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Quantitative phosphoproteomic analysis of T-cell receptor signaling

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Summary

TCR signaling critically depends on protein phosphorylation across many proteins. Localization of each phosphorylation event relative to the T cell receptor (TCR) and canonical T cell signaling proteins will provide clues about the structure of TCR signaling networks. Quantitative phosphoproteomic analysis by mass spectrometry provides a wide-scale view of cellular phosphorylation networks. However, analysis of phosphorylation by mass spectrometry is still challenging due to the relative low abundance of phosphorylated proteins relative to all proteins and the extraordinary diversity of phosphorylation sites across the proteome. Highly selective enrichment of phosphorylated peptides is essential to provide the most comprehensive view of the phosphoproteome. Optimization of phosphopeptide enrichment methods coupled with highly sensitive mass spectrometry workflows significantly improves the sequencing depth of the phosphoproteome to over 10,000 unique phosphorylation sites from complex cell lysates. Here we describe a step by step method for phosphoproteomic analysis that has achieved widespread success for identification of serine, threonine, and tyrosine phosphorylation. Reproducible quantification of relative phosphopeptide abundance is provided by intensity-based label-free quantitation. An ideal set of mass spectrometry analysis parameters is also provided that optimize the yield of identified sites. We also provide guidelines for the bioinformatic analysis of this type of data to assess the quality of the data and to comply with proteomic data reporting requirements.

Keywords

immunoaffinity purification; label-free quantitation; phosphoproteomics; T cell signaling; tyrosine phosphorylation; mass spectrometry

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[7]Cut 1 cm of the 200 μL tips when pick up the beads slurry.

1. Introduction

Among the many post translational modifications (PTMs) that occur in cells, protein phosphorylation is a critically important modification found in T-cell receptor (TCR) signaling. Determination that a protein is phosphorylated is not enough to understand the precise role that the phosphorylation plays in cellular signaling. Therefore, identification and quantitation of the exact position of the phosphorylation site is essential. A single protein can be targeted by multiple kinases thus phosphorylated in multiple sites with different dynamics (1). In the past, identification of multiple phosphorylation sites on target proteins was a very laborious task involving *in vivo* or *in vitro* labeling with radioactive phosphate, separation of labeled peptides by column or thin-layer chromatography, and Edman sequencing. However, the recent and rapid advancement of highly sensitive and accessible mass spectrometry based techniques makes this an invaluable addition to the toolbox. Analysis of intact purified proteins (top down proteomics) or complex mixtures of phosphopeptides from a tryptic digest of cellular lysates (bottom up proteomics) can provide direct information about the position and relative abundance of phosphorylation sites within a protein sequence. In particular, the ability of bottom up proteomics to provide identification and quantification of tens of thousands of phosphopeptide sequences from a cell lysate at high confidence from a single 180 min LC/MS analysis enables new approaches for the exploration of signaling pathways. Analysis of phosphopeptides by mass spectrometry is substantially more difficult compared to detection of unphosphorylated peptides due to a number of physiochemical properties of the modified peptides. For example, small phosphopeptides are very hydrophilic and may not be retained well on reversed-phase matrices typically used in LC-MS(2). Furthermore, some phosphorylation sites are often extremely difficult to detect due to the low abundance of the phosphopeptide and low stoichiometry of phosphorylation in a cellular context of abundant background proteins. Lastly, the presence of the phosphorylation site on a peptide decreases its ionization efficiency when detected in positive ion mode, making detection of phosphopeptides even more difficult. The phosphopeptides must be enriched to achieve optimal sequencing depths of the phosphoproteome.

Many methods have been developed to enrich the total phosphoproteome from the rest of the proteome including Fe(III) and Ga(III) immobilized metal affinity chromatography (IMAC) (3), or metal oxide affinity chromatography (MOAC) based on enrichment with TiO_2 beads (4) or titanium(IV) functionalized soluble nanopolymer (PolyMAC-Ti) (5). Sequencing depths in excess of 10,000 unique phosphorylation sites can be expected from complex cell lysates using MOAC. Of critical importance in maximization of sequencing depth and quantitative reproducibility is the use of sub 2 μm reversed-phase beads in the LC/MS acquisition of data. The use of these particles is accompanied by higher backpressures and typically requires ultrahigh performance liquid chromatography (UHPLC) to achieve sufficient flow at the electrospray tip. Here we describe an effective protocol for TiO_2 enrichment of phosphopeptides that has achieved widespread success.

