Experimental Section

Animals

Inbred male Lewis (RT-1^{l}) rats weighing 250–300 g were purchased from Envigo RMS, Inc. (Indianapolis, IN). Animals were maintained in laminar flow cages in a pathogen-free animal facility at the University of Pittsburgh and given a standard diet and water *ad libitum*. All procedures were performed according to the guidelines of the Institutional Animal Care and Use Committee at the University of Pittsburgh and the National Research Council's Guide for the Humane Care and Use of Laboratory Animals.

Ex vivo lung perfusion (EVLP) in Rats

EVLP was performed using a commercially available rodent EVLP system (IL-2 Isolated Perfused Rat or Guinea Pig Lung System; Harvard Apparatus, Holliston, MA) as described previously. (1) Acellular Steen solution was used for perfusate and medicated with methylprednisolone (Solu-Medrol®; Pfizer, Inc., New York, NY) and cephalosporin (Cefazolin; WG critical care LLC, Paramus, NJ) equally in all experimental groups using EVLP. Perfusion flow was started at 10% of target flow and gradually increased for 1 hour toward a target flow rate that was calculated as 20% of cardiac output (75 mL/min/250 g donor body weight). Pulmonary artery pressure, peak airway pressure, and airway flow were monitored continuously, and dynamic lung compliance and pulmonary vascular resistance were also analyzed. Ac_4GalNAz for bioorthogonal metabolic labeling was dissolved in DMSO as a stock solution and administered into perfusate at a final concentration of 100 μM at the time of priming. To administer cyclosporine A (CyA) during EVLP, CyA (Sigma-Aldrich) was administered into the perfusate at a final concentration of 1 mM at the time of priming.

Cellular Protein Extraction and Biotinylation of the Rat Lungs

Rat lungs after EVLP or LTx were dissected into small pieces and stored at -80 °C. The frozen samples were minced into fine pieces with surgical scissors and homogenized in 2 mL tubes pre-filled with glass beads (Benchmark Scientific, D1031-10). The homogenized tissues were washed with PBS to remove the perfusate residues. The cellular fractions were extracted with 8 mM CHAPS buffer (with 1 M NaCl, 1% protease inhibitor in PBS, pH 5.50) at 4 °C. The extracts were reacted to Alkyne-PEG4-biotin under the CuAAC condition for Western blot analysis. The protein samples were quantified by BCA protein assay kit (Pierce, 23225) for equal protein input amount and analyzed using SDS-PAGE.

Chemoselective Enrichment of Azide-tagged proteins

The cellular protein extracts of the lungs were reacted with Alkyne-PEG4-desthiobiotin under the CuAAC condition. The proteins were precipitated with four volumes of ice-cold acetone at -20 °C overnight to remove unreacted Alkyne-PEG4-desthiobiotin. The protein pellets were air-dried and re-dissolved in 2% SDS in PBS (pH 7.40), then saved as *Input* samples. Following quantification using BCA protein assay kit (Pierce, 23225), an aliquot of each *Input* sample containing 1.7 mg of protein was introduced to 75 μL streptavidin-conjugated resins (Thermo Fisher Scientific, 53117) for desthiobiotinylated protein pull-down. Following 3-hour binding, the *Supernatants* were collected and the resins were washed 7 times, 15 mins each, with 2% SDS, 40 mM additional NaCl in PBS (pH 7.40). The pulled-down proteins were eluted with 30 mM biotin in 2% SDS, 40 mM additional NaCl in PBS (pH 7.40) (Eluting buffer) at room temperature for 1 hour. The eluates were concentrated with a 10 kDa molecular cut-off filter (Millipore Sigma, UFC5010) to ~67 μL. These *Eluate* samples had their protein concentrations determined by BCA assay and then were stored at -80 °C for SDS-PAGE and proteomic analysis.

