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Supporting Information

Oncogenic KRAS creates an aspartate metabolism signature in colorectal cancer cells

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Bottom-up sample preparation

For each DLD-1 isogenic cell line, 25 µg of protein was acetone precipitate overnight at -20 °C, and resuspended in 25 mM Tris-HCl pH=7.8, 6 M urea and 2 M thiourea, 1 mM $MgCl_2$ (Sigma-Aldrich). Reduction of disulfide bonds was carried out at 40 °C for one hour by addition of DTT to a final concentration of 10 mM. Cysteine alkylation was performed by incubation of the protein solution with 15 mM iodoacetamide (Sigma-Aldrich) for 45 min in the dark at room temperature. Alkylation was quenched with the addition of DTT (Sigma-Aldrich), and lysates were diluted to 2 M urea using Tris-HCl pH=7.8. Lysates were then digested with rLys-C protease (Promega) at a 1:25 protease-to-protein ratio at 37°C for 4 hours under shaking (450 rpm). Lysates were then diluted four-fold in 25 mM Tris-HCl and mass spectrometry grade trypsin (Trypsin Gold, Promega, Madison, WI) was added at 1:50 protease-to-protein ratio. The digestion proceeded for 12 hours, rocking, at 37ºC. Digests were separately quenched by addition of TFA to final 0.1% (v/v). Proteolytic peptides were cleaned using C18 spin columns (Thermo Fisher Scientific), flash-frozen and lyophilized. Dried peptides from DLD-1 cells were resuspended in 0.1% TFA immediately before analysis.

Quantitative bottom-up mass spectrometry and data analysis

On-line reversed phase chromatography was performed using an EasyLC 1200 UPLC system (Thermo Scientific, Sunnyvale). Mobile Phase A consisted of 0.1% formic acid (FA) in water, while Mobile Phase B was 99.9% acetonitrile and 0.1% FA. Peptides (~1 µg) were loaded onto a

C18 EasySpray analytical column (500 mm, 75 µm i.d., Thermo Scientific), keeping constant pressure of 600 bar. After sample loading, the chromatographic gradient was run at 0.3 µL/min and consisted of a ramp from 0 to 35% Mobile Phase B in 68 min, followed by a wash at 90% Solution B in 7 min total, and a final re-equilibration step of 15 min (90 min total).

Samples were analyzed using a label-free quantitation strategy. Two biological replicates of each cell line were prepared, and each sample was analyzed in triplicate. The sample sequence was randomized. Mass spectrometry measurements were carried out on an Orbitrap QE HF-X mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a EasySpray ESI source (Thermo Scientific). Instrument parameters were set as follows: broadband MS^1 scans were recorded over a 375-1200 *m/z* window at 120,000 resolving power (at 200 *m/z*), with a maximum injection time of 50 ms and an AGC target of 1e6 charges. The RF of the ion funnel was set at 40% of maximum. Data-dependent MS^2 scans were obtained via HCD fragmentation (NCE=28) at 15,000 resolving power (at 200 *m/z*), with a maximum injection time of 32 ms and an AGC target of 1e5 charges. Precursor peptides were quadrupole isolated using a 1.2 *m/z* window. Dynamic exclusion was set to 20 s, and peptides with charges from 2+ to 5+ were considered for fragmentation.

Raw data was searched against the Swiss-Prot human database (Release 2017_04) with the MSAmanda node in the original Proteome Discoverer v2.2 software (and not MS Amanda plus IMP Vienna PD nodes in Proteome Discoverer) in a target-decoy approach [2] and subsequently filtered to HUPO reporting standards (1% FDR at the protein level). Integrated intensity values and statistics were handled by Proteome Discoverer and validated in R3.5.1.

Quantitative metabolomics:

For each biological replicate, 2e6 cells were seeded on 10 cm^2 plates two days prior to sample collection and shASS1 was induced with 500 ng/mL doxycycline. Cells were incubated with U13C6-glucose-labeled RPMI for 18 hours. Cells were washed with warm 0.9% saline and lysed on dry ice with -80ºC, 80% MeOH. Samples were flash frozen and thawed once and then the soluble metabolome was collected after centrifugation at 21,000 x g for 10 min at 4ºC. Metabolite extracts were evaporated to dryness and reconstituted in 250 µL of 90% acetonitrile / 10% water. A pooled sample was generated by combining 10 µL of each unlabeled sample in the study and was used for quality control purposes. A total of 2 µL was injected for each sample.

Instrument and method setup

Samples were analyzed using the Thermo Scientific™ Orbitrap ID-X™ Tribrid™ mass spectrometer (San Jose, CA) coupled to a Thermo Scientific™ Vanquish™ Horizon UHPLC system (Germering, Germany). Instrumentation was controlled by Thermo Scientific™ Xcalibur™ 4.2 software, Thermo Scientific™ Instrument Control Software 3.1 SP1 and Thermo Scientific™ SII software for Xcalibur 1.4. Chromatographic separation was achieved with a Sequant™ ZIC™-pHILIC column (15 cm × 2.1 mm ID). Metabolites were eluted into the mass spectrometer using a flow rate of 300µL/min and the following gradient: 90% B at 0 min; 30% B from 15 to 18 min; 90% B from 20 to 36 min. Solvent A consisted of water with 10 mM ammonium acetate, pH 9.8 and solvent B consisted of acetonitrile. The column temperature was set to 45°C.

Each sample (unlabeled and labeled) was analyzed using the “MS1 Profiling” method template. The method consisted of a Full MS scan in negative polarity with a scan range of *m/z* 67-1000, applying a resolving power of 120,000, with AGC target of 1e5 and a maximum inject time of 50 ms. The samples were analyzed in a randomized order and the pooled sample (QC) was injected

every 9 samples. For unknown identification, the AcquireX Deep Scan workflow within the Xcalibur Software was used. In brief, the method template named "AcquireX Inclusion-Exclusion Reference" was used for the analysis of a solvent blank and the pooled sample for the automatic generation of target exclusion and target inclusion lists, respectively. The method consisted of a Full MS scan in negative polarity with a scan range of *m/z* 67-1000, applying a resolving power of 120,000, with AGC target of 1e5 and a maximum inject time of 50 ms. Then, the pooled sample was iteratively analyzed using the "AcquireX identification MS2" method template. The method consisted of a Full MS scan in negative polarity with a scan range of *m/z* 67-1000, applying a resolving power of 60,000, with AGC target of 1e5 and a maximum inject time of 50 ms and data dependent MS2. MS2 was performed using stepped HCD and NCE values of 20%, 35% and 50%. Isolation width was set to 1.5 and fragment ions were detected in the Orbitrap with resolving power of 30,000, AGC target of 5e4 and maximum inject time of 54ms. Inclusion list targets determined by AcquireX were prioritized over other precursors. After each reinjection of the pooled sample the inclusion and exclusion lists were updated based on the precursors fragmented to allow for more unique and less abundant sample components to be analyzed. Other parameters were set as follows: spray voltage 3000 V, sheath gas 40 AU, auxiliary gas 8 AU, sweep gas 1 AU, ion transfer tube temperature 275°C, vaporizer temperature 320°C and S-lens RF amplitude of 35%.

Data Processing

Untargeted metabolomics data were processed using Thermo Scientific™ Compound Discoverer™ 3.0 software. A modified version of the "Stable Isotope labeling w/ metabolika Pathways and ID using online databases" was employed to provide unknown and labeled peak detection followed by differential analysis, exhaustive compound annotation, and pathway

mapping. Briefly, the workflow included retention time alignment, unknown compound detection, and compound grouping across all samples, elemental composition prediction for all compounds, labeled compound detection and fractional label incorporation calculation after natural abundance correction for each compound. The workflow also included database searching at the precursor level against ChemSpider and a custom small molecule database, mzLogic to rank putative candidates generated from ChemSpider, as well as MS2 spectral matching against mzCloud and a local mzVault spectral library. Differential analysis and compound mapping to biological pathways against Metabolika, KEGG and BioCyc were also performed within Compound Discoverer software.

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