Reversible tyrosine phosphorylation which has been estimated to represent less than 1% of all human phosphorylation events (6,7) plays a key role in many aspects of regulating several essential molecular mechanisms and processes including gene transcription, cell

growth, cell cycle, differentiation and motility in mammalian cells (8). Therefore identification of tyrosine phosphorylated residues and quantification of the relative phosphorylation level is critical for understanding their contribution to signaling networks and consequently, to pathological processes (9). Although a number of methods have been adopted for enrichment and analysis of tyrosine phosphorylation (10–15,9), quantitative proteomic analysis of tyrosine phosphorylation by mass spectrometry is still challenging, due to the low occurrence of this post-translational modification compared to serine and threonine phosphorylation in mammalian cells (11).

Immunoaffinity purification using a pan-specific phosphotyrosine antibody is commonly used to enrich tyrosine phosphorylated peptides. Recently a comparison between the two most commonly used antibodies showed that the P-Tyr-100 anti-phosphotyrosine antibody performs superiorly when compared to 4G10 antibody for label-free phosphotyrosine-based phosphoproteomics (15). This study also indicated that optimization of phosphotyrosine peptide capture protocol coupled with mass spectrometry methods can potentially enhance the identification of phosphotyrosine containing peptides. Here we provide an optimized protocol that can be used to identify and quantify phosphotyrosine containing peptides from human T cells. The optimized protocol is able to detect and quantify 934 unique tyrosine phosphorylation sites from Jurkat T cells using the Orbitrap Velos mass spectrometer (16) and 1557 unique tyrosine phosphorylation sites on the Q-Exactive mass spectrometer (manuscript in preparation).

The measurement of statistically significant quantitative changes in the phosphoproteome from cells lacking T cell signaling proteins provides critical information on the prospective role of each phosphorylation site in T cell signaling (17–19,16,20). Bottom up proteomics is a powerful approach for quantitation of relative changes in peptide and protein abundance across different cellular states or treatments. Both label-free and label-based strategies can be employed to quantitate relative peptide abundance (19). Currently, the two main strategies for label-free quantitation are spectral counting and signal intensity of detected peptides (19). In the spectral counting approach, the rate at which a peptide precursor ion is selected for fragmentation in a mass spectrometer is correlated to its abundance. Spectral counts can then be averaged into a protein abundance index. This approach is not appropriate for phosphoproteomic analysis due to the necessary phosphopeptide enrichment steps which would skew the spectral counts according to the number of phosphorylation sites on each protein which may not correlate to the protein’s abundance. In the signal intensity quantitation approach, the selected ion chromatogram for each peptide is calculated from a LC-MS/MS run and the peak areas are integrated over the chromatographic time scale. Retention time alignment of replicate analyses and accurate mass greatly facilitates the comprehensive quantitation and statistical analysis of each phosphopeptide.

Label-based approaches rely on the assumption that an isotope-labeled peptide is chemically identical to its native counterpart and thus behave identically during chromatographic and MS analysis. In stable isotope labeling by amino acids in cell culture (SILAC), stable isotopes of amino acids are metabolically incorporated into the proteomes of cells, which enables mixing of the experimental groups being compared and consequently, minimization of errors in quantitation that can occur through sample handling (21). MS/MS-based

quantitation can also be performed by various isotope-labeling techniques, such as isobaric tags for relative and absolute quantitation (iTRAQ) (22) and tandem mass tags (TMT) (23).

In the protocols described here, we focus on utilization of intensity-based label-free quantitation because it combines a high degree of quantitative reproducibility and a large dynamic range with less limitation on the number of comparisons between cellular states and requires the least amount of method development due to the lack of necessity to optimize the labeling parameters.

