Electronic Supplementary Material (ESI) for RSC Chemical Biology.


# Supporting Information

# Monitoring GAPDH activity and inhibition with cysteine-reactive chemical probes

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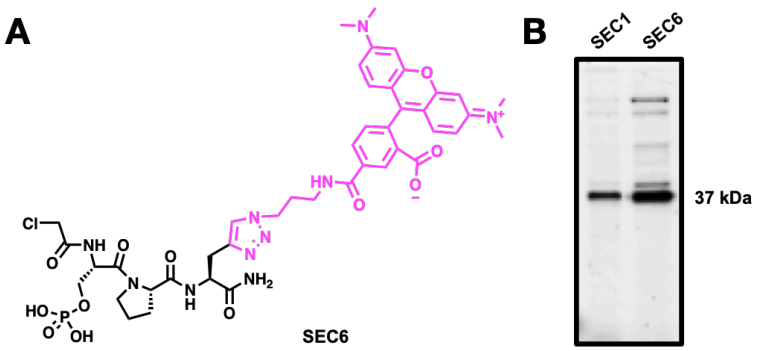
**Supplemental Table 1.** Complete ReDiMe abundance dataset for Jurkat cells treated with vehicle (light) and 100 μM SEC1 (heavy) (n=3, s.d. <25%).

**Supplemental Table 2.** Filtered cysteine reactivity datasets for Jurkat cells treated with vehicle (IA-heavy) and KA (IA-light) at 1, 5, and 10 μM (n=3, s.d. <25%).

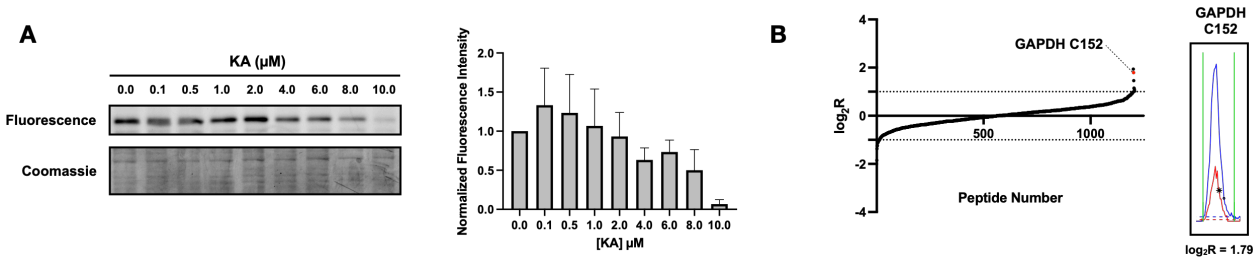
**Supplemental Table 3.** Filtered cysteine reactivity dataset for HeLa cells treated with vehicle (IA-heavy) and KA (IA-light) at 10 μM (n=3, s.d. <25%).

**Supplemental Table 4. Thermal shift analysis (TSA) of GAPDH in the presence of $NAD^+$ and koningic acid (KA).** GAPDH is stabilized by the cofactor $NAD^+$ in a concentration-dependent manner and is destabilized by KA in a concentration-independent manner even with addition of excess $NAD^+$. Neither $NAD^+$ nor KA has an effect on the thermal stability of GAPDH that has the active site cysteine mutated to either serine or alanine. ([a]average of 6 experiments, [b]average of 3 experiments, [c]average of 2 experiments, [d]single experiment)

