SUPPLEMENTARY MATERIAL

Establishing calyculin A treatment conditions

To define conditions for elevating phosphorylation, HeLa cells were treated with various doses of calyculin A for increasing amounts of time. Phosphorylation was elevated with 10-20 nM calyculin A treatment as early as 15 min (Supplementary Fig 1). To minimize potential toxicity from exposure to this potent phosphatase inhibitor, treatment with 10 nM calyculin A for 15 min was chosen to prepare samples for proteomics analysis.

SDS-PAGE and western blotting

For analysis of phospho-T and elongation factor 2 (EF2), cells were lysed in (Tris-buffered saline (TBS) with 2% SDS, 5 μM zinc acetate, 1 mM sodium orthovanadate, 2 mM β-glycerophosphate, 1 mM PMSF, EDTA-free protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), 100 nM microcystin) and boiled for 5 min. Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL). Samples (25 μg) were subjected to SDS-PAGE using either a 6% resolving gel (phospho-T blot) or a 12% resolving gel (EF2 blots), then proteins were transferred to nitrocellulose membranes (Schleicher and Schuell Bioscience). The phospho-T blot was incubated with phospho-T primary antibody (Cell Signaling Technology, Danvers, MA, 1:500) and bound antibody detected with chemiluminescence (ECL plus Western Blotting Detection System, Amersham Biosciences, Pittsburgh, PA). This phospho-T antibody exhibits some cross reactivity towards phospho-S and phospho-Y.

Antibodies for total and phospho-T56 EF2 (a gift from Dr. Angus Nairn, Yale University, School of Medicine) were rabbit antibodies, so a single blot containing duplicate lanes for each sample was prepared and cut in half for analysis in parallel. One half was incubated with phospho-T56 EF2 primary antibody (1:500), and the other half was incubated with total EF2 primary antibody (1:500). Each half was also probed using mouse monoclonal antibody for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Ambion, Austin, TX, 1:16000) as a loading control. Primary antibodies were visualized with

goat anti-rabbit antibody conjugated to IR-680 dye 1:10000) or goat anti-mouse antibody conjugated to IR-680 dye (1:10000) (Molecular Probes), and signals quantified using an Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE). Fluorescent signals for phospho- and total EF2 were normalized to their respective GAPDH signals, then the ratio of phospho-T56 EF2: total EF2 was calculated.

For analysis of MYPT1, cells were lysed in TBS with 2% SDS, 5 μM zinc acetate, 1 mM PMSF, EDTA-free protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) and phosphatase inhibitor cocktail sets 1 and 2 (EMD Biosciences, Inc. San Diego, CA)). Ten μg of each sample was subjected to SDS-PAGE using a 10% resolving gel and transferred to nitrocellulose. As for EF2, a single blot containing duplicate lanes for control and treated samples was prepared and cut in half for parallel processing. Each half was probed with either total MYPT1 (Upstate, Charlottesville, VA, 1:500 in TBST) or phospho-T696 MYPT1 (Upstate, 1:1000), then bound antibody detected with goat anti-rabbit antibody conjugated to IR-680 dye (1:5000, Molecular Probes, Eugene, OR). Each half was also probed for GAPDH. The ratio of phospho-T696 MYPT1: total MYPT1 was performed as described above for EF2.

Protein extraction, digestion and methylation

For each sample, protein was extracted using Trizol reagent (Invitrogen, Inc.) according to the manufacturer's instructions with modifications as described.[17] The final protein pellet was resolubilized in 6 M guanidine hydrochloride. Two batches of cells (from 5 nearly confluent 100 mm plates) were independently treated and harvested, followed by protein extraction. Proteins from the two independent biological samples were pooled. Protein concentration was determined using the BCA reaction and the extracts were stored at -80°C until further use. Samples were diluted 10-fold with 50 mM NH_4HCO_3 (pH 7.4) and digested with modified trypsin (Promega, Madison, WI) at a trypsin/protein ratio of 1:20 at 37 °C for 4 h, then for an additional 16 h at room temperature after addition of a second aliquot of trypsin. Proteolysis was terminated by acidifying the reaction mixture with glacial CH_3COOH. The tryptic digest

was desalted using a SPE C18 column (Supelco, Bellefonte, PA), dried under vacuum, then methylated according to the published protocol[4] with the exception that the 1 h methylation reaction was repeated a second time to ensure complete methylation.