2. Materials

For preparation of solutions and buffers, LC-MS grade water is used. Solutions and buffers are freshly prepared at room temperature and used immediately. All sample preparation procedures involving intact proteins are performed at 4°C unless otherwise specified.

2.1. Reagents and solutions for cell lysis and in solution trypsin digestion

1. Protein sample: 1×10^8 cells or 10 mg protein
2. Cell lysis buffer: 9 M urea, 1 mM Na orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate in 20 mM HEPES, pH 8.0 (*see* **Note** [1])
3. 45 mM dithiothreitol and 100 mM iodoacetimide
4. Trypsin, sequencing grade, modified, TPCK treated, affinity purified (Cat, V5113, Promega, NJ, USA)

2.2. Reagents and solutions for C18 column (Sep-Pack) purification

1. Solvent A containing 0.1% of trifluroacetic acid (TFA)
2. Solvent B containing 0.1% of TFA in 40% acetonitrile
3. Sep-Pack light C18 cartridges (WAT020515, Waters, MA, USA) and extraction manifold apparatus (WAT200677, Waters)
4. 10 mL disposable syringe

2.3. Reagents and solutions for TiO_2 enrichment

1. TiO_2 Buffer containing 0.1% formic acid in 30% acetonitrile
2. Solution A containing 40% TFA in 50% acetonitrile
3. Solution B containing 6% TFA, 25% lactic acid in 67.5% acetonitrile
4. Buffer A containing 25% solution A in acetonitrile
5. Buffer B containing 75% of buffer A and 25% of solution B
6. Buffer C containing 26.7% ammonium hydroxide

[1]It is extremely important to freshly prepare and use the urea solution within 4 h to prevent artefactual carbamylation of peptides caused by the decomposition of the urea.

7. Buffer D containing 26.7% ammonium hydroxide in 40% acetonitrile
8. Buffer E containing 50% glacial acetic acid
9. Titansphere Phos-TiO Kit (GL Sciences, Japan)
10. A stock of 100 pmol/μL of a custom synthesized phosphoserine containing peptide FQpSEEQQQTEDELQDK (*see* **Note** [2])

2.4. Reagents and solutions for immune affinity purification (IAP)

1. IAP buffer containing 10 mM sodium phosphate, 50 mM NaCl in 50 mM MOPS, pH 7.2
2. Solvent A and B (see in section 2.2)
3. Protein G agarose (Roche, USA)
4. Phospho-tyrosine mouse mAb (P-Tyr-1000) (Cell Signaling, MA, USA)
5. A stock of 100 pmol/μL of a custom synthesized pTyr peptide LIEDAEpYTAK (*see* **Note** [2])
6. A stock of 100 pmol/μL of a custom synthesized human angiotensin II peptide DRVpYIHPF (*see* **Note** [2])
7. PBS buffer

2.5. Reagents and solutions for LC-MS/MS

1. HPLC Solution A: 0.1 M acetic acid in H_2O
2. HPLC Solution B: 0.1 M acetic acid in acetonitrile (99.8%; HPLC grade)

3. Methods

3.1. Protein extraction and trypsin digestion of the samples

1. Lyse the cells with 2 mL of ice-cold cell lysis buffer and incubate on ice for 20 min (*see* **Note** [1]).
2. Vortex the cell lysates vigorously for 1 min.
3. Sonicate the cell lysates on ice using a microtip sonicator (Q55 Sonicator, Qsonica, USA) with 6, 5-second bursts (Sonication setup amplification 25%)
4. Centrifuge the cell lysates at 20,000×g for 15 min at 15°C.
5. Collect the supernatant for BCA protein quantification and subsequent trypsin digestion.
6. Proteins are reduced with DTT (final concentration = 4.5 mM) for 30 min at 60°C.

[2]Synthetic peptides should be dissolved in 0.1 M Acetic acid.

7. Complete digestion is facilitated by alkylation of cysteine residues. For protein alkylation, add iodoacetamide (final concentration = 10 mM) for 20 min at room temperature in the dark.
8. Dilute cell lysate five-fold with 100 mM ammonium bicarbonate, pH 8.9.
9. Add affinity purified, TPCK-treated trypsin to protein in ratio 1:100 (w/w) trypsin/total protein and incubate overnight at 37°C.

