Supplementary methods

All chemicals, if not otherwise mentioned, were bought with the highest available purity from Sigma-Aldrich, Taufkirchen, Germany.

Supplementary methods 1a

Cell culture, lysis and protein digestion

*D. melanogaster* Kc167 cells were grown in Schneiders *Drosophila* medium (Invitrogen, Auckland, New Zealand) supplemented with 10 % fetal calf serum, 100 U penicillin (Invitrogen, Auckland, New Zealand) and 100 µg/ml streptomycin (Invitrogen, Auckland, New Zealand) in an incubator at 25 °C. $10^9$ cells were washed twice with ice cold phosphate buffered saline (PBS) and were resuspended in ice cold lysis buffer containing 10 mM HEPES, pH 7.9, 1.5 mM MgCl$_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT) and a protease inhibitor mix (Roche, Basel, Switzerland). In order to preserve the protein phosphorylation, several phosphatase inhibitors were added to a final concentration of 20 nM calyculin A, 200 nM okadaic acid, 4.8 µm cypermethrin (all bought from Merck KGaA, Darmstadt, Germany), 2 mM vanadate, 10 mM sodium pyrophosphate, 10 mM NaF and 5 mM EDTA. After 10 min incubation on ice, cells were lysed by douncing. Cell debris and nuclei were removed by centrifugation for 10 min at 4 °C using 5,500 xg. Then the cytoplasmic and membrane fraction were separated by ultracentrifugation at 100,000 xg for 60 min at 4 °C. The protein of the cytosolic fraction (supernatant) was subjected to acetone precipitation. The protein pellets were resolubilized in 3 mM EDTA, 20 mM TrisHCl pH 8.3 and 8 M urea. The disulfide bonds of the proteins were reduced with tris(2-carboxyethyl)phosphine (TCEP) at a final concentration of 12.5 mM at 37 °C for 1 h.
The produced free thiols were alkylated with 40 mM iodoacetamide at room temperature for 1 h. The solution was diluted with 20 mM TrisHCl (pH 8.3) to a final concentration of 1.0 M urea and digested with sequencing-grade modified trypsin (Promega, Madison, Wisconsin) at 20 μg per mg of protein overnight at 37 °C. Peptides were desalted on a C18 Sep-Pak cartridge (Waters, Milford, Massachusetts) and dried in a speedvac. For every isolation experiment 1.5 mg peptide was used as starting material.

**Phosphopeptide isolation**

*Isolation of phosphopeptides using phosphoramidate chemistry:* 1.5 mg dry peptide was reconstituted in 700 μL of methanolic HCl which was prepared by adding 160 μL of acetyl chloride to 1 mL of anhydrous methanol. The methyl esterification was allowed to proceed at 12 °C for 120 minutes. Solvent was quickly removed in a speedvac, and peptide methyl esters were dissolved 40 μL methanol, 40 μL water and 80 μL acetonitrile (ACN). Then 250 μL of the reaction solution containing 50 mM N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide (EDC), 100 mM imidazole, pH 6.0, 100 mM 2-(N-Morpholino)ethanesulfonic acid (MES) pH 5.8 and 2 M cystamine were added. Then the pH of the solution was, if necessary, adjusted to pH 5.6. The reaction was allowed to stand at room temperature with vigorous shaking for 8 hrs and then the solution was loaded onto a C18 Sep-Pak cartridge. The derivatized peptides were washed with 0.1 % trifluoroacetic acid (TFA), and then treated with 10 mM TCEP in 100 mM phosphate buffer (pH 6.0) for 8 min in order to produce free thiol groups. After washing with 0.1 % TFA to remove TCEP and phosphate buffer, peptides were eluted with 80 % ACN, 0.1 % TFA. Phosphate buffer was added to adjust final pH to 6.0. Then ACN was partially removed in the speedvac (final concentration of ACN was around 30-40 %) and the derivatized phosphopeptides
were incubated with 5 mg maleimide functionalized-glass beads for 1 h at pH 6.2 (Maleimide functionalized glass beads were prepared by 2 hrs reaction between 3 equivalents of 3-maleimidopropionic acid N-hydroxy succinimide ester and 1 equivalent of aminopropyl controlled pore glass (CPG) beads (Proligo Biochemie, Hamburg, Germany)). Then the beads were washed sequentially several times with 3 M NaCl, water, methanol and finally 80 % ACN to remove non-specifically bound peptides. In the last step, the beads were incubated with 20 % TFA, 30 % ACN for 1 h to recover phosphopeptides. The recovered sample was dried in the speedvac and reconstituted in 0.1 % TFA for LC-MS(/MS) analysis.

