

Supporting Information:

SP3 FAIMS chemoproteomics for high coverage profiling of the human cysteinome

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(A) Supplementary Figures

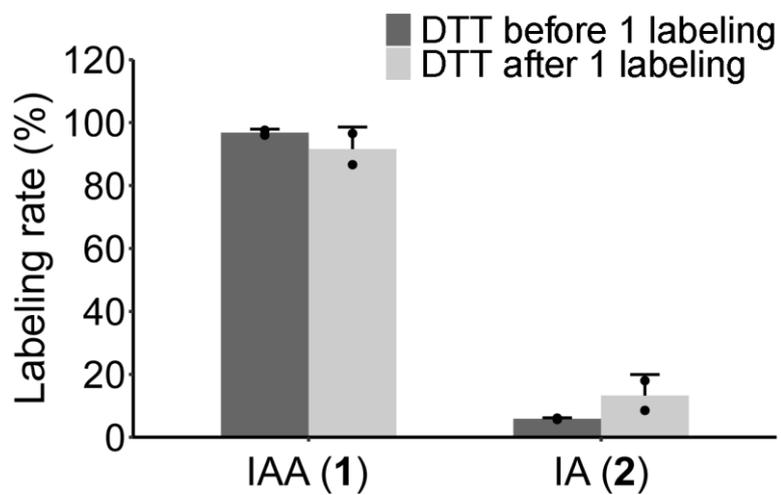


Figure S1. Percentage of cysteines labeled with IA-alkyne (1) or IA (2) when treated with 1 mM DTT (before), 2 mM IAA and 20 mM IA, or 2mM IA, 10mM DTT (after) and 20 mM IA. Experiments were performed in duplicate.

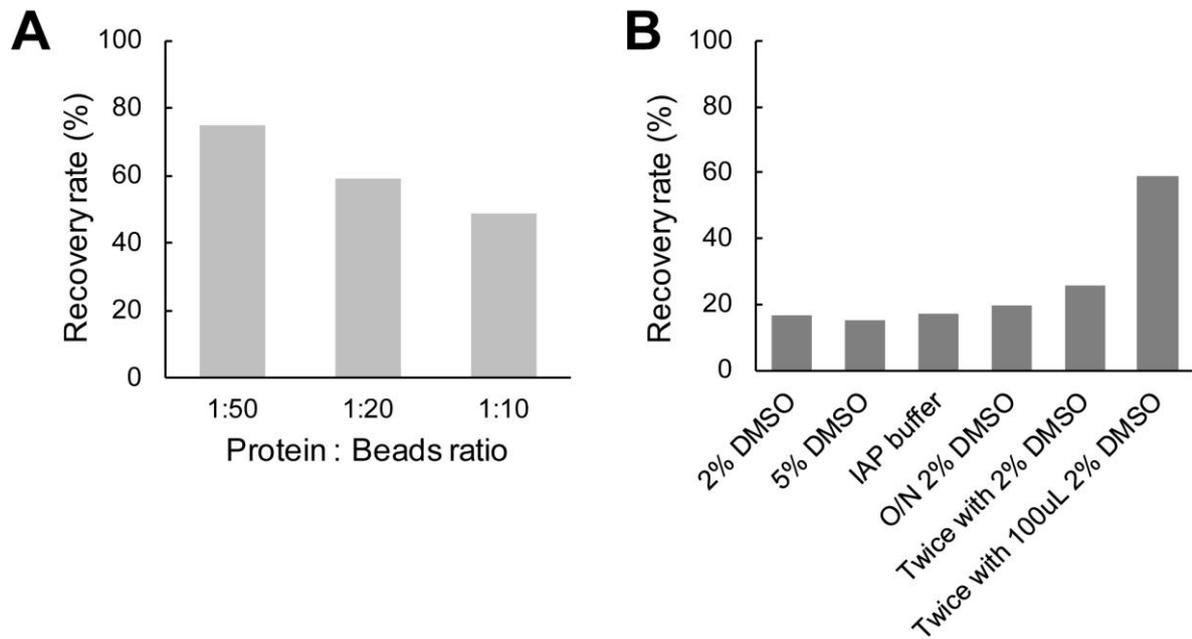


Figure S2. Optimization of SP3 protocols. (A) Recovery rate (amount of eluted peptides / amount of starting material * 100%) of SP3 with different protein to beads ratios. (B) Recovery rate of SP3 with different elution conditions. Elution condition is 50 μ L at 37°C for 30 min if not indicated.

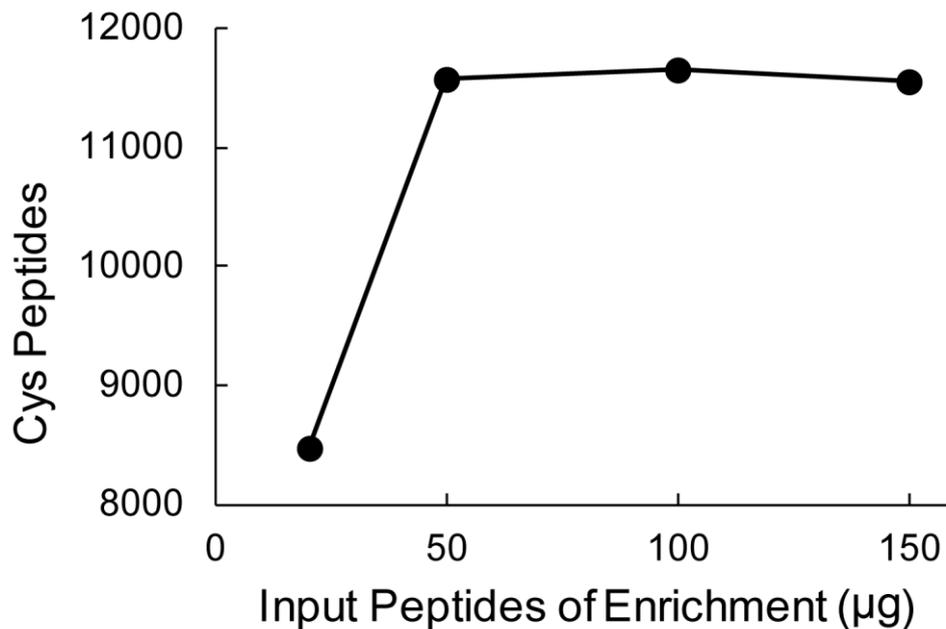


Figure S3. Number of cysteine peptides identified with different amounts of input peptides of NeutrAvidin enrichment.