3.2. Peptide desalting using C18 column

1. Add TFA solution to each of the trypsin digested sample to a final concentration of 1% TFA.
2. Centrifuge the acidified peptide solutions for 5 min at 1800×g.
3. Transfer the peptide containing supernatant into a 15 mL conical tube.
4. Set up the C18 Sep-Pak column and the 10 mL syringe to the Extraction manifold reservoir. Equilibrate C18 Sep-Pak column by washing with 5 mL of 100% acetonitrile and then wash twice with 3.5 mL of Solvent A (see section 2.2).
5. Load the acidified cleared digested samples to the Sep Pak column and turn on the vacuum slowly (1–2 psi) to pass the solutions through the column.
6. Wash the column three times with 5 mL of Solvent A (see section 2.2).
7. Elute peptides from column with 10 mL of elution buffer Solvent B (see section 2.2) and collect the peptide containing solution into a fresh 15 mL conical tube.
8. Split the eluents (10:90) for phosphopeptide enrichment by TiO_2 (~1×10^7 cell equivalents) and enrichment of phosphotyrosine peptide by immune affinity purification (~9×10^7 cell equivalents).
9. Lyophilize the digested, frozen peptide samples overnight (*see* **Note** [3]).

3.3. Phosphopeptides enrichment

3.3.1. Global phosphopeptides enrichment by TiO_2

1. Spin down the trypsin digested dried peptide sample at 1800×g for 5 min at room temperature and reconstitute the dried peptide samples in 100 μL TiO_2 Buffer from the 1×10^7 cell equivalent sample (*see* **Note** [4]).
2. Centrifuge the samples at 12000×g for 5 min at 15°C and collect the clear supernatant into a new eppendorf tube.
3. Add 500 fmol of synthetic pSer and angiotensin II standard peptides to every 100 μg of peptide sample (1×10^6 cell equivalents) (*see* **Note** [5]).

[3]To achieve optimal phosphotyrosine identification yields the frozen purified peptides should be lyophilized.
[4]Mix well by pipetting. Do not vortex.
[5]Add 5 μL of each standard from a 100 fmol/μL stocks

4. Set a TiO_2 tips within the centrifugal adaptor (provided by the vendor) and set it on top of the 2 mL waste fluid tube.
5. Wash tip with 20 μL of Buffer A to the (see section 2.3) to the top of the TiO_2 phospoTips and centrifuge at 3000×g for 2 min at room temperature.
6. Wash tip with 20 μL of Buffer B (see section 2.3) to the TiO_2 phospoTips and centrifuge at 3000×g for 2 min at room temperature and discard all the liquid from the waste fluid tube.
7. Dilute the 110 μL of peptide solution from step 3 with 150 μL of Buffer B and then add to the TiO_2 phospoTips (*see* **Note** [6]) and centrifuge the samples at 1000×g for 10 min at room temperature (*see* **Note** [6]).
8. Wash the TiO_2 phospoTips by adding 20 μL of Buffer B followed by a centrifugation 3000×g for 2 min at room temperature.
9. Wash the TiO_2 phospoTips three times by adding 20 μL of Buffer A followed by a centrifugation 3000×g for 2 min at room temperature.
10. Transfer the TiO_2 phospoTips with the centrifugal adaptor to a 1.7 mL recovery collection tube provided in kit and elutes phosphopeptides with 50 μL of buffer C (see section 2.3) and centrifuge at 1000×g for 5 min at room temperature.
11. Elute phosphopeptides again with 50 μL of Buffer D (see section 2.3) and centrifuge at 1000×g for 5 min at room temperature into the same collection tube.
12. Acidify the eluted phosphopeptides with 5 μL Buffer E (see section 2.3).
13. Dry the samples in the SpeedVac for 2 h and store the dried peptides at −80°C until analysis on the mass spectrometer.

