Detailed description of TMT-Mass Spectrometry method

Labelled peptides were fractionated using Pierce™ High pH Reversed-Phase Peptide Fractionation Kit into 8 fractions. Each fraction was re-solubilized in 0.1% aqueous formic acid and 2 µg of each was loaded onto a Thermo Acclaim Pepmap (Thermo, 75 µm ID X 2 cm C18 3 µm beads) precolumn and then onto an Acclaim Pepmap Easyspray (Thermo, 75 µm X 15 cm with C18 2 µm beads) analytical column separation using a Dionex Ultimate 3000 uHPLC at 250 nL/min with a gradient of 2-35% organic (0.1% formic acid in acetonitrile) over 3h running the default settings for MS3-level SPS TMT quantitation [123], on an Orbitrap Fusion instrument (Thermo Fisher Scientific) which was operated in DDA-MS3 mode. Briefly, MS1 scans were collected at 120,000 resolution, scanning from 375-1500 m/z, collecting ions for 50ms or until the AGC target of 4e5 was reached. Precursors with a charge state of 2-5 were included for MS2 analysis, which were isolated with an isolation window of 0.7 m/z. Ions were collected for up to 50 ms or until an AGC target value of 1e4 was reached and fragmented using CID at 35% energy; these were then read out on the linear ion trap in rapid mode. Subsequently, the top 10 (height) sequential precursor notches were selected from MS2 spectra for MS3 quantitative TMT reporter ion analysis, isolated with a m/z window of 2 m/z, and fragmented with HCD at 65% energy. Resulting fragments were read out in the Orbitrap at 60,000 resolution, with a maximum injection time of 105 ms or until the AGC target value of 1e5 was reached.

LC-MS/MS data were searched with Proteome Discoverer 2.5 against a Uniprot human database (20359 entries). Precursor and fragment tolerances were set to 10 ppm and 0.02 Da, respectively. Carbamidomethylation (C) and TMT16plex (N-ter and K) were set as fixed modification, whereas deamidation (NQ), oxidation (M) and phosphorylation (STY) were set as variable modifications. Trypsin was selected as Enzyme. GO term enrichment and pathway analyses were

performed with DAVID (https://david.ncifcrf.gov/) and STRING (https://string-db.org/), respectively.

[123] McAlister, G.C., et al., MultiNotch MS3 enables accurate, sensitive, and multiplexed detection of differential expression across cancer cell line proteomes. Anal Chem, 2014. 86(14): p. 7150-8.