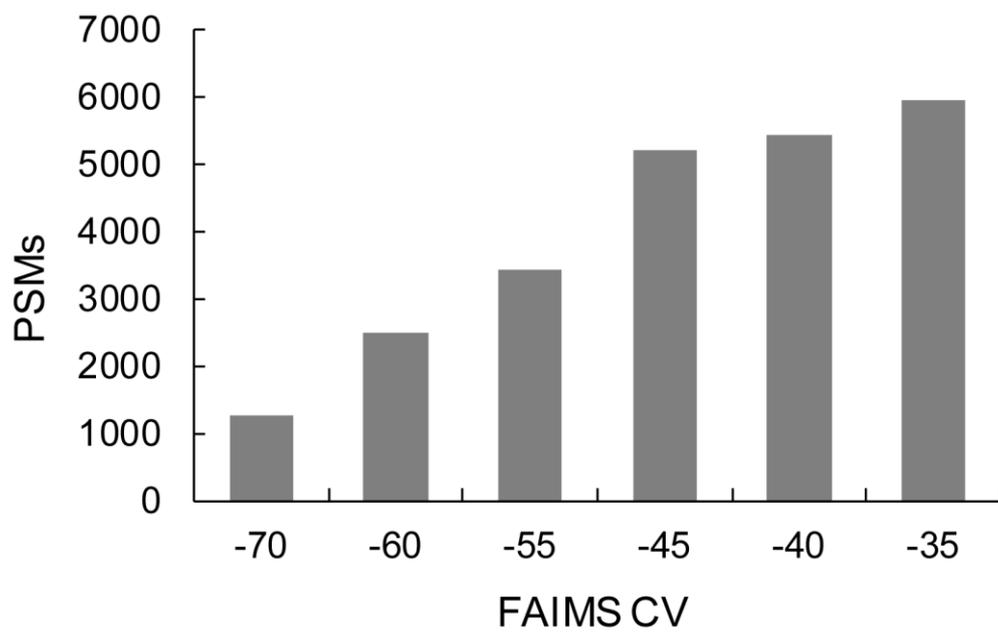


Figure S4. PSMs identified with different FAIMS CV (-70, -60, -55, -45, -40, -35 V).

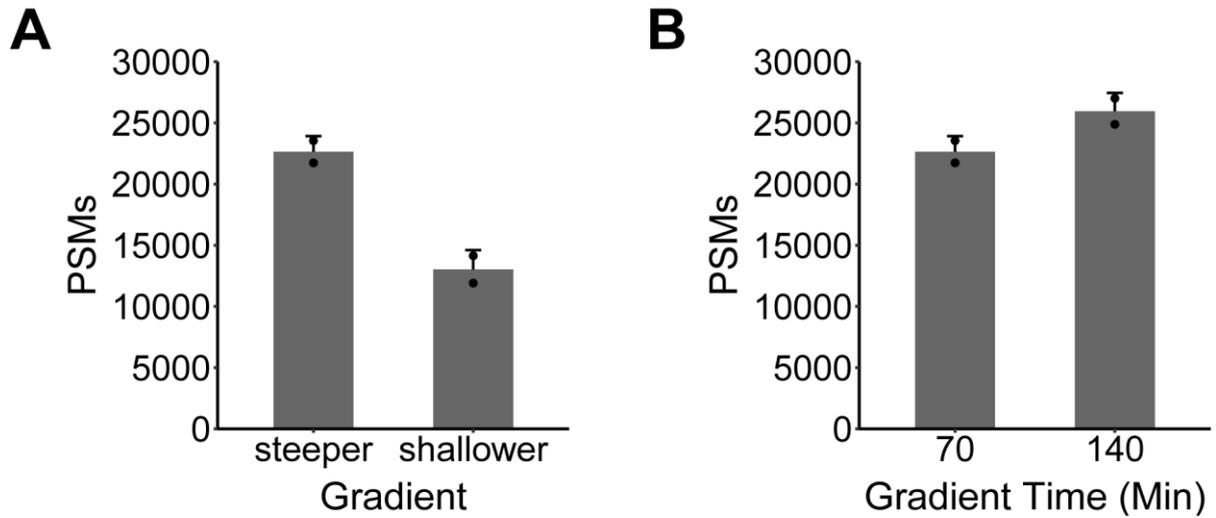


Figure S5. PSMs identified with different (A) gradient settings and (B) gradient time of liquid chromatography (LC) in LC-MS/MS analysis of cysteine enriched peptides. Experiments were performed in duplicate.

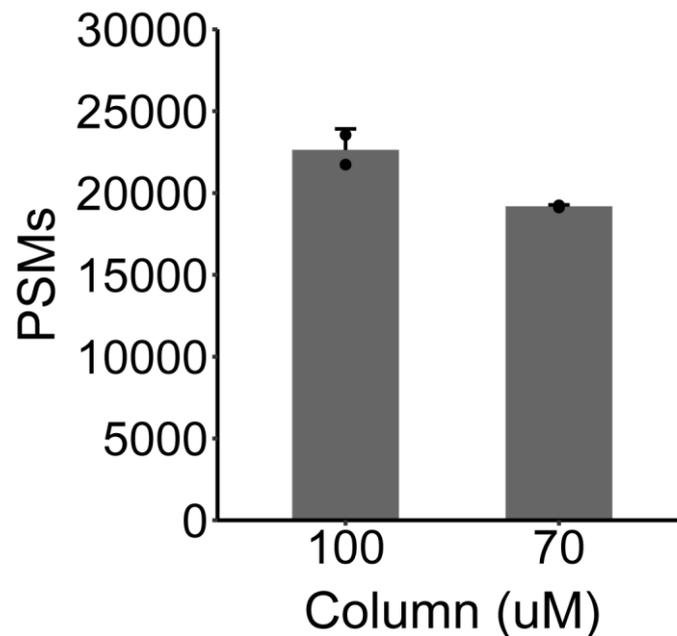


Figure S6. PSMs identified with different C18 column internal diameters, used for LC-MS/MS analysis of cysteine enriched peptides. Experiments were performed in duplicate.