3.3.2. Phosphotyrosine peptide enrichment with IAP

3.3.2.1. Antibody coupling

1. Wash PTMScan P-Tyr-1000 Kit beads twice with 1.0 mL cold PBS buffer (see section 2.4) (*see* **Note** [8]). After each wash step, centrifuge at 1500×g for 2 min at 4°C and carefully discard the supernatant.
2. Wash the beads three times with 1.0 mL cold IAP buffer (see section 2.4). After each wash step, centrifuge at 1500×g for 2 min at 4°C and carefully discard the supernatant.
3. Store the beads on ice for subsequent use.

[6]Mix well in the tips by pipetting. Discard the waste fluid from the waste fluid tube and then Centrifuge again at 1000×g for 10 min at room temperature to remove all of the buffers from the Tips.

[8]After adding PBS, invert the tube to avoid bead precipitation. Supernatant should be removed with micropipette. To avoid the loss of bead, do not completely suck the soup.

3.3.2.2. Elution of phosphotyrosine peptides

1. Briefly centrifuge the lyophilized peptide at 1500×g for 5 min at room temperature.
2. Reconstitute the 9×10^7 cell equivalent dried peptide samples (see section 3.2.9) in 1 mL of IAP buffer (see section 2.4) and keep on ice for 5 min (*see* **Note** [9]).
3. Remove any particulate material by centrifugation 1500×g for 5 min at 4°C.
4. Add 1 pmol of synthetic pTyr peptide standard (see section 2.4) to the peptide solution.
5. Transfer the peptide solution to the bead slurry (see section 3.3.2.1**)** and incubate for 2 h on a Barnstead/Thermolyne LABQUAKE rotator (8 rpm) at 4°C.
6. Centrifuge the mixture at 1500×g for 2 min at 4°C and discard the supernatant (*see* **Note** [10]).
7. Wash the beads three times with 1mL IAP buffer and remove the supernatant by centrifugation at 1500×g for 2 min at 4°C (*see* **Note** [11]).
8. Wash the beads with 1 mL of ice cold water, mix by inverting tube 5 times and remove the supernatant by centrifugation at 1500×g for 2 min at 4°C (*see* **Note** [12]).
9. Elute the tyrosine phosphorylated peptides with 55 μL of 0.15% TFA for 10 min at 22 °C followed by collection of the eluent by centrifugation of the mixture at 1500×g for 2 min at 4 °C into a new collection tube (*see* **Note** [13]).
10. Elute the peptides a second time with 45 μL of 0.15% TFA and collect the eluent in a different collection tube (as above).
11. Wet a ZipTip with 50 μL of solvent B (see the recipe in section 2.2) (*see* **Note** [14]).
12. Equilibrate the tip with 50 μL solvent A, twice (see the recipe in section 2.2) (*see* **Note** [15]).
13. Pipette the first eluted phosphopeptide sample aliquot from step 9 (55 μL) with micropipette into the ZipTip by repeatedly pipetting the solution 10 times and then expelling the liquid into the original tube.

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[9] Do not vortex or mixing by pipet. Dissolve peptide by hand by gentle shaking (or use a VWR model 200 rocker, speed 2~3 rpm or similar rocker) for 30 min at room temperature. Use sonication water bath to aid the dissolve (~10 sec).

[10] Remove supernatant using 250 μL tips.

[11] Mixing by inverting tube 5 times. Remove supernatant with 1mL micropipette (aspirate the supernatant as much as possible, using 1 mL, 250 μL and 20 μL tips respectively). Minimize the loss of beads during aspiration.

[12] In this step, unbuffered LC-grade water should be used. Remove the supernatant completely with insulin syringe placing the angled edge of the needle against the plastic and inserting the needle into the beads while keeping the angled opening against the plastic. The insulin syringe is only mandatory when all the liquid needs to be removed from the beads.

[13] Tap the bottom of the tube with fingers for several times (gently touch using the palm side of finger. Do not vortex. Use an insulin syringe to collect the eluted peptides from the beads.

[14] Spin down the supernatant 1500×g, 2 min, 4°C. Split the solution into two tubes. (Because ZipTip can only hold 50 μL, yet for the whole 100 μL sample, use the same ZipTip.) Spin down the solution.