Isolation of phosphopeptides using IMAC: 1.5 mg dry peptide was reconstituted in 700 µL of methanolic HCl which was prepared by adding 160 µL of acetyl chloride to 1 mL of anhydrous methanol. The methyl esterification was allowed to proceed at 12 °C for 120 minutes. Then solvent was quickly removed in a Speedvac. The dry, methylated peptides were reconstituted in 30 % ACN, 250 mM acetic acid at pH 2.7 and mixed with 30 µl equilibrated PHOS-Select™ gel (Sigma-Aldrich) in a blocked mobicol spin column (MoBiTec, Göttingen, Germany). Then the pH of the solution was, if necessary, adjusted to pH 2.65. This peptide solution was incubated for 2 hrs with end-over-end rotation. Then the resin was washed two times with 250 mM acetic acid, 30 % ACN and once with ultra pure water. Finally phosphopeptides were eluted once with 50 mM and once with 100 mM phosphate buffer, pH 8.9 and pH was quickly adjusted to 2.75 after elution. Finally the peptides were cleaned with C18 ultra micro spin columns (Harvard Apparatus Ltd, Edenbridge, United Kingdom).
For the influence of peptide to resin ratio see Supplementary Figure 2.
Isolation of phosphopeptides using \( pTiO_2 \): 1.5 mg of peptide was reconstituted in a solution containing 80 % ACN, 2.5 % TFA and was saturated with phthalic acid. Then the peptide solution was added to 4 mg equilibrated TiO\(_2\) (GL Science, Saitama, Japan) in a blocked mobicol spin column (MoBiTec, Göttingen, Germany) and was incubated for 15 min with end-over-end rotation. The column was washed two times with the saturated phthalic acid solution, two times with 80 % ACN, 0.1 % TFA and finally two times with 0.1 % TFA. The peptides were eluted with a 0.3 M NH\(_4\)OH solution. If applicable, the recovered peptides were methylated at 12 °C using a similar methanolic HCl to peptide ratio as used for the methylation prior to the isolation of the phosphopeptides using IMAC and PAC. Then peptides were dried and reconstituted in 0.1 % TFA before LC-MS\(^n\) analysis as before.

For the influence of peptide to resin ratio see Supplementary Figure 1.

Isolation of phosphopeptides using \( dhbTiO_2 \): 1.5 mg of peptide was reconstituted in a solution containing 200 mg/ml 2,5-dihydroxybenzoic acid (ABCR, Karlsruhe, Germany), 80 % ACN, 2.5 % TFA. Then the peptide solution was added to 4 mg equilibrated TiO\(_2\) (GL Science, Saitama, Japan) in a blocked mobicol spin column (MoBiTec, Göttingen, Germany) and was incubated for 15 min with end-over-end rotation. The column was washed two times with the 2,5-dihydroxybenzoic acid solution, two times with 80 % ACN, 0.1 % TFA and finally two times with 0.1 % TFA. The peptides were eluted with a 0.3 M NH\(_4\)OH solution. If applicable, the recovered peptides were methylated (as described for pTiO\(_2\)), dried and reconstituted in 0.1 % TFA before LC-MS\(^n\) analysis as before.
Comparison of pTiO2 and IMAC without methyl esterification of starting peptide material and without methyl esterification of product (see also Supplementary Table 3).

*Isolation of phosphopeptides using pTiO2:* 1.5 mg of peptide was reconstituted in a solution containing 80 % ACN, 2.5 % TFA and was saturated with phthalic acid. Then the peptide solution was added to 4 mg equilibrated TiO₂ (GL Science, Saitama, Japan) in a blocked mobicol spin column (MoBiTec, Göttingen, Germany) and was incubated for 15 min with end-over-end rotation. The column was washed two times with the saturated phthalic acid solution, two times with 80 % ACN, 0.1 % TFA and finally two times with 0.1 % TFA. The peptides were eluted with a 0.3 M NH₄OH solution. Then peptides were dried and reconstituted in 0.1 % TFA before LC-MS analysis as before.

*Isolation of phosphopeptides using IMAC:* 1.5 mg was reconstituted in 30 % ACN, 250 mM acetic acid at pH 2.7 and mixed with 30 μl equilibrated PHOS-Select™ gel (Sigma-Aldrich) in a blocked mobicol spin column (MoBiTec, Göttingen, Germany). Then the pH of the solution was, if necessary, adjusted to pH 2.65. This peptide solution was incubated for 2 hrs with end-over-end rotation. Then the resin was washed two times with 250 mM acetic acid, 30 % ACN and once with ultra pure water. Finally phosphopeptides were eluted once with 50 mM and one with 100 mM phosphate buffer, pH 8.9. pH was adjusted to 2.75 after elution. Finally the peptides were cleaned with C18 ultra micro spin columns (Harvard Apparatus Ltd, Edenbridge, United Kingdom).
Supplementary methods 1b