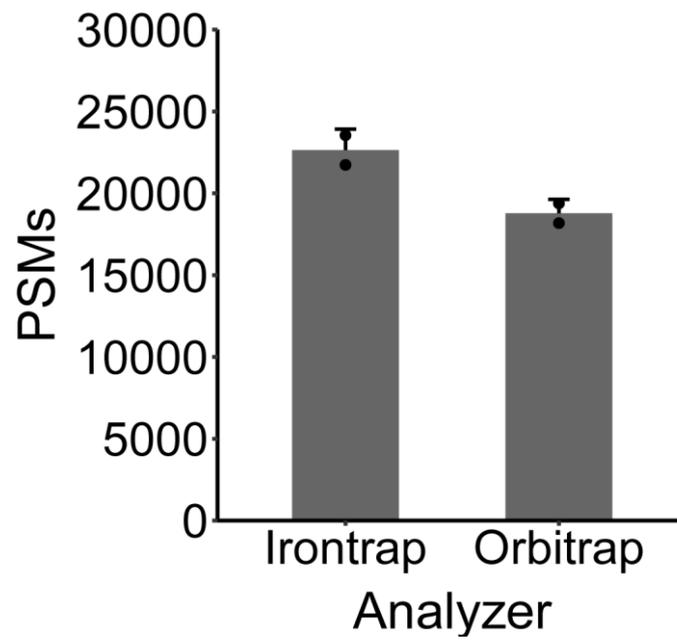


Figure S7. PSMs identified with different MS/MS fragmentation mass analyzer in LC-MS/MS analysis of cysteine enriched peptides. Experiments were performed in duplicate.

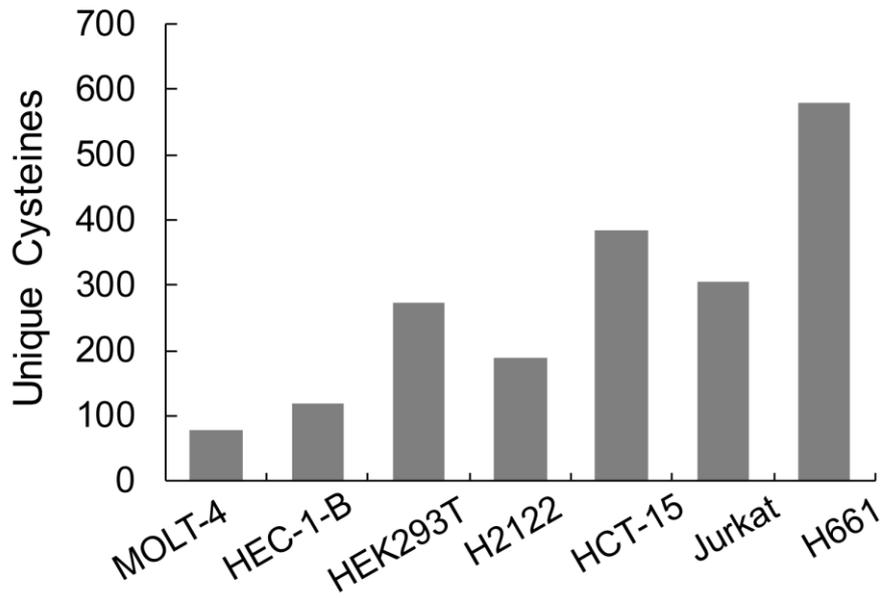


Figure S8. Number of unique cysteines identified only in a specific single cell line and in both replicates.

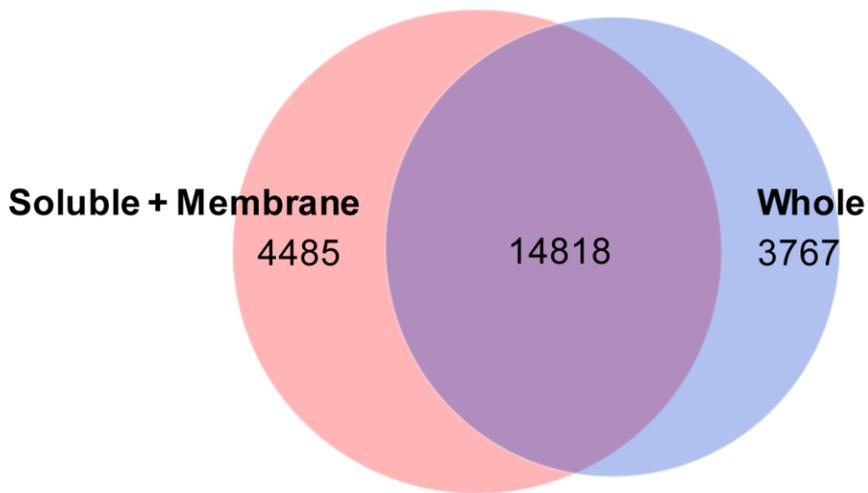


Figure S9. Venn diagram of unique cysteines identified with and without soluble/membrane fractionation of HEK293T cells.