[15] Cut a 250 μL tip to fit ZipTip, the pipette setting could be higher than 50 μL to make the aspiration and dispensing easier. Expelling the solution to a lint-free tissue (Kimwipes), briefly touch the liquid at the end to the tissue.

14. Pipette the second phosphopeptide aliquot (45 μL) from step 10 onto the same ZipTip according to procedure in step 13.

15. Wash the tip twice with 50 μL solvent A (*see* **Note** [16]).

16. Elute the peptide with 10 μL of solvent B (*see* **Note** [17]).

17. Dry the peptides using a SpeedVac for 30 min at 22°C (*see* **Note** [18]).

3.4. LC-MS/MS analysis

LC-MS/MS can be performed as described previously (16). Many proteomic core facilities will be able to provide basic capabilities to collect this type of data according to the parameters specified here. Tryptic peptides can be analyzed by a fully automated phosphoproteomic technology platform developed in the Salomon lab called HTAPP (24) and Peptide Depot(25) or using commercial software such as Mascot(26), PEAKS 7.5 (http://www.bioinfor.com) (27), and Scaffold PTM 3.0 (http://www.proteomesoftware.com/products/ptm/). The nanoLC-MS/MS experiments are performed with an Agilent 1200 Series Quaternary HPLC system (Agilent Technologies, Santa Clara, CA) connected to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Waltham, MA). For the TiO_2 analysis, reconstitute the lyophilized phosphopeptides from step 3.3.1.13 that were derived from 1×10^7 cell equivalents in 100 μL HPLC Solution A (see section 2.5) and inject 10 μL for each analysis using LC-MS/MS. For the phosphotyrosine IAP analysis, reconstitute the dried peptide from step 3.3.2.17 with 10 μL of HPLC Solution A (see section 2.5) and inject 5 μL for each analysis. Prior to LC-MS/MS analysis of either TiO_2 and phosphotyrosine IAP samples, add 500 fmol of human phospho-angiotensin II peptide as an internal standard.

For LC-MS/MS analysis, the peptides are separated through a linear reversed-phase gradient from 0% to 40% HPLC Solution B (see section 2.5) over 60 min with a total 90 min run time. For optimal sensitivity, the electrospray analytical column can be fabricated with new objective self-pack PicoFrit columns (New Objective Inc, MA, USA) packed with 3 μm ReproSil-Pur 120 C18 reversed-phase particles (Dr. Maisch HmbH, Germany). The use of 1.9 μm particles will improve sequencing depth and quantitative reproducibility dramatically but may necessitate the use of UHPLC. The electrospray ion source is operated at 2.0 kv in a split flow configuration, as described previously (28). The Q Exactive plus is operated in data dependent mode using a top-9 data dependent method. Survey full scan MS spectra (m/z 400–1800) are acquired at a resolution of 70,000 with an AGC target value of 3×10^6 ions or a maximum ion injection time of 200 ms. Peptide fragmentation is performed via higher-energy collision dissociation (HCD) with the energy set at 28 NCE. The MS/MS spectra are acquired at a resolution of 17,500, with a targeted value of 2×10^4 ions or a maximum integration time of 200 ms. The underfill ratio, which specifies the minimum percentage of the target value likely to be reached at maximum fill time, is defined as 1.0%.

[16]Pipette the solvent A with a micropipette. Expel to a lint-free tissue. After the last wash dab the tip well, but not to excess, on the lint-free tissue.

[17]Use a 20 μL pipette which exactly fits the ZipTip without an adapter. Pipette 10 μL of solvent B into a new microcentrifuge tube. Draw the solvent into the tip and expel. Repeat this for 8–10 times. During this process, the tip is always in the solution. Expel the entire volume into a sample vial.

[18]Leave the cap on the tube, but use needle to make a hole.

The ion selection abundance threshold is set at 8.0×10^2 with charge state exclusion of unassigned and z =1, or 6–8, 8> ions and dynamic exclusion time of 30 seconds.

