**Photolabeling and Target Engagement Studies**

For gel-based experiments, cells were grown in 6-well plates to ∼80% confluence at the time of treatment. Cells were carefully washed with Dulbecco’s phosphate buffered saline (DPBS) and replenished with fresh serum-free media containing indicated probe, and, if applicable, competitors or DMSO vehicle. Following incubation at 37°C for 30 min, cells were directly exposed to 365 nm light for 10 minutes at 4°C. For negative control experiments without UV irradiation, cells were incubated at 4°C for 10 min under ambient light. For MS-based experiments, cell labeling was performed in a similar manner as described above. Modifications to this protocol included using isotopically ‘light’ and ‘heavy’ SILAC cells that were grown to ∼80% confluence prior to treatment in 10 cm plates. In probe-versus-control probe and probe-versus-probe experiments, isotopically light cells were treated with indicated fragment probe, while the heavy cells were treated with control or additional photoreactive probe to be compared, at indicated concentrations. In competition experiments, heavy and light cells were co-treated with the indicated FFF probe and competitor or DMSO, respectively. Following treatments and photocrosslinking, cells were harvested in cold DPBS by scraping, centrifuged (1,300 g, 3 min, 4°C), and pellets washed with cold DPBS (2X) and then aspirated. Pellets were either directly processed or snap frozen in liquid N2 and kept at −80°C until use.

**Preparation of Probe-labeled Proteome for Gel- and MS-based Protein Analyses**

Cells pellets were lysed in cold 0.8% NP-40 Lysis Buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 0.8% Igepal, 5% glycerol and sonicated using a Branson Sonifier probe (10 pulses, 30% duty cycle, output setting = 4). For experiments requiring enrichment of mitochondria, cells were processed as described in the respirometry section. Protein concentration was determined using the DC Protein Assay (Bio-Rad) and absorbance read using a Tecan, Infinite F500 plate reader following manufacturer’s instructions. For SILAC experiments, isotopically heavy and light whole cell lysates were adjusted to 1.5 mg/mL and were then mixed in equal proportions (500 μL each) in cold DPBS.

**Gel-based Analysis of In Situ Photolabeling**

Proteomes from treated cells were diluted to 1 mg/mL. To each sample (50 μL), 6 μL of a freshly prepared “click” reagent mixture containing 0.1 mM tris(benzyltriazolylmethyl)amine (TBTA) (3 μL/sample, 1.7 mM in 1:4 DMSO:t-ButOH), 1 mM CuSO4 (1 μL/sample, 50 mM in H2O), 25 μM tetramethylrhodamine (TAMRA) azide (1 μL/sample, 1.25 mM in DMSO), and freshly prepared 1 mM tris(2-carboxyethyl)phosphine HCl (TCEP) (1 μL/sample, 50 mM in H2O) was added to conjugate the fluorophore to probe-labeled proteins. Upon addition of the click mixture, each reaction was immediately mixed by vortexing and then allowed to react at ambient temperature for 1 hr before quenching the reactions with SDS loading buffer (4X stock, 17 μL). Proteins (25 μg total protein loaded per gel lane) were resolved using SDS-PAGE (10% acrylamide) and visualized by in-gel fluorescence on a Bio-Rad ChemiDoc MP flatbed fluorescence scanner. Gel fluorescence and imaging was processed using Image Lab (v 5.2.1) software.

**Preparation of TMT-Labeled Proteome for MS-based Analysis**

Profiling experiments were adapted from methods previously reported (Niphakis et al., 2015). For SILAC experiments, heavy and light soluble proteomes (1.5 mg) were combined in 1 mL DPBS. A mixture of TBTA (60 μL/sample, 1.7 mM in 1:4 DMSO:t-BuOH), CuSO4 (20 μL/sample, 50 mM in H2O), TCEP (20 μL/sample, 50 mM in DPBS) and Biotin-N3 (10 μL/sample, 10 mM in DMSO) was added and each sample was rotated at room temperature. After 1 hr, the mixture was transferred to a 15 mL falcon tube and a cold 4:1 mixture (2.5 mL) of methanol (MeOH)/chloroform (CHCl3) was added followed by cold PBS (1 mL) on ice. The resulting mixture was centrifuged (5,000 x g, 10 min, 4°C) to fractionate the protein interphase from the organic and aqueous solvent layers. After washing the protein disc carefully with cold 1:1 MeOH:CHCl3 (3 × 1 mL), the protein disc was sonicated in cold 4:1 MeOH:CHCl3 (3 mL), and the remaining precipitate was pelleted by centrifugation (5,000 x g, 10 min, 4°C). The pellet was aspirated and resuspended in a freshly prepared solution of proteomics-grade urea (500 μL, 6 M in DPBS) containing 10 μL of 10% SDS and then dissolved by sonication. Disulfides were reduced by adding 50 μL of a 1:1 mixture containing TCEP (200 mM in DPBS) pre-neutralized with potassium carbonate (600 mM DPBS) for 30 min at 37°C. Reduced thiols were then alkylated by addition of iodoacetamide (70 μL of 400 mM in DPBS) for 30 min at ambient temperature protected from light. To each solution, 130 μL of 10% SDS (in DPBS) was added and then diluted to ~0.2% SDS with DPBS (5.5 mL) and incubated with pre-equilibrated streptavidin agarose resin (100 μL 1:1 slurry, Pierce) for 1.5 hr at ambient temperature on a rotator. The streptavidin beads were collected by centrifugation (1,300 g, 5 min) and sequentially washed with 0.2% SDS in DPBS (1 × 5 mL), detergent-free DPBS (2 × 5 mL), and H2O (2 × 5 mL) to remove unbound protein, excess detergent, and small molecules. The resin was transferred to a Protein LoBind tube (Eppendorf) and bound proteins were digested on-bead overnight at 37°C in ∼200 μL total volume containing sequencing grade porcine trypsin (2 μg, Promega) in the presence of urea (2 M in DPBS) and CaCl2 (1 mM). The proteolyzed supernatant was transferred to a new Protein LoBind tube, acidified with formic acid (5% final) and stored at –20°C until analyzed.