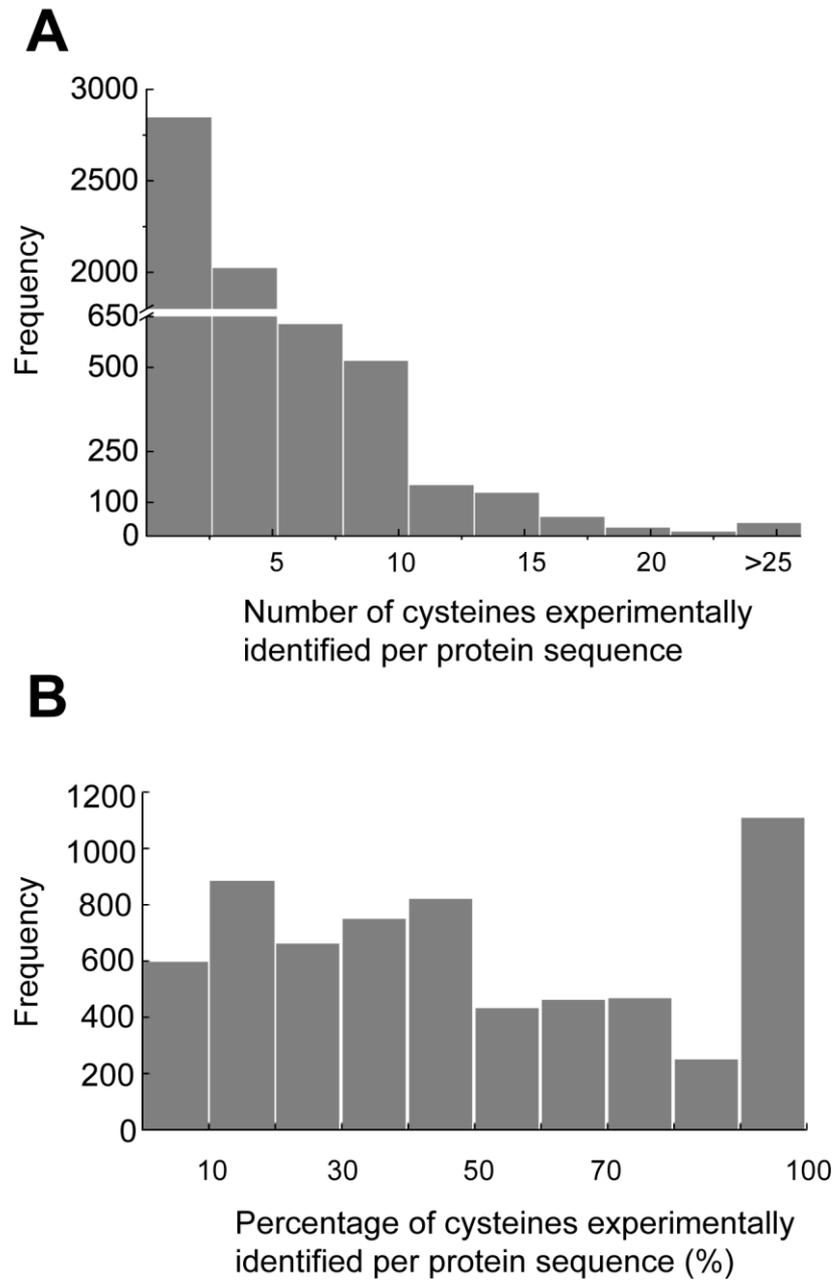


Figure S10. Histograms of (A) number of cysteines and (B) percentage of cysteines experimentally identified per protein sequence.

(B) Supplementary Tables

Table S1-6 can be found as excels attached.

Table S7. Files in PRIDE datasets.

PRIDE dataset	Figure	File name	Experiment
PXD023056 (Figure 1)	1B	2020-11-19-kb-70min_FAIMS_100micron_3cv_-35_-45_-55--OTIT_SY33-1	0.1 mM-1
		2020-11-19-kb-70min_FAIMS_100micron_3cv_-35_-45_-55--OTIT_SY33-9	0.1 mM-1
		2020-11-19-kb-70min_FAIMS_100micron_3cv_-35_-45_-55--OTIT_SY33-2	1 mM-1
		2020-11-19-kb-70min_FAIMS_100micron_3cv_-35_-45_-55--OTIT_SY33-10	1 mM-1
		2020-11-19-kb-70min_FAIMS_100micron_3cv_-35_-45_-55--OTIT_SY33-4	20 mM-1
		2020-11-19-kb-70min_FAIMS_100micron_3cv_-35_-45_-55--OTIT_SY33-12	20 mM-1
	1B, S1	2020-11-19-kb-70min_FAIMS_100micron_3cv_-35_-45_-55--OTIT_SY33-3	2 mM-1
		2020-11-19-kb-70min_FAIMS_100micron_3cv_-35_-45_-55--OTIT_SY33-11	2 mM-1
	1C	2020-09-24-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY42-3-1	2 mM-1
		2020-09-24-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY42-3-3	2 mM-2
		2020-09-23-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY43-1-1	2 mM-3
		2020-09-23-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY43-1-2	2 mM-4
2020-09-21-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY44-1-1		2 mM-5	
2020-09-21-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY44-1-2		2 mM-6	
1C, D	2020-09-24-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY42-3-2	4 mM-1	
	2020-09-23-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY43-1-4	4 mM-2	
	2020-09-21-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY44-1-3	4 mM-3	
	2020-09-21-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY44-1-4	4 mM-4	
1D	2020-09-21-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY44-2-1	0.1 mM-1	
	2020-09-21-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY44-2-2	0.1 mM-2	
S1	2020-11-19-kb-70min_FAIMS_100micron_3cv_-35_-45_-55--OTIT_SY33-7	DTT after IAA-1	
	2020-11-19-kb-70min_FAIMS_100micron_3cv_-35_-45_-55--OTIT_SY33-15	DTT after IAA-2	
PXD023057 (Figure 2)	2B	2020-08-23-KB-70min_FAIMS_3cv-35-45-60_OTIT_steeper_SY40-sp3-1-6uL	SP3-1