3.5. Data analysis

Peptide spectrum matching of MS/MS spectra is performed against a human-specific database (UniProt; complete proteome set) using MASCOT v. 2.4 (Matrix Science, Ltd, London W1U 7GB UK)(26). A concatenated database containing an equal number of "target" and reversed "decoy" sequences is employed to estimate the false discovery rate (FDR) (29). Msconvert from ProteoWizard (30), using default parameters and with the MS2Deisotope filter on, is used to create peak lists for Mascot. Mascot database searches are performed with the following parameters: trypsin enzyme cleavage specificity, 2 possible missed cleavages, 7 ppm mass tolerance for precursor ions, 20 mmu mass tolerance for fragment ions. Search parameters permit variable modification of methionine oxidation (+15.9949 Da), static modification of carbamidomethylation (+57.0215 Da) on cysteine and variable modification of phosphorylation (+79.9663 Da) on serine, threonine, and tyrosine residues. The resulting peptide spectrum matches (PSMs) are reduced to sets of unique PSMs by eliminating lower scoring duplicates. Peptide assignments from the database search are filtered down to 1% false discovery rate (FDR) by Mascot MOWSE score thresholding. To validate the position of the phosphorylation sites, the Ascore algorithm (31) can be applied to all data, and the reported phosphorylation site position should reflect the top Ascore prediction. The use of other algorithms may be acceptable but some sort of probabilistic score should be reported for each phosphopeptide to estimate the likelihood that the phosphorylation site position is confidently determined.

3.6. Quantitation of relative phosphopeptide abundance

Here we provide a detailed description of the quantitative analysis parameters used in the Salomon lab HTAPP (24) and Peptide Depot (25) software which may be adapted to commercially available software available in many proteomic core facilities. Label-free relative quantification of phosphopeptide abundance is performed via calculation of selected ion chromatogram (SIC) peak areas. Retention time alignment of individual replicate analyses can be performed as previously described (32). Peak areas can be calculated by inspection of SICs using in-house software programmed in R 3.0 based on the Scripps Center for Metabolomics' XCMS package (33). This approach performs multiple passes through XCMS's central wavelet transformation algorithm (implemented in the centWave function) over increasingly narrower ranges of peak widths, and used the following parameters: mass window of 10 ppm, minimum peak widths ranging from 2 to 20 seconds, maximum peak width of 80 seconds, signal to noise threshold of 10 and detection of peak limits via descent on the non-transformed data enabled. For cases when centWave did not identify an MS peak, we use the getPeaks function available in XCMS to integrate in a pre-defined region surrounding the maximum intensity signal of the SIC. SIC peak areas are determined for every phosphopeptide that is identified by MS/MS. In the case of a missing MS/MS for a particular peptide, in a particular replicate, the SIC peak area is calculated according to the peptide's isolated mass and the retention time calculated from retention time alignment. A minimum SIC peak area equivalent to the typical spectral noise level of 1000 is required of all data reported for label-free quantitation. All individual SIC peak areas

are normalized to the peak area of the exogenously spiked phosphopeptide FQpSEEQQQTEDELQDK or LIEDApYTAK added prior to phosphopeptide enrichment and reversed-phase elution into the mass spectrometer. The *p*-values are calculated from at least three replicate analyses. To overcome the possibility of missing data caused by the stochastic peak selection in data dependent scanning in the mass spectrometer, the collection of at least 5 biological replicate analyses is strongly recommended. To select phosphopeptides that show a statistically significant change in abundance between stimulated cells and control, two-tailed unpaired Student's *t* tests and *q*-values for multiple hypothesis tests can be calculated based on the determined *p*-values using the R package QVALUE as previously described (34,35). Other methods such as ANOVA with post-hoc Tukey HSD (36) may be used to correct for multiple hypothesis. Some form of multiple hypothesis correction is required for publication of this type of data. Determination of the significantly altered set of phosphopeptides can be facilitated by selection of a FDR and fold change threshold for the quantitative data visualized with a volcano plot. The proteomic community favors a 1% or lower FDR threshold on peptide sequence assignment and a 5% or lower FDR threshold on the quantitative data.

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