SUPPLEMENTAL METHODOLOGY

1. Proteomics analysis

1.1 Sample preparation

Protein extraction and quantification

Plasma samples were diluted with phosphate-buffered saline (PBS) containing proteases and phosphatase inhibitors and vortexed vigorously. Then, samples were sonicated with 30s pulse at 50% amplitude and centrifuged at 14000g for 15 min. Supernatants were collected for protein precipitation with the addition of 10% TCA/acetone. The protein pellets were resuspended in 6M urea/50 mM ammonium bicarbonate (ABC) and quantified by Bradford's method.

Protein digestion

550 µg of total protein were reduced with 4 mM 1.4-Dithiothreitol (DTT) for 1h at 37°C and alkylated with 8 mM iodoacetamide (IAA) for 30 min at 25ºC in the dark. Afterwards, samples were overnight digested (pH 8.0, 37ºC) with sequencing-grade Trypsin/Lys-C (ThermoFisher Scientific, CA, USA) at enzyme:protein ratio of 1:50. Digestion was quenched by acidification with 1% (v/v) formic acid and each sample was divided in two, 50 µg of initial total protein were used for proteomics analysis and 500 µg were used for phosphosproteomics. Before peptide TMT labelling and phosphopeptide enrichment, samples were desalted on Oasis HLB SPE column (Waters, Massachusetts, USA).

1.2 Proteomics analysis

Peptide 11-plex TMT labelling

After digestion, 50 µg was used for proteomics analysis and the TMT 11-plex labelling (ThermoFisher Scientific, CA, USA) was performed according to the manufacturer's instructions. To normalise all samples in the study along the different TMT-multiplexed batches used, a pool containing all the samples was labelled with TMT-126 tag and included in each TMT batch. The different TMT 11-plex batches were desalted on Oasis HLB SPE columns before the nanoLC-MS analysis.

nanoLC-(Orbitrap)MS/MS analysis

Labelled and multiplexed peptides were loaded on a trap nano-column (100 µm I.D.; 2cm length; 5µm particle diameter, ThermoFisher Scientific, CA, USA) and separated onto a C-18 reversed phase (RP) nano-column (75µm I.D.; 15cm length; 3µm particle diameter, Nikkyo Technos Co. LTD, Japan) on an EASY-II nanoLC from ThermoFisher. The chromatographic separation was performed with a 180 min gradient using Milli-Q water (0.1% formic acid) and acetonitrile (0.1% formic acid) as mobile phase at a flow rate of 300 nL/min. Each sample was analysed in triplicate to increase proteome coverage.

Protein identification/quantification

Protein identification/quantification was performed on Proteome Discoverer software v.1.4.0.288 (ThermoFisher Scientific, CA, USA) by Multidimensional Protein Identification Technology (MudPIT) combining the 3 raw data files obtained.

For protein identification, all MS and MS/MS spectra were analysed using Mascot search engine (v.2.5). The workflow was set up using the Mascot node combing Homo Sapiens database (74449 entries) and the contaminants database (247 entries), both searches assuming trypsin digestion. Two missed cleavages were allowed and an error of 0.02 Da for a FT-MS/MS fragmentation mass and 10.0 ppm for a FT-MS parent ion

mass were allowed. TMT-10plex was set as quantification modification and oxidation of methionine and acetylation of N-termini were set as dynamic modifications, whereas carbamidomethylation of cysteine was set as static modifications. The false discovery rate (FDR) and protein probabilities were calculated by Percolator.

For protein quantification, the ratios between each TMT-label against 126-TMT label were used and quantification results were normalized based on the protein median.

Mass spectrometry analyses were performed on an LTQ-Orbitrap Velos Pro from ThermoFisher by an enhanced FT-resolution MS spectrum (R=30,000 FHMW) followed by a data-dependent FT-MS/MS acquisition (R=15,000 FHMW, 40% HCD) from the most intense ten parent ions with a charge state rejection of one and dynamic exclusion of 0.5 min.

1.3 Phosphoproteomics analysis

Phosphopeptide Enrichment

After digestion, 500 μg were used for phosphoproteomic analysis Peptide mixtures were enriched with TiO2 particles according to the High-Select TiO2 Phosphopeptide Enrichment Kit protocol (ThermoFisher Scientific, CA, USA). Briefly, peptides were suspended with Binding/Equilibration Buffer and were added to the TiO2 Spin Tip column, previously conditioned. After phosphopeptide binding the column was washed and phosphopeptides were eluted with Elution Buffer. Samples were dried in a speed vacuum before.

nanoLC-(Orbitrap)MS/MS analysis

Mass spectrometry analyses were performed on an EasyII nanoLC coupled to an LTQ-Orbitrap Velos Pro both from ThermoFisher by an enhanced FT-resolution MS

spectrum (R=30,000 FHMW) followed by a data-dependent FT-MS/MS acquisition (R=7,500 FHMW, 40% HCD) from the most intense ten parent ions with a charge state rejection of one and dynamic exclusion of 0.5 min. chromatographic separation was performed in reverse phase mode onto a C-18 reversed phase (RP) nano-column (75µm I.D.; 15cm length; 3µm particle diameter, Nikkyo Technos Co. LTD, Japan) with a 180 min gradient using Milli-Q water (0.1% formic acid) and acetonitrile (0.1% formic acid) as mobile phase at a flow rate of 300 nL/min.

Protein identification/quantification

Protein and peptide identification/quantification were performed on Proteome Discoverer software v.1.4.0.288 (ThermoFisher Scientific, CA, USA). For peptide identification, all MS and MS/MS spectra were analysed using the Mascot search engine (v.2.5). The workflow was set up using the Mascot node combing Homo Sapiens database (74449 entries) and the contaminants database (247 entries), both searches assuming trypsin digestion. Two missed cleavages were allowed and an error of 20 ppm for a FT-MS/MS fragmentation mass and 10 ppm for a FT-MS parent ion mass were allowed.

Oxidation of methionine, acetylation of N-termini and phosphorylation of serine, threonine and tyrosine were set as dynamic modifications, whereas carbamidomethylation of cysteine was set as static modifications. The false discovery rate (FDR) and protein probabilities were calculated by Fixed PSM value.

Quantification was performed by a label-free approach using the chromatographic peak area of identified peptides.