Supplementary Methods

CRISPR-SpCas9 genome editing in cell lines

We used sgRNAs targeting NCOA4 as follows: sgNCOA4-1: (ACAGACCTGACTGTTCTCCA) (1) sgNCOA4-2: (GCATCACTACACCTCAAAAG) (2), and sgNCOA4-3 (TGAGGTGTAGTGATGCACGG). For a control cutting guide, we used sgROSA26 (sgControl): (ACAGCAAGTTGTCTAACCCG) (3). PaTu-8988T NCOA4 knockout (KO) clones were generated using a sgRNA targeting *NCOA4* (GTCTTAGAAGCCGTGAGGT) cloned into px330 (Addgene).

Quantitative Proteomics

Mass spectrometry-based proteomics was performed as previously described (4). The following reagents were employed: Isobaric TMT reagents (Thermo Fisher Scientific), BCA protein concentration assay kit (Thermo Fisher Scientific), Empore-C18 material for in-house made StageTips (3 M), Sep-Pak cartridges (100 mg, Waters), solvents for Liquid chromatography (LC) (J.T. Baker), mass spectrometry (MS)-grade trypsin (Thermo Fisher Scientific), Lys-C protease (Wako), and cOmplete protease inhibitors (Millipore Sigma). Unless otherwise noted, all other chemicals were purchased from Thermo Fisher Scientific.

Cell pellets from TCC-Pan2 cells or *KPC* or *KPCN* tumors were lysed using 8 M urea, 200 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (EPPS) at pH 8.5 with protease inhibitors. Samples were further homogenized, and DNA was sheared via sonication using a probe sonicator (20, 0.5 sec pulses; level 3). Total protein was determined using a BCA assay and stored at -80ºC until future use. A total of 50-100 µg

of protein was aliquoted for each condition and TMT channel for further downstream processing. Protein extracts were reduced using tris(2-carboxyethyl)phosphine (TCEP) for 15 min at room temperature. Next, samples were alkylated with 10 mM iodoacetamide for 45 min in the dark at room temperature. To facilitate the removal of incompatible reagents, proteins were precipitated using chloroform/methanol. Briefly, to 100 µL of each sample, 400 µL of methanol was added, followed by 100 µL of chloroform with thorough vortexing. Next, 300 µL of HPLC grade water was added and samples were vortexed thoroughly. Each sample was centrifuged at 14,000 x g for 5 min at room temperature. The upper aqueous layer was removed, and the protein pellet was washed twice with methanol and centrifuged at 14 000 x g for 5 min at room temperature. Protein pellets were re-solubilized in 200 mM EPPS buffer and digested overnight with Lys-C (1:100, enzyme: protein ratio) at room temperature. The next day, trypsin (1:100 ratio) was added and incubated at 37ºC for an additional 6 hr in a ThermoMixer set to 1,200 RPM. Digested samples were labeled immediately or stored at -80ºC until future use.

To each digested sample, 30% anhydrous acetonitrile was added and 50 µg of peptides were labeled using 100 µg of TMT reagent. To equalize protein loading, a ratio check was performed by pooling 2 µg of each TMT-labeled sample. Samples were combined and desalted using in-house packed C18 StageTips and analyzed by LC-MS/MS. Normalization factors were calculated from this label check, samples were mixed 1:1 across all TMT channels and desalted using a 100 mg Sep-Pak solid phase extraction cartridge. Eluted pooled peptides were fractionated immediately or stored at -80ºC until future use.

Pooled TMT-labeled peptide samples were resuspended in 10 mM ammonium bicarbonate, 5% acetonitrile, pH 8.0 buffer and were fractionated with basic-pH reverse-phase (bRP) HPLC using an Agilent 300 extend C18 column and collected into a 96 deep-well plate. Peptides were subjected to a 50 min linear gradient from 13 to 43% buffer B (10 mM ammonium bicarbonate, 90% acetonitrile, pH 8.0) at a flow rate of 0.25 mL/min. Samples were consolidated into 24 fractions as previously described, and 24 fractions were desalted using StageTips prior to analyses using LC-MS/MS.