	2020-09-22-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY41-1-2-60	SP3-2
2B, C	2020-09-21-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY44-3-3-NA	MeOH/CHCl ₃ -1 (0.1 mM)
	2020-09-21-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY44-3-4-NA_20200922184424	MeOH/CHCl ₃ -2 (0.1 mM)
2C	2020-10-27-kb-70min_FAIMS_3cv_-35_-45_-55--OTIT-SY43-3-3-rerun	4 mM-1
	2020-10-27-kb-70min_FAIMS_3cv_-35_-45_-55--OTIT-SY43-3-4-rerun	4 mM-2
S3	2020-10-06-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY45-1-20	20 µg
	2020-10-05-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY45-2-50	50 µg
	2020-10-05-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY45-3-100	100 µg
	2020-10-05-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY45-4-150	150 µg
3A, B, S4, 5, 6	2020-09-22-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY40-1	3 CVs, -35 -45 -55, steeper, 70 min, 100 µm, irontrap -1
	2020-10-05-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY41-1	-2
	2020-09-22-KB-70min_FAIMS_cv_-35_OTIT_SY40-1	1 CV-1
3A	2020-09-22-KB-70min_FAIMS_2cv_-35_-45_OTIT_SY40-1	1 CV-2
	2020-10-05-KB-70min_FAIMS_2cv_-35_-45_OTIT_SY41-1	2 CVs-1
3B	2020-09-22-KB-70min_FAIMS_3cv_-30_-40_-50_OTIT_SY40-1	2 CVs-2
	2020-10-05-KB-70min_FAIMS_3cv_-30_-40_-50_OTIT_SY41-1	-30 -40 -50-1
	2020-10-05-KB-70min_FAIMS_3cv_-35_-50_-65_OTIT_SY41-1	-30 -40 -50-2
	2020-10-05-KB-70min_FAIMS_3cv_-35_-50_-65_OTIT_SY41-2	-35 -50 -65-1
PXD023060 (Figure 3)	2020-08-23-KB-70min_FAIMS_3cv-35-45-60_OTIT_steeper_SY39-7-100-sp3-6uL_FS-35	-35 -50 -65-2
	2020-08-18-KB-70min_FAIMS_2cv-35-60_SP3-SY39-7-100-OTIT-steeper_FS-40	-35
	2020-08-23-KB-70min_FAIMS_3cv-35-45-60_OTIT_steeper_SY39-7-100-sp3-6uL_FS-45	-40
	2020-08-23-KB-70min_FAIMS_3cv-35-45-60_OTIT_steeper_SY39-7-100-sp3-6uL_FS-45	-45
	2020-08-23-KB-70min_FAIMS_3cv-35-45-60_OTIT_steeper_SY39-7-100-sp3-6uL_FS-55	-45
	2020-08-19-KB-70min_FAIMS_2cv-35-60_actual_SP3-SY39-7-100-OTIT-steeper_FS-60	-55
	2020-08-19-KB-70min_FAIMS_2cv-35-60_actual_SP3-SY39-7-100-OTIT-steeper_FS-60	-60
	2020-08-19-KB-70min_FAIMS_2cv-35-70_actual_SP3-SY39-7-100-OTIT-steeper_FS-70	-60
	2020-08-19-KB-70min_FAIMS_2cv-35-70_actual_SP3-SY39-7-100-OTIT-steeper_FS-70	-70
	2020-08-19-KB-70min_FAIMS_2cv-35-70_actual_SP3-SY39-7-100-OTIT-steeper_FS-70	-70
S4A	2020-09-22-KB-140min_FAIMS_3cv_-35_-45_-55_OTIT_SY40-1	140 min-1

	2020-10-05-KB-140min_FAIMS_3cv_-35_-45_-55_OTIT_SY41-1	140 min-2	
S4B	2020-10-07-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY41-1-shallower	Shallower-1	
	2020-10-07-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY41-2-shallower	Shallower-2	
S5	2020-10-11-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY41-1-70um	70 µm-1	
	2020-10-11-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY41-2-70um	70 µm-2	
S6	2020-10-05-KB-70min_FAIMS_3cv_-35_-45_-55_OTOT_SY41-1	Orbitrap-1	
	2020-10-05-KB-70min_FAIMS_3cv_-35_-45_-55_OTOT_SY41-2	Orbitrap-2	
4A, B	2020-10-09-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY-41-4-1	HCT-15-1	
	2020-10-09-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY-46-2	HCT-15-2	
	2020-10-09-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY-41-4-2	H661-1	
	2020-10-09-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY-46-1	H661-2	
	2020-10-31-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY-49-1-5-na	MOLT-4-1	
	2020-11-01-kb-70min_FAIMS_3cv_-35_-45_-55--OTIT-SY49-1-6-na-rerun	MOLT-4-2	
	2020-10-09-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY-46-3	HEC-1-B-1	
	2020-10-09-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY-46-4	HEC-1-B-2	
	2020-10-31-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY-49-1-3-na	Jurkat-1	
	2020-10-31-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY-49-1-4-na	Jurkat-2	
	2020-11-01-kb-70min_FAIMS_3cv_-35_-45_-55--OTIT-SY49-1-1-na-rerun	H2122-1	
	2020-10-31-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY-49-1-2-na	H2122-2	
	4A, B, C, S7	2020-08-23-KB-70min_FAIMS_3cv-35-45-60_OTIT_steeper_SY40-sp3-1-6uL	HEK293T, Trypsin-1
		2020-09-22-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY41-1-2-60	HEK293T, Trypsin-2
4C	2020-09-05-KB-70min_FAIMS_3cv_-35_-45_-60_OTIT_SY41-3-2	LysC-1	
	2020-09-22-KB-70min_FAIMS_3cv_-35_-45_-55_OTIT_SY42-2-2-LysC	LysC-2	
	2020-10-31-kb-70min_FAIMS_3cv_-35_-45_-55--OTIT-SY49-2-1-na	Chymotrypsin-1	
	2020-10-31-kb-70min_FAIMS_3cv_-35_-45_-55--OTIT-SY49-2-2-na	Chymotrypsin-2	
4D, S7	2020-09-05-KB-70min_FAIMS_3cv_-35_-45_-60_OTIT_SY41-2-S	Soluble-1	

PXD023059
(Figure 4)