**Preparation of Unenriched Proteomes for MS-based Protein Analyses**

3x10 cm plates per cell line were grown in complete DMEM, treated with indicated compound or control and harvested at ~80% confluence in cold DPBS by scraping. Cells were centrifuged (1,300 g, 3 min, 4°C), and pellets washed with cold DPBS (2X). Pellets were then snap frozen in liquid N2 and kept at −80°C until the day prior to analysis. Cell pellets were resuspended in 0.8% NP-40 Lysis Buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 0.8% Igepal, 5% glycerol, 1x protease inhibitor cocktail (Roche), and sonicated using a Branson Sonifier probe (3x10 pulses, 40% duty cycle, output setting = 4). Samples were spun down (1000xg for 10min) and reduced for 60 min at 37°C. Samples were alkylated with iodoacetamide (50 mM final) for 45 min in the dark and quenched for 15 min with DTT (5mM final) in the dark. Protein was isolated from the lysates via methanol-chloroform protein extraction. The isolated protein was reconstituted in 8M urea via sonication and spun down at 2,000xg for 5 min. A DC Protein Assay (Bio-Rad) was used to measure protein concentration of each sample in triplicate. 1 mg of each sample was digested overnight at 37 °C in 2 M urea in 200mM EPPS buffer containing sequencing grade porcine trypsin (Promega, V5111) in the presence of CaCl2 (1 mM). Samples were acidified with 20% acetic acid (pH was reduced to ∼2-3), desalted in C18 Sep-Pak columns (Waters) and eluates were dried via speedvac. Dried peptides were resuspended in 200mM EPPS pH 8.0 and peptide level was quantified using the microBCA Peptide Assay (Pierce) in triplicate. Anhydrous acetonitrile was added to 25 µg peptide in 35 µL EPPS to 30% final volume. 6 µL (20 µg/µL) of respective 10-plex TMT tag (Thermo Scientific, cat # 90110) was added and the reaction was incubated at room temperature for 1 hour with occasional vortex before quenching with 6 µL of 5% hydroxylamine for 15 min followed by 4 µL formic acid. 5 µg of each channel was combined, vacuum-centrifuged to near dryness, and reconstituted in Buffer A (95% H2O, 5% acetonitrile, 0.1% formic acid) prior to high pH fractionation.

**Preparation of Tandem Mass Tag (TMT) Labeled Samples for Multiplexed MS-based Analysis**

After streptavidin enrichment, the beads were centrifuged (1,300 g, 5 min) and washed sequentially with 0.2% SDS in DPBS (1 x 5 mL), DPBS (2 x 5 mL) and 200mM EPPS (Sigma Aldrich, E9502, pH 8, 1 x 5 mL). The beads were transferred to a low-binding 1.5 mL microfuge tube (Axygen, CNT-1.5FL) and the enriched proteins were digested on-bead overnight at 37°C in ~200 µL of 2 M urea in 200mM EPPS buffer containing 2 µg sequencing grade porcine trypsin (Promega, V5111) in the presence of CaCl2 (1 mM). The samples were centrifuged to separate the beads and supernatant, then anhydrous acetonitrile was added to the supernatant to 30% final volume. 6 µL (20 µg/µL) of respective 10-plex TMT tag (Thermo Scientific, cat # 90110) was added and the reaction was incubated at room temperature for 1 hour with occasional vortex before quenching with 6 µL of 5% hydroxylamine for 15 min followed by 4 µL formic acid. All 10-plex samples were vacuum-centrifuged to near dryness, reconstituted in Buffer A (95% H2O, 5% acetonitrile, 0.1% formic acid), combined and stored at -80°C until analysis.