IMAC

Methylated peptides from each sample (~1.5 mg) were resuspended in 600 µL of 0.1% $CH_3COOH/CH_3CN/CH_3OH$ (1:1:1), and loaded onto Fe^{+3} -activated IMAC MacroTrap cartridges (3 mm i.d. × 8 mm length) (Michrom, Auburn, CA) custom packed with POROS 20 MC resin (Applied Biosystems, Foster city, CA). The IMAC cartridges were washed first with 300 µL of 25/74/1 $CH_3CN/H_2O/CH_3COOH$ containing 100 mM NaCl, then with 300 µL 0.1% CH_3COOH, and were eluted with 150 µL of 50 mM Na_2HPO_4 (pH~8.5). Eluted peptides were immediately acidified with glacial CH_3COOH to pH~4.

Management of the False Discovery Rate

The scan number of the peak apex of each unique peptide identified by SEQUEST in each LC-MS/MS dataset was extracted using the MASIC program (http://ncrr.pnl.gov/software). All the LC-MS/MS datasets were then normalized by aligning them against one LC-MS/MS baseline analysis in the elution time dimension, and scan numbers were converted to values between 0 and 1 (the so-called normalized elution time (NET)). As a result, a database of peptides was formed and each identified peptide was characterized by its theoretical mass, NET, and peptide sequence.

LC-MS features were aligned against the SEQUEST results using the LCMSWARP algorithm.[16] Peak matching was then performed by matching the features against the peptide database established above in terms of mass and NET. Each match was then associated with a mass error and a NET error. The mass error was the mass difference between the LC-MS feature and the theoretical monoisotopic mass of the peptide and the NET error was the difference between the aligned NET of the LC-MS feature and the normalized scan number of the peptide.

The management of FDR was achieved via the decoy approach [20] at both the stage of building the peptide database and the filtering of the peak matching results. It has been shown recently [20] that high mass measurement accuracy information can be used with the decoy database approach to reduce the overall FDR, which was estimated as 2 × (# of reverse matches) / (# of reverse matches + # of matches to forward sequences). The high mass accuracy information used for this calculation was obtained from the LC-FTICR analysis. In addition, by using an additional tight NET error cutoff, we were able to further reduce the FDR, compared with that of the estimated FDR using mass error only.

After peak matching, the distribution of mass and NET errors of the correct matches typically showed a Gaussian-like distribution. For a 5% FDR, a 2.3 standard deviation of the mass and NET error was chosen so that all the matches which fell within this region were considered correct matches. This translated to a cutoff of 12 ppm and 0.06 in mass and NET error, respectively, in filtering the peak matching results. Supplementary Figure 2 illustrates the management of FDR using the decoy approach.

Reviewing spectra using the SpectrumLook software package

We have written a software package called SpectrumLook that allows readers to inspect the fragmentation (MS/MS) spectra for the phosphopeptides identified in this study. Using this software, readers can visually browse the MS/MS spectra that led to the phosphopeptide identifications, including viewing annotations for the identified b- and y-ions, and neutral loss ions where appropriate. This software is supported by the Microsoft Windows platform. There are six files included with the SpectrumLook package that can be accessed at http://ncrr.pnl.gov/Rossie_SpectrumLookSoftwarePackage.zip:

Note: To access the file, right click on the above link and select “Open Weblink in Browser”.

1. SpectrumLook_Installer.msi - the installer. To install, double click on the file and follow the installation prompts. During installation, a shortcut to run the SpectrumLook program is placed at Start->Programs->PAST Toolkit->SpectrumLook. Alternatively, navigate to the C:\Program Files\SpectrumLook\ folder and double-click file "SpectrumLook.exe".

2. SRData_grouped.mzXML - the phosphopeptide spectra in mzXML format. Note that this file includes both the MS/MS spectra and the preceding MS1 spectrum for each MS2 spectrum.
3. SRData_grouped_syn.txt - a summary of the identifications determined by SEQUEST. See the Readme.txt file for a description of the columns in this file.
4. SRData_grouped.ini - a parameter file that specifies the appropriate parameters for these data when browsing them with SpectrumLook.
5. Readme.txt and RevisionHistory.txt - text files that describe the SpectrumLook software.

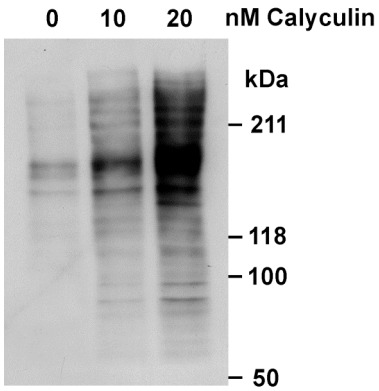
SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Elevation of protein phosphorylation in response to calyculin A treatment. Samples treated with DMSO vehicle control (0 nM), 10 nM or 20 nM calyculin A were subjected to SDS-PAGE and western blot analysis for phospho-T. Each lane was loaded with 25 μg protein. The migration of molecular weight standards is marked.