		2020-10-19-kb-70min_FAIMS_3cv_-35_-45_-55--OTIT-SY-47-S	Soluble-2
		2020-09-05-KB-70min_FAIMS_3cv_-35_-45_-60_OTIT_SY41-2-M	Membrane-1
		2020-10-19-kb-70min_FAIMS_3cv_-35_-45_-55--OTIT-SY-47-M	Membrane-2
PXD023058 (Figure 6)	6B	2020-11-10-kb-70min_FAIMS_100micron_3cv_-35_-45_-55--OTIT_HD-1	isoTOP-ABPP-1
		2020-11-10-kb-70min_FAIMS_100micron_3cv_-35_-45_-55--OTIT_HD-2	isoTOP-ABPP-1
		2020-11-10-kb-70min_FAIMS_100micron_3cv_-35_-45_-55--OTIT_HD-3	isoTOP-ABPP-1
		2020-10-28-kb-70min_FAIMS_3cv_-35_-45_-55--OTIT-CF3_SP3_isoTOP-HD-3	isoTOP-ABPP-4

(C) Methods

Cell culture and preparation of cell lysates. Cell culture reagents including Dulbecco's phosphate-buffered saline (DPBS), Dulbecco's modified Eagle's medium (DMEM)/high glucose media, Roswell Park Memorial Institute (RPMI) media, trypsin-EDTA and penicillin/streptomycin (Pen/Strep) were purchased from Fisher Scientific. Fetal Bovine Serum (FBS) were purchased from Avantor Seradigm (lot # 214B17).

All cell lines were obtained from ATCC and were maintained at a low passage number (< 20 passages). HEK293T (ATCC: CRL-3216) cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotics (Penn/Strep, 100 U/mL). H661 (ATCC: HTB-183), HCT-15 (ATCC: CCL-225), Jurkat (ATCC: TIB-152), MOLT-4 (ATCC: CRL-1582) and H2122 (ATCC: CRL-5985) cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% antibiotics (Penn/Strep, 100 U/mL). HEC-1-B (ATCC: HTB-113) cells were cultured in EMEM supplemented with 10% FBS and 1% antibiotics (Penn/Strep, 100 U/mL). Media was filtered (0.22 μ m) prior to use. Cells were maintained in a humidified incubator at 37 °C with 5% CO₂. Cell lines were tested for mycoplasma using the Mycoplasma Detection Kit (InvivoGen).

Cells were harvested by centrifugation (4,500 g, 5 min, 4 °C), washed twice with cold DPBS, resuspended in DPBS, sonicated, and clarified by centrifuging (21,000 g, 10 min, 4 °C). The lysates were then transferred to a new microcentrifuge tube. Protein concentrations were determined using a Bio-Rad DC protein assay kit from Bio-Rad Life Science (Hercules, CA) and the lysate diluted to the working concentrations indicated below.

Fractionation of the lysate. 500 μ L of HEK293T lysates (2 mg/mL) were fractionated with ultracentrifugation (45,000 g, 1 hr, 4 °C). Supernatant was saved as the soluble fraction. Pellet was resuspended with 500 μ L PBS, sonicated and saved as the membrane fraction. Protein concentrations of both fractions were determined using a Bio-Rad DC protein assay kit and the lysates were diluted to the working concentrations.

Proteomic sample preparation - IA-alkyne labeling. HEK293T proteome (200 μ L of 1 mg/mL, prepared as described above) was dried with SpeedVac and resuspended with 200 μ L 2M Urea. Samples were then labeled with IA-alkyne **1** for 1h at ambient temperature. Reduction with DTT was performed either before (1 μ L of 200 mM stock in water, final concentration = 1mM) or after (10 μ L of 200 mM stock in water, final concentration = 10 mM) the IA-alkyne labeling for 15 min at 65 °C. To this iodoacetamide (Acros Organics, 122270050, 10 μ L of 400 mM stock in water, final concentration = 20 mM) was added and the mixture was incubated for 30 min at 37 °C with shaking. Trypsin (Worthington Biochemical, LS003740, 2 μ L, 1 mg/mL in 666 μ L of 50 mM acetic acid and 334 μ L of 100 mM CaCl₂, final weight = 2 ng) was then added. After overnight digestion, 5% FA was added and samples were desalted using C18 Tips (Pierce, 87782), following the manufacturer's instructions. The samples were dried (SpeedVac) and then reconstituted in Molecular Biology Grade (MB) water containing

5% acetonitrile and 1% FA. The samples were then analyzed by LC-MS/MS, as described below.

Proteomic sample preparation - CuAAC. HEK293T proteome (200 μ L of 1 mg/mL, prepared as described above) was first labeled with IA-alkyne **1** (4 μ L of 100 mM stock solution in DMSO, final concentration = 2 mM) for 1h at ambient temperature. CuAAC was performed with biotin-azide **2** (4 μ L of 200 mM stock in DMSO, final concentration = 4 mM), TCEP (4 μ L of fresh 50 mM stock in water, final concentration = 1 mM), TBTA (12 μ L of 1.7 mM stock in DMSO/*t*-butanol 1:4, final concentration = 100 μ M), and CuSO₄ (4 μ L of 50 mM stock in water, final concentration = 1 mM) for 1h at ambient temperature.

Chloroform/methanol precipitation for proteomic sample preparation. After CuAAC, 3 mL methanol, 1 mL chloroform, and 3 mL water were added into each sample and samples were centrifugated (4,200 *g*, 10 min, 4 °C). The upper liquid was aspirated and the resulting pellet was washed with cold methanol (3 mL). The pellet was then solubilized in 0.5 mL 0.6% SDS/PBS with sonication and heating (5 min, 95 °C) and any insoluble material was removed by an additional centrifugation at ambient temperature (14,000 *g*, 1 min).

Neutravidin enrichment of labeled proteins. For each sample, 100 μ L of NeutrAvidin-agarose beads slurry (Pierce, 29200) was washed twice in 10 mL PBS and then resuspended in 2.5 mL PBS. The proteins were added to the suspension of streptavidin-agarose beads (final concentration 0.1 % SDS/PBS) and the bead mixture was rotated for 2h at ambient temperature. After incubation, the beads were pelleted by centrifugation (1,400 *g*, 3 min) and were washed (2 \times 10 mL PBS and 2 \times 10 mL water).

