8. Suspend the beads in 1mL binding buffer and transfer to a 2mL glass vial.
9. Prepare a silica capillary (45cm long, i.d. 530µm) with a fritted end
    a. Prepare the frit solution by combining 80µL Kasil with 20µL formamide and vortexing 1 minute.
    b. Use capillary action to draw about 0.5cm of frit solution into the silica capillary.
    c. Heat the capillary to 50^{o}C for 10 minutes, then use a heat gun for 15 seconds to condition the frit.
    d. Carefully use a helium bomb to flow 100% MeCN over the frit for 10 seconds without causing the liquid to spray (requires extremely low pressure).
    e. Use a hand-drill to drill out one side of a 2.5cm Teflon tubing (i.d. 110µm) so it will form a tight seal with the 530µm i.d. capillary, then use a silica capillary (o.d. 360µm) to widen the rest of the connector and remove debris from the drilling. Attach the connector to the fritted end of the capillary.
10. Use the helium bomb to load beads onto the silica capillary. Do not allow the column to dry. When the solvent is exhausted add additional binding buffer and continue loading. Repeat until >95% of the beads have been loaded.
11. Wash the beads with 0.1% acetic acid for 5 minutes flowing at 200µL/min.
12. Prepare a pre-column with POROS R2 resin.
    a. The column is a silica capillary 13cm long (i.d. 100µm) packing with 10cm of POROS R2 resin. It should have 0.5cm frits on both ends and be pre-conditioned with 5pmol of a short control peptide (ex. angiotensin). Immediately before use the column must be equilibrated on an HPLC with at least one gradient from 0 to 70% MeCN in 0.2M acetic acid.
13. Connect the R2 pre-column to the SulfoLink capillary using the Teflon connector.
14. Adjust the pressure for a flow-rate of 4µL/min using 0.1% acetic acid.
15. Flow Oxone (DuPont, 2mg/mL) for 10 minutes, followed by 0.1% acetic acid for 25 minutes.
16. Remove the R2 pre-column and wash 5 minutes with 0.2M acetic acid at 150 PSI.
17. Prepare an IMAC column.
    a. IMAC column is a 13cm silica capillary (i.d. 200µm) packed with 10cm POROS IMAC resin and fritted on both ends. The column must be pre-conditioned with 10pmol of digested β-casein.
18. The IMAC column is washed and charged with Fe^{3+} as described.[3]
19. Connect the R2 column to the IMAC column and flow 70% MeCN 0.2M acetic acid at 1µL/min. Flow must not exceed this rate.
20. Wash the IMAC column as described and elute peptides to a C18 pre-column using phosphate buffer.
    a. The C18 pre-column is a 13cm silica capillary packed with 10cm of C18 beads with average diameter 10µm and fritted on both ends. The column must be pre-conditioned with 5pmol

control peptide (ex. angiotensin) and equilibrated immediately before use on an HPLC with at least one gradient from 0 to 70% MeCN in 0.2M acetic acid.

21. Wash the C18 pre-column with 0.2M acetic acid for 10 minutes at 600 PSI.

Mass Spectrometry

1. Analytical columns are packed as conditioned as described.[2] The tip is 1µm pulled with a laser-puller such that flow rate on an HPLC at 40 bar pressure is approximately 20nL/min.
2. Use a Teflon connector to connect the C18 pre-column in-line with a nano-flow HPLC and the analytical column.
3. Mass spectrometer is an OrbiTrap XL (Thermo Scientific) running in data-dependent mode.
    a. MS1 is at maximum resolution (100,000) using the Orbitrap mass analyzer, followed by up to 11 data-dependent MS/MS scans using LTQ mass analyzer (CID fragmentation at 35% activation energy, isolation window 3 m/z units, dynamic exclusion for 150 seconds).
    b. HPLC gradient is 2-hours piece-wise linear from 0 to 70% MeCN with 0.2M acetic acid (8 min: 9.1%; 100 min: 28%; 115 min: 42%; 120 min: 70%).
4. MS/MS spectra are extracted using DTASuperCharge v1.9 (http://msquant.sourceforge.net/) and searched using Mascot v2.1 (searched against the NCBI mouse proteome, parent mass tolerance 12ppm, MS/MS tolerance 0.7 Da, 1 missed cleavage, variable modifications: SILAC Arg/Lys, pS/pT, Met oxidation, Met di-oxidation).

Western Blotting

Prepare samples using the *in vitro* labeling reaction described above.

1. Heat-kill the kinase reaction at 95^{o}C for 5 minutes (denaturing SDS can also be used, absolutely no BME or DTT).
2. Prepare *p*-nitrobenzyl mesylate (PNBM): 100 mM stock in DMSO, dilute 1:2 in DMSO (to 50mM), then dilute 5 times in ddH2O to a final concentration 25mM (mix after each addition to avoid precipitating PNBM). This is a 10x reaction solution.
3. Add 10x PNBM to each sample for a final concentration 2.5mM and let the reaction incubate 2 hours at room temperature.
4. Add Western blot loading buffer and boil that samples according to standard Western blot protocol.
5. Run the Western blot using rabbit mAb against haptenylated thiophosphate (blocking and primary incubation in 5% milk with TBS 0.3% Tween). The antibody can be extremely difficult to strip so loading control may be blotted first.

Buffers

Lysis/Kinase buffer:

20 mM Hepes pH 7.5
137 mM NaCl
0.5 mM EGTA (not EDTA)
25 mM $MgCl_2$
0.2% Triton X-100
25 mM beta-glycerophosphate
10% Glycerol
2 mM TCEP (added after cell lysis to avoid interfering with protein assay)
1 mM PMSF (added immediately before lysis)
Halt Protease/Phosphatase (Pierce/Thermo, #78441)

Trypsin buffer – 100 mM ammonium acetate pH 8.9 + 1 mM $CaCl_2$

Binding buffer – 25mM HEPES 50% MeCN pH 7.0 (pH 8.5 when quenching SulfoLink beads with DTT)

Non-standard Reagents

N6-PhEt-ATPγS – Biolog (www.biolog.de, #P026, products #B072 and #F008 may work better with some AS-kinases)

RmAb for thiophosphate ester – Epitomics (www.epitomics.com, #2686-1)

p-nitrobenzyl mesylate – Epitomics (#3700-1)

SILAC media and amino acids – Pierce/Thermo (www.piercenet.com)

DMEM for SILAC - #89985

Dialyzed FBS - #89986

L-Arginine HCl - #89989

$^{13}C_6$ L-Arginine HCl – #88210

L-Lysine 2HCl – #89987

$^{13}C_6$ L-Lysine 2HCl – #89988

L-Proline - #88211

SulfoLink beads – Pierce/Thermo (#20401)

POROS 20 MC IMAC beads – Applied Biosystems (#1-5428-02)

POROS R2 beads – Applied Biosystems (#1-1118-02)

10 µm C18 beads – YMC Europe (ODS-A)

Fused silica capillary – Polymicro Technology (www.polymicro.com, TSP product line)

References

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