All mass spectrometry data was acquired using an Orbitrap Lumos mass spectrometer in-line with a Proxeon NanoLC-1200 UHPLC system (RRID:SCR_020562). Peptides were separated using an in-house 100 μm capillary column packed with 40 cm of Accucore 150 resin (2.6 μm, 150 Å) (ThermoFisher Scientific) using a 120 min LC gradient from 4 to 24% acetonitrile in 0.125% formic acid per fraction. Eluted peptides were acquired using a synchronous precursor selection (SPS-MS3) method for TMT quantification as previously described (5). Briefly, MS1 spectra were acquired at 120K resolving power with a maximum of 50 ms ion injection in the Orbitrap. MS2 spectra were acquired by selecting the top 10 most abundant features via collisional induced dissociation (CID) in the ion trap using an automatic gain control (AGC) setting of 15K, quadrupole isolation width of 0.5 m/z and a maximum ion accumulation time of 50 ms. These spectra were passed in real time to the external computer for online database searching.

Intelligent data acquisition (IDA) using real-time searching (RTS) was performed using Orbiter (6). Whole proteome peptide spectral matches were analyzed using the Comet search algorithm (release_2019010) designed for spectral acquisition speed (7,8).

The same forward- and reversed-sequence human or mouse protein databases were used for both the RTS search and the final search (Uniprot). The RTS Comet functionality has been released and is available here: http://cometms.sourceforge.net/. Real-time access to spectral data was enabled by the Thermo Scientific Fusion API (https://github.com/thermofisherlsms/iapi). The core search functionalities demonstrated here have also been incorporated into the latest version of the Thermo Scientific instrument control software (Tune 3.3 on Orbitrap Eclipse) (9). Next, peptides were filtered using simple initial filters that included the following: not a match to a reversed sequence, maximum PPM error of <50, minimum XCorr of 0.5, minimum deltaCorr of 0.10 and minimum peptide length of 7. If peptide spectra matched to above criteria, an SPS-MS3 scan was performed using up to 10 *b*- and *y-type* fragment ions as precursors with an AGC of 200K for a maximum of 200 ms with a normalized collision energy setting of or 55 (TMTPro 16) (10).

All acquired data were processed using Comet (7) and a previously described informatics pipeline (11). Briefly, peptide spectral libraries were first filtered to a peptide false discovery rate (FDR) of less than 1% using linear discriminant analysis employing a target decoy strategy. Spectral searches were done using a custom fasta-formatted database which included common contaminants, reversed sequences (Uniprot Human, 2020 or Uniprot Mouse, 2020) and the following parameters: 50 ppm precursor tolerance, fully tryptic peptides, fragment ion tolerance of 0.9 Da and a static modification of TMT (+304.2071 Da) on lysine and peptide N-termini, carbamidomethylation of cysteine residues (+57.021 Da) were set as static modifications, while oxidation of methionine residues (+15.995 Da) was set as a variable modification. Resulting peptides were further

filtered to obtain a 1% protein FDR and proteins were collapsed into groups. Reporter ion intensities were adjusted to correct for impurities during synthesis of different TMT reagents according to the manufacturer's specifications. For quantitation, a total sum signal-to-noise of all report ion ions of 160 was required for analysis. Lastly, protein quantitative values were normalized so that the sum of the signal for all protein in each channel was equal to account for sample loading.

RNAi-mediated NCOA4 knockdown

Target sequences are as follows: ishControl: ATCTCGCTTGGGCGAGAGTAAG; ishNcoa4-1: GCCCTAAGGACGTGCCTAATA; ishNcoa4-2: TCCTACAAACCTCGGCTTTAA, ishNcoa4-3: CGGGCTGAACAGCAAATTAAA. For RNAi, siRNAs were obtained from Horizon Discovery (Dharmacon) as follows: siNCOA4: ACAAAGAUCUAGCCAAUCA (D-010321-01) and control non-targeting siRNA: siControl, D-001210-01-05.

References

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