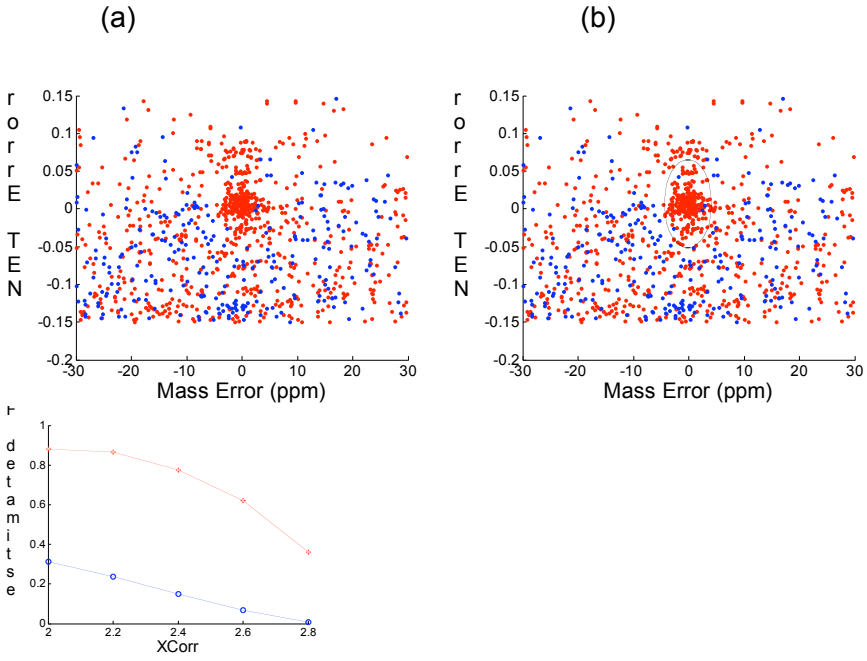
Supplementary Figure 2. An LC-FTICR analysis of a control sample was aligned against an LTQ LC-MS/MS analysis of a control sample and matches between LC-MS features, and peptides identified by SEQUEST were generated using mass and elution time tolerances at different XCorr values. **(a)** Mass and elution time differences between LC-MS features and matching peptides of charge 3+ with an XCorr greater than or equal to 2.6. Peptides corresponding to reverse sequences are shown in blue, while peptides corresponding to forward sequences are shown in red. It can be seen that the mass and NET error of peptides from reverse sequences show a uniform-like distribution since the matches are random. The distribution of the mass and NET error of peptides from the forward sequences is the summation of a uniform-like component which spreads across the mass and NET error tolerance region and a Gaussian-like component, which is dense, centering on zero mass and NET error, respectively. (b) Filtering of peak matching results. Peak matches that fell within the elliptical region were considered to be correct with a certain FDR. (c) Relationship between XCorr cutoff and FDR. An increase in XCorr cutoff reduces the FDR. The line in red represents the FDR when all the matches in the entire rectangular window of 30 ppm and 0.15 NET were used. The line in blue repesents the FDR when only the matches in the central elliptical area were considered.

Supplementary Figure 3. Elevated phosphorylation on EF2 T56 and MYPT1 (PP1 regulatory subunit 12) T696 in response to calyculin A treatment. Samples from cells treated with vehicle

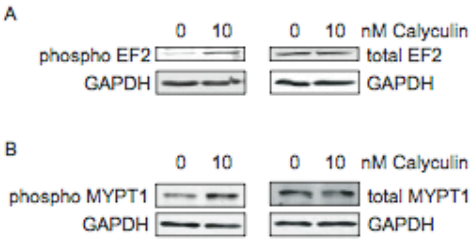
control (0 nM) or 10 nM calyculin A were subjected to SDS-PAGE and western blot analysis for phospho-T56 EF2 and EF2 (A) (25 μg protein per sample) or phospho-T696 MYPT1 or MYPT1 (B) (10 μg protein per sample) respectively. In both cases, GAPDH was monitored as the loading control. Blots were quantified using fluorescent secondary antibodies and Li-Cor fluorescence based imaging. The average ratio from 3 independent sets of treated and control samples showed a 3-fold increase in EF2 phospho-T56 levels and a 3-fold increase in MYPT1 phospho-T696 levels following treatment with 10 nM calyculin A.



Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3