Trypsin digest on NeutrAvidin-agarose beads. The beads were first centrifuged (21,000 *g*, 2 min), and resuspended in 500 μ L 6 M urea/PBS. DTT (10 μ L of 200 mM stock in water, final concentration = 10 mM) was added into each sample and the sample was incubated at 65 °C for 15 min. Then, iodoacetamide (10 μ L of 400 mM stock in water, final concentration = 20 mM) was added and incubated for 30 min at 37 °C with shaking in dark. The bead mixture was diluted with 900 μ L PBS, pelleted by centrifugation (21,000 *g*, 1 min), and resuspended in PBS containing 200 μ L 2M urea/PBS followed by the addition of trypsin solution (Worthington Biochemical, LS003740, 2 μ L, 1 mg/mL in 666 μ L of 50 mM acetic acid and 334 μ L of 100 mM CaCl₂, final weight = 2 ng). The digestion was overnight at 37 °C with shaking. The beads were then separated from the digest with Micro Bio-Spin columns (Bio-Rad) by centrifugation (21,000 *g*, 1 min), washed (2 \times 1 mL PBS and 3 \times 1 mL water) and transferred to fresh eppendorf tubes with 1 mL water. Bound peptides were eluted twice with 60 μ L of 80% acetonitrile in MB water containing 0.1% FA. The first 10 min incubation at RT and the second one at 72 °C. The combined elution was dried (SpeedVac) and resuspended in 100 μ L water containing 0.1% FA. Samples were then desalted using C18 Tips (Pierce, 87782). The samples were dried (SpeedVac) and reconstituted in Molecular Biology Grade (MB) water containing 5% acetonitrile and 1% FA. The samples were analyzed by LC-MS/MS.

SP3 proteomic sample preparation.^[1] After CuAAC labeling, each sample was treated with 20 μ L 10% SDS/PBS and then 0.5 μ L benzonase (Fisher Scientific, 70-664-3) for 30 min at 37 °C. For each 200 μ L sample (1 mg/mL protein concentration), 20 μ L Sera-Mag SpeedBeads Carboxyl Magnetic Beads, hydrophobic (GE Healthcare, 65152105050250, 50 μ g/ μ L, total 1 mg) and 20 μ L Sera-Mag SpeedBeads Carboxyl Magnetic Beads, hydrophilic (GE Healthcare, 45152105050250, 50 μ g/ μ L, total 1 mg) were mixed and washed with water for three times. The bead slurries were then transferred to the CuAAC samples, incubated for 5 min at RT with shaking (1000 rpm). Absolute ethanol (400 μ L) was added to each sample, and the samples were incubated for 5 min at RT with shaking (1000 rpm). Samples were then placed on a magnetic rack, washed three times with 80% ethanol in water (400 μ L). After washing, beads were resuspended in 200 μ L 2 M urea in 0.5% SDS/PBS. DTT (10 μ L of 200 mM stock in water, final concentration = 10 mM) was added into each sample and the sample was incubated at 65 °C for 15 min. Then, iodoacetamide (10 μ L of 400 mM stock in water, final concentration = 20 mM) was added and the solution was incubated for 30 min at 37 °C with shaking in dark. After that, absolute ethanol (400 μ L) was added to each sample, and the samples were incubated for 5 min at RT with shaking (1000 rpm). Beads were then again washed three times with 80% ethanol in water (400 μ L). Next, beads were resuspended in 200 μ L 2 M urea in PBS and 2 μ L trypsin solution was added. Digest was overnight at 37 °C with shaking. After digestion, ~ 4 mL acetonitrile (> 95% of the final volume) was added to each sample and the mixtures were incubated for 10 min at RT with shaking (1000 rpm). The beads were then washed (3 \times 1 mL acetonitrile) with magnetic rack. Peptides were eluted from SP3 beads with 100 μ L of 2% DMSO in MB water for 30 min at 37 °C with shaking (1000 rpm). The elution was repeated again with 100 μ L of 2% DMSO in MB water. Peptide concentration assay (Pierce, 23275) was performed to test the concentration of the peptide and calculate the recovery rate of the samples with SP3. The elution can be used for NeutrAvidin enrichment or analyzed by LC-MS/MS.

NeutrAvidin enrichment of labelled peptides. For each sample, 50 μ L of NeutrAvidin Agarose resin slurry (Pierce, 29200) was washed three times in 10 mL IAP buffer (50 mM MOPS pH 7.2, 10 mM sodium phosphate, and 50 mM NaCl buffer) and then resuspended in 800 μ L IAP buffer. Peptide solutions eluted from SP3 beads were then transferred to the NeutrAvidin Agarose resin suspension, and the samples were rotated for 2h at RT. After incubation, the beads were pelleted by centrifugation (21,000 g, 1 min) and washed (3 \times 1 mL PBS, 6 \times 1 mL water). Bound peptides were eluted twice with 60 μ L of 80% acetonitrile in MB water containing 0.1% FA. The first 10 min incubation at RT and the second one at 72 °C. The combined eluants were dried (SpeedVac), then reconstituted with 5% acetonitrile and 1% FA in MB water and analyzed by LC-MS/MS.

IsoTOP-ABPP with SP3. IsoTOP-ABPP samples were prepared as described previously.^[2,3] Briefly, Jurkat cells were harvested and lysed by sonication in PBS. Proteomes were adjusted to 2 mg/mL. Samples were labeled for 1 h at ambient temperature with either 500 μ M CF3 compound or DMSO (CF3, 2 μ L of 50mM stock in DMSO). Samples were chased with 200 μ M iodoacetamide alkyne (IA-alkyne, 0.4 μ L of 100 mM stock in DMSO) for 1 h at ambient temperature. Samples were conjugated by CuAAC to either the light (DMSO treated) or heavy

(fragment treated) TEV tags (4 μ L of 5 mM stocks in DMSO, final concentration = 100 μ M), TCEP, TBTA ligand and CuSO₄. After 1h, the heavy and light treated samples were combined followed by the addition of 20 μ L of 10% SDS and 0.5 μ L benzonase. The samples were incubated for 20 min at 37C. After SP3 cleaning and elution as previously described, samples were then enriched on Streptavidin resin (100 μ L slurry) in PBS (1 mL) with rotating for 2 hours. Beads were then washed (2x PBS and 2x water) and then resuspended in 75 μ L TEV buffer (50 mM *Tris*, pH 8, 0.5 mM EDTA, 1 mM DTT). 5 μ L TEV protease (70 μ M) was added, and the reactions were rotated for 7h at 29 °C. The samples were then cleaned using micro bio-spin columns, desalted using Pierce C18 100 μ L bed zip-tips, speedvac'd and reconstituted in 20 μ L 5% ACN, 1% formic acid.