MS analysis

Samples were analyzed on a hybrid LTQ-FTICR mass spectrometer (Thermo, San Jose, CA) interfaced with a nanoelectrospray ion source. Chromatographic separation of peptides was achieved on an Agilent Series 1100 LC system (Agilent Technologies, Waldbronn, Germany), equipped with a 11 cm fused silica emitter, 150 μm inner diameter (BGB Analytik, Böckten, Switzerland), packed in-house with a Magic C18 AQ 5 μm resin (Michrom BioResources, Auburn, CA, USA). Peptides were loaded from a cooled (4°C) Agilent auto sampler and separated with a linear gradient of ACN/water, containing 0.15 % formic acid, with a flow rate of 1.2 µl/min. Peptide mixtures were separated with a gradient from 2 to 30 % ACN in 90 minutes. Three MS/MS spectra were acquired in the linear ion trap per each FT-MS scan, the latter acquired at 100,000 FWHM nominal resolution settings with an overall cycle time of approximately 1 second. Charge state screening was employed to select for ions with at least two charges and rejecting ions with undetermined charge state. For each peptide sample a standard data dependent acquisition (DDA) method on the three most intense ions per MS-scan was used and a threshold of 200 ion counts was used for triggering an MS/MS attempt.

Between all LC-MS runs 200 fmol of [Glu]fibrinopeptide B (GluFib) (from 5-45 % ACN in 25 min) were measured in order to monitor the column performance and to confirm that no cross contamination between the LC-MS runs of the isolates occurred. In order to achieve the highest reproducibility all samples were measured using the same column.

With every phosphopeptide isolation method three independent isolations were performed. Two LC-MS/MS runs were performed per isolation.
Supplementary methods 1c

Data analysis

LC/MS pre-processing, alignment and similarity assessment was performed by the novel program Superhirn, which is written in C++ and will be freely downloaded from its project homepage (http://tools.proteomecenter.org/SuperHirn.php) after publication. Initially, Superhirn performs a peak detection routine on all input LC/MS runs, where biological signals are separated from background noise. The features are separated from the background noise through a filter which discards all features that do not elute continuously over a minimal time interval. The features are then scored by the product between the feature intensity and the quality of fit to the theoretical isotope distribution pattern. For the analysis only features passing a low score threshold of 1,000 were considered for further analysis. Increasing the threshold yields features with high quality but reduces the number of features detected. After filtering, the MS1 features are defined by their charge state, mass to charge ratio and apex retention time. In all measurements, only MS1 features within the retention time range of 30 to 102 min were analyzed because nearly all peptides eluted between this time points of the ACN gradient. Following data pre-processing, retention time fluctuations between two LC/MS runs were removed in an alignment step and LC/MS similarity assessment was performed, which comprises the computation of a LC/MS similarity score and the overlap of MS1 features. The similarity score reflects the reproducibility of intensity and retention time values between common MS1 features, where the overlap is the percentage of features found in both LC/MS runs. These two steps are repeated for all possible pairs of LC/MS runs and similarity scores as well as feature overlap are represented in a 2D color matrix (see figure 2a and b).
The MS/MS data were searched against the *D. melanogaster* NCI non redundant database (NCI database drosophila (August 3rd, 2005); drosophila_nci_20050803, containing 38,872 proteins) using SORCERER-SEQUEST(TM) v3.0.3 which was run on the SageN Sorcerer (Thermo Electron, San Jose, CA, USA). For the *in silico* digest trypsin was defined as protease, cleaving after K and R (if followed by P the cleavage was not allowed). Two missed cleavages and one non-tryptic terminus were allowed for the peptides which had a maximum mass of 6,000 Da. The precursor ion tolerance was set to 50 ppm and fragment ion tolerance was set to 0.5 Da. Before searching the neutral loss peaks were removed and indicated as described previously\(^2\). These data were also used to perform the neutral loss event analysis. Only neutral losses with an intensity of at least 50 % of the maximum peak intensity in a DTA spectrum were considered. Furthermore a mass window of 3 Da was allowed for the neutral loss ion. The 3 Da mass tolerance is a deconvoluted tolerance. As implemented, the program will consider an actual mass tolerance of +/- 3.0 for singly charged precursor ions, +/- 1.5 for doubly charged precursor ions, and +/- 1.0 for triply charged precursor ions.

Then data were searched allowing phosphorylation (+79.9663 Da) of serine, threonine and tyrosine as a variable modification and carboxyamidomethylation of cysteine (+57.0214 Da) residues as well as methylation (+14.0156 Da) of all carboxylate groups as a static modification. In the end the search results were subjected to statistical filtering using Peptide Prophet (V3.0)\(^3\). Peptides with a p value of equal or bigger than 0.9, which in this case equals to a probability of ~98 % of being correct, were used for all further analysis.

For figure 3 QualBrowser 2.0 from Thermo Electron Corporation was used.

For figure 4 the Venn diagram was made with VennMaster 0.17a.
For figure 5b, 5c and Supplementary Figure 3 the root mean square deviations were calculated as $$\left(\frac{p(1-p)}{n}\right)^{0.5}$$.

For figure 5b the molecular mass distribution and for figure 5c the amino acid occurrence was derived from an in silico digest (fully tryptic) of the *D. melanogaster* drosophila_nci_20050803 database.