Supporting Information

Supplementary figure legend

**Figure S1.** Physicochemical properties of phosphopeptide isomers separated by reverse phase LC. Differences in physicochemical properties (hydrophobicity and electrostatic forces) of 64 phosphopeptide isomers identified in IEC-6 and J774 cells. A) Difference in absolute local hydrophobicity scale between the region centered on the phosphorylated sites (windows of 3 amino acids, amino acids scale values from Kyte J., Doolittle R.F. ¹). Difference in proximity (in amino acids) of the phosphate group to an acidic residue (B) or a basic residue (C) that could affect electrostatic interactions.

**Figure S2.** The order of elution of phosphopeptide isomers is related to proximity of the phosphorylated residue to the N-terminus. For 73% of isomeric pairs, phosphopeptides with the modified residue closest to the N-terminus (N-ter) elute last, suggesting that electrostatic interactions occur between the N-ter amino group and the negatively charge phosphate moiety.

**Figure S3.** Overlap in phosphopeptide identification between CID, HCD and ETD for *D. melanogaster* S2 phosphoproteome. Unique peptide identification with a Mascot score > 20 and a phosphosite position confidence > 0.75 were selected. A) Number of unique peptides identified during the DDA experiment. B) Number of unique peptides identified in the targeted experiment using inclusion list and all three fragmentation modes for each precursor ion.
**Figure S4.** Difference of retention time for 117 phosphopeptide isomers of *D. melanogaster* S2 cells using DDA (survey) and targeted MS/MS analyses. Data were obtained using the LC-MS elution profile detection algorithm and assignments were confirmed by MS/MS. The histogram shows the common and uniquely detected isomers from the survey and the targeted analyses.

**Figure S5.** Properties of 117 phosphopeptide isomers of *D. melanogaster* S2 cells. Histograms for the distribution of the peptide length (A), distance in amino acids between phosphorylated site position (B), retention time difference at peak top (C), and chromatographic resolution of isomer peaks for an intensity threshold of 10 000 counts (D).

**Figure S6.** Isobaric peptide artifacts. Isobaric peptide artifacts can be confounded with phosphopeptide isomers. Alternative enzymatic cleavage, peptide sequence, other modification (e.g. alternate position of oxidation) and conformers are peptides with the same mass that can be separated by LC.

**Figure S7.** LC separation of phosphopeptide conformers. Example of separated phosphopeptide conformers: GIMEEEMRPSPLSDR (m/z: 625.63+) with both methionines oxidized. A) LC-MS profile showing two separated conformers and the acquired HCD MS/MS spectra for the targeted analysis (green circle). B) Heatmap of the observed peptide fragments. Each row represents a fragment and each column a MS/MS scan. Fragments marked with red localize the phosphorylated residue. The vertical black line separates both conformers.
Figure S8. MS/MS spectra of four phosphopeptide isomers from IPSSSSDFSK. CID, ETD and HCD MS/MS spectra are shown for the four phosphopeptide isomers of IPSSSSDFSK. Mascot score and phosphorylation localization confidence are reported for each spectrum.
Supplementary figures

Figure S1

A

B

C

Difference in hydrophobicity
Difference in distance to an acidic residue
Difference in distance to a basic residue
Figure S3

A. No inclusion
4783 unique phosphopeptides
(≥75% confidence, Mascot score ≥ 20)

B. Inclusion
1633 unique phosphopeptides
(≥75% confidence, Mascot score ≥ 20)
Figure S4

A histogram showing the frequency of retention time difference for different analyses. The histogram is color-coded, with red for "Both," green for "Survey," and blue for "Targeted." The x-axis represents the retention time difference, and the y-axis represents the frequency.
Figure S5

A

B

C

D

Peptide Length

Phosphorylation sites distance

Retention time difference

LC Peaks resolution

Frequency

Frequency

Frequency

Frequency
<table>
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<tr>
<th>Phosphopeptide positional isomers</th>
<th>Alternative enzymatic cleavage</th>
<th>Alternative peptide sequence</th>
<th>Alternative modification shift</th>
<th>Alternative peptide conformers</th>
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<td>EDGMSDSPVERK</td>
<td>GMSEEDDSPVMRK</td>
<td>GMSEEDDSP_cotrans_VM RK</td>
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<td>GMSEEDDSP_trans_VM RK</td>
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</tbody>
</table>
Supplementary tables

Table S1. Phosphorylation sites identified in S2, IEC-6 and J774 cells. Accession, gene symbol, description, species, residue, position, localization confidence, peptide sequence, peptide modifications and mascot score are reported for each phosphorylated site.

Table S2. Phosphopeptide isomers identified in mouse J774 and rat IEC6 cells. Peptide sequence, modification site, m/z, localisation confidence, score, retention time, resolution of LC separation, distance between phosphorylated amino acid, distance to the nearest acid or basic residues and local hydropathicity scale are reported.

Table S3. Synthetic phosphopeptides analysis. List of the 9 synthetic phosphopeptides used to test the separation of isomers by LC and the algorithm performance. Peptide sequence, modifications, m/z, localisation confidence, score, retention time and resolution of LC separation are reported.

Table S4. Phosphopeptide isomers identified in D. melanogaster S2 cells. List of the 117 isomers detected from the phosphoproteome dataset and included peptide ions for the targeted analysis. In addition to the data fields reported in Table S2, this table also provides inclusion m/z, inclusion time window, SCX fraction, capture efficiency from inclusion list, true isomers and ambiguous cases, and artifacts from the survey (DDA) and targeted analyses. MS/MS identification from CID/ETD/HCD fragmentation modes for the phosphorylated peptide IPSSSSDFS is also reported.