Liquid-chromatography tandem mass-spectrometry (LC-MS/MS) analysis. The samples were analyzed by liquid chromatography tandem mass spectrometry using a Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer coupled with a High Field Asymmetric Waveform Ion Mobility Spectrometry (FAIMS) Interface. Peptides were resuspended in 5% formic acid and fractionated online using a 18cm long, 100 μ M inner diameter fused silica capillary packed in-house with bulk C18 reversed phase resin (particle size, 1.9 μ m; pore size, 100 Å; Dr. Maisch GmbH). The 70 or 140-minute water-acetonitrile gradient was delivered using a Thermo Scientific™ EASY-nLC™ 1200 system at different flow rates (Buffer A: water with 3% DMSO and 0.1% formic acid and Buffer B: 80% acetonitrile with 3% DMSO and 0.1% formic acid). The detailed gradient includes 0 – 5 min from 3 % to 10 % at 300 nL/min, 5 – 64 min from 10 % to 50 % at 220 nL/min, and 64 – 70 min from 50 % to 95 % at 250 nL/min buffer B in buffer A. Data was collected with charge exclusion (1, 8,>8). Data was acquired using a Data-Dependent Acquisition (DDA) method comprised of a full MS1 scan (Resolution = 120,000) followed by sequential MS2 scans (Resolution = 15,000) to utilize the remainder of the 3 second cycle time. Time between master scans was set 1 s. HCD collision energy of MS2 fragmentation was 30 %. Different FAIMS compensation voltage (CV) was applied as described.

The MS data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE^[4,5] partner repository with the dataset identifier PXD023056 (Figure 1), PXD023057 (Figure 2), PXD023060 (Figure 3), PXD023059 (Figure 4) and PXD023058 (Figure 6). File details were listed in **Table S7**.

Protein and peptide identification. Raw data collected by LC-MS/MS were searched with MSFragger (version 3.1.1).^[6,7] Proteomic workflow of FragPipe and its collection of tools was set as default. Precursor and fragment mass tolerance was set as 50 ppm and 600 ppm respectively. Missed cleavages were allowed up to 1. Peptide length was set 7 - 50 and peptide mass range was set 500 - 5000. Cysteine residues were searched with a static modification for carboxyamidomethylation (+57.02146) and a differential modification at cysteine residues, which is +406.21509 for cysteine enrichment, +80.0626 for IA-alkyne labelling. MS data of isoTOP-ABPP were searched using the ProLuCID algorithm (publicly available at http://fields.scripps.edu/yates/wp/?page_id=17) using a reverse concatenated, nonredundant variant of the Human UniProt database (release-2020_01).

Cysteine residues were searched with a static modification for carboxyamidomethylation (+57.02146) and differential modification with +464.28595 and +470.29976 at cysteines for isotopic tags. The rest of parameters were set the same as MSFragger.

The MS search results have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE^[4,5] partner repository with the dataset identifier PXD023056 (Figure 1), PXD023057 (Figure 2), PXD023060 (Figure 3), PXD023059 (Figure 4) and PXD023058 (Figure 6). File details were listed in **Table S7**.

Data analysis and processing. Custom python and R scripts were implemented to compile labeled peptide datasets. Unique proteins, unique cysteines, and unique peptide-spectrum matches (PSMs) were quantified for each dataset, using unique identifiers. Unique proteins were established based on UniProt protein IDs. Unique cysteines were classified by an identifier consisting of a UniProt protein ID and the amino acid number of the modified cysteine; residue numbers were found by aligning the peptide sequence to the corresponding UniProt protein sequence. Unique peptides were found based on sequences containing a modified cysteine residue. The number of shared unique cysteines and PSMs were calculated for each pair of cell lines and are represented as heatmaps. Additionally, venn diagrams were constructed to display the number of common cysteine identifiers shared between various experiments. A total number of unique cysteines and proteins was computed by aggregating the unique identifiers from the cell line, fractionation, and protease datasets. These total counts were compared to previously published datasets from Backus et al.^[3]

An *in silico* digestion was performed using custom python scripts and the Human UniProt database (release 2020_08). Canonical protein sequences were cut after lysine residues and arginine residues that were not followed by a proline residue. Truncated protein sequences between 6 to 45 amino acids in length were utilized for further analysis. The total number of theoretical proteins or cysteines one could detect by MS were determined by counting the number of protein IDs or cysteines associated with the remaining truncated protein sequences. From the resulting trypsin digested protein sequences, the percentage of cysteines experimentally identified by MS was calculated. A total of 1,073 proteins did not have a cysteine that was detectable by the previously mentioned criteria. A histogram depicts the percentages of experimentally labeled cysteines per protein. In addition, the number of cysteine amino acids per protein sequence in the Human UniProt database (release 2020_08) is plotted in a histogram.

For isoTOP-ABPP, ratios were calculated with MS1 signal intensity of light isotopically labeled cysteines over that of heavy labeled cysteines. 4 replicates were performed and ratios were averaged. Within each replicate dataset, peptides bearing the same unique identifier, including those with mis-cleavage events, were grouped together and the median ratio was kept for downstream calculations. The mean +/- SD of the ratios for each replicate are reported along with the number of PSMs for each listed peptide in all replicates. Peptides with multiple cysteines were counted as one liganded cysteine.

Synthesis of reagents. Compound **1** and **4** were prepared as has been reported^[8] and synthesis of compound **4** was performed according to the previous studies.^[3]

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