**High pH Fractionation**

The spin columns for high pH fractionation were pre-equilibrated prior to use. Briefly, the columns were placed in Eppendorf tubes (2 mL), spun down to remove the storage solution (5,000 g, 2 min), and washed with CH3CN (2 × 300 μL, 5,000 g, 2 min) and buffer A (2 × 300 μL, 95% H2O, 5% CH3CN, 0.1% FA, 5,000 g, 2 min). TMT labeled peptides were re-dissolved in buffer A (300 μL, 95% H2O, 5% CH3CN, 0.1% FA) and loaded onto pre-equilibrated spin columns for high pH fractionation. The columns were spun down (2,000 g, 2 min) and the flow through was used to wash the original Eppendorf tube and passed through the spin column again (2,000 g, 2 min). The column was then washed with buffer A (300 μL, 2,000 g, 2 min) and 10 mM aqueous NH4HCO3 containing 5% CH3CN (300 μL, 2,000 g, 2 min), and the flow through was discarded. The peptides were eluted from the spin column into fresh Eppendorf tubes (2.0 mL) with a series of 10 mM NH4HCO3 / CH3CN buffers (2000 g, 2 min). The following buffers were used for peptide elution (% CH3CN): 7.5, 10, 12.5, 15, 17.5, 20, 22.5, 25, 27.5, 30, 32.5, 35, 37.5, 40, 42.5, 45, 47.5, 50, 52.5, 55, 75. Every 7th fraction was combined into a new clean Eppendorf tube (2 mL) and the solvent was removed using SpeedVac vacuum concentrator. The resulting 7 combined fractions were re-suspended in buffer A (24 μL) and analyzed on the Orbitrap Fusion mass-spectrometer (5 μL injection volume).

**Liquid Chromatography-Mass-Spectrometry (LC-MS) Analysis**

Samples were analyzed by liquid chromatography tandem mass-spectrometry using an Orbitrap Fusion mass spectrometer (Thermo Scientific) coupled to an UltiMate 3000 Series Rapid Separation LC system and autosampler (Thermo Scientific Dionex). The peptides were eluted onto a capillary column (75 μm inner diameter fused silica, packed with C18 (Waters, Acquity BEH C18, 1.7 μm, 25 cm) and separated at a flow rate of 0.25 μL/min using the following gradient: 5% buffer B in buffer A from 0-15 min, 5%–35% buffer B from 15-155 min, 35%–95% buffer B from 155-160 min, 95% buffer B from 160-169 min, 95%–5% buffer B from 169-170 min, and 5% buffer B from 170-200 min (buffer A: 95% H2O, 5% acetonitrile, 0.1% FA; buffer B: 5% H2O, 95% CH3CN, 0.1% FA). The voltage applied to the nano-LC electrospray ionization source was 1.9 kV. Data was acquired using an MS3-based TMT method adapted from Wang et al. (2019). Briefly, the scan sequence began with an MS1 master scan (Orbitrap analysis, resolution 120,000, 400−1700 m/z, RF lens 60%, automatic gain control [AGC] target 2E5, maximum injection time 50 ms, centroid mode) with dynamic exclusion enabled (repeat count 1, duration 15 s). The top ten precursors were then selected for MS2/MS3 analysis. MS2 analysis consisted of: quadrupole isolation (isolation window 0.7) of precursor ion followed by collision-induced dissociation (CID) in the ion trap (AGC 1.8E4, normalized collision energy 35%, maximum injection time 120 ms). Following the acquisition of each MS2 spectrum, synchronous precursor selection (SPS) enabled the selection of up to 10 MS2 fragment ions for MS3 analysis. MS3 precursors were fragmented by HCD and analyzed using the Orbitrap (collision energy 55%, AGC 1.5E5, maximum injection time 120 ms, resolution was 50,000). For MS3 analysis, we used charge state–dependent isolation windows. For charge state z = 2, the MS isolation window was set at 1.2; for z = 3-6, the MS isolation window was set at 0.7. The MS2 and MS3 files were extracted from the raw files using RAW Converter (version 1.1.0.22; available at http://fields.scripps.edu/rawconv/), uploaded to Integrated Proteomics Pipeline (IP2), and searched using the ProLuCID algorithm (publicly available at http://fields.scripps.edu/downloads.php) using a reverse concatenated, non-redundant variant of the Human UniProt database (release-2012\_11). N-termini and lysine residues were also searched with a static modification corresponding to the TMT tag (+229.1629 Da). Peptides were required to be at least 6 amino acids long, to have at least one tryptic terminus, and to contain the DTB modification. ProLuCID data was filtered through DTASelect (version 2.0) to achieve a peptide false-positive rate below 1%.

**TMT-ABPP Ratio Calculation for Target Engagement Studies**

At the individual TMT experiment level, the following filters were applied to remove low-quality peptides: removal of non-unique peptides, removal of half-tryptic peptides, removal of peptides with more than one internal missed cleavage site, removal of peptides with low (< 10,000) sum of reporter ion intensities in both expanded or activated channels, removal of peptides with high variation (coefficient of variance > 0.5) between the replicate expanded or activated channels if their sum of reporter ion intensities is greater than 5,000. R values (For Competition based studies) for each peptide were calculated using the average reporter ion intensities of activated and expanded TMT channels.