Proteomic Analysis with Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

The *Eluate* samples were further concentrated by vacuum evaporation to ~36 μL total volume. A 4.5 μL aliquot of each *Input* sample was diluted to 36 μL with 4% SDS. Disulfide bonds were reduced by adding 1.5 μL of 233 mM dithiothreitol (DTT) (Sigma) and heating to 55 ºC for 15 mins after cooling, proteins were alkylated in the dark for 20 mins by adding 2.3 μL of 0.5 M iodoacetamide

(Sigma). Samples were acidified by adding 3.9 µL of 27.5% phosphoric acid, and subsequently diluted 6.5-fold with 240 µL of 0.1 M Tris buffer in 90% methanol. Samples were loaded into S-Trap™ micro columns (Protifi) in two aliquots by centrifuging after each addition; columns were washed four times with 150 µL of 0.1 M Tris buffer in 90% methanol. Proteolytic digestion occurred within the S-traps at 47 ºC for 75 mins after adding 1 µg of trypsin (Promega) in 20 µL of 50 mM ammonium bicarbonate (ABC, Sigma). Peptides were eluted in 40 µL of 50 mM ABC, followed by 40 µL of 0.2% formic acid (Sigma), followed by 40 µL of 1:1 acetonitrile:water. Peptide samples were evaporated to dryness and then reconstituted with 10 µL of 5% acetonitrile containing 0.2% formic acid. LC-MS/MS analysis (below) was performed with 2.6 µL of each *Eluate* sample. A variable amount (1.0 to 2.0 µL) of each *Input* sample was injected to equalize their protein amounts based upon BCA results.

Samples were analyzed using a UPLC-MS/MS system consisting of an Easy-nLC 1200 ultra-high-pressure liquid chromatography system and an Orbitrap Fusion Lumos mass spectrometer (ThermoFisher Scientific). Injected peptide samples were loaded at a pressure of 300 bar onto a 20 cm long fused silica capillary nano-column packed with C18 resin (1.7 µm-diameter, 130 Å pore size from Waters). Peptides eluted over 120 mins at a flow rate of 350 nL/min with the following gradient, where buffer A was aqueous 0.2% formic acid and buffer B was 80% acetonitrile with 0.2% formic acid: time 1 min–5% buffer B; time 52 mins–30% buffer B; time 80 mins–42% buffer B; time 90 mins–55% ACN; time 95 to 100 mins–85% buffer B; time 101 to 120 mins–equilibrate at 0% buffer B. The nano-column was held at 60 ºC using a column heater constructed in-house.

The nanospray source voltage was set to 2200 V. Full-mass profile scans were performed in the orbitrap between 375-1500 m/z at a resolution of 120,000, followed by MS/MS HCD scans in the orbitrap of the highest intensity parent ions in a 3 second cycle time at 30% relative collision energy and 15,000 resolution, with a 2.5 m/z isolation window. Charge states 2-6 were included and dynamic exclusion was enabled with a repeat count of one over a duration of 30 seconds and a 10 ppm exclusion width both low and high. The AGC target was set to “standard”, maximum inject time was set to “auto”, and 1 µscan was collected for the MS/MS orbitrap HCD scans.

Proteomic Data Analysis

LC-MS/MS data were analyzed with the free and open-source search software program MetaMorpheus (version 0.0.320, https://github.com/smith-chem-wisc/MetaMorpheus/releases). The Swiss-Prot Rat XML (canonical) database containing 8,137 protein entries (downloaded from UniProt 2/09/2022) was utilized, along with MetaMorpheus’s contaminants database. MetaMorpheus was used to calibrate the 24 raw files and then subject them to Global Post-Translational Modification Discovery (GPTMD) (2) in order to identify PTMs not annotated in the reference database including: the Common Biological, Common Artifact, and Metal Adduct categories within MetaMorpheus; as well as two custom modifications involving the azido sugar labels (on serine/threonine with and without the alkyne-biotin at +244.0808 Da and +671.3490, respectively). Next, the mass spectra files were searched against the GPTMD databases using the following search settings: protease = trypsin; maximum missed cleavages = 2; minimum peptide length = 7; maximum peptide length = unspecified; initiator methionine behavior = Variable; fixed modifications = Carbamidomethyl on C, Carbamidomethyl on U; variable modifications = Oxidation on M; max mods per peptide = 2; max modification isoforms = 1024; precursor mass tolerance = ±5 PPM; product mass tolerance = ±20 PPM; report PSM ambiguity = True.

References:

1. Noda K, Shigemura N, Tanaka Y, Bhama JK, D'Cunha J, Luketich JD, et al. Successful prolonged ex vivo lung perfusion for graft preservation in rats. European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery. 2014;45(3):e54-60.

2. Solntsev SK, Shortreed MR, Frey BL, Smith LM. Enhanced Global Post-translational Modification Discovery with MetaMorpheus. J Proteome Res. 2018;17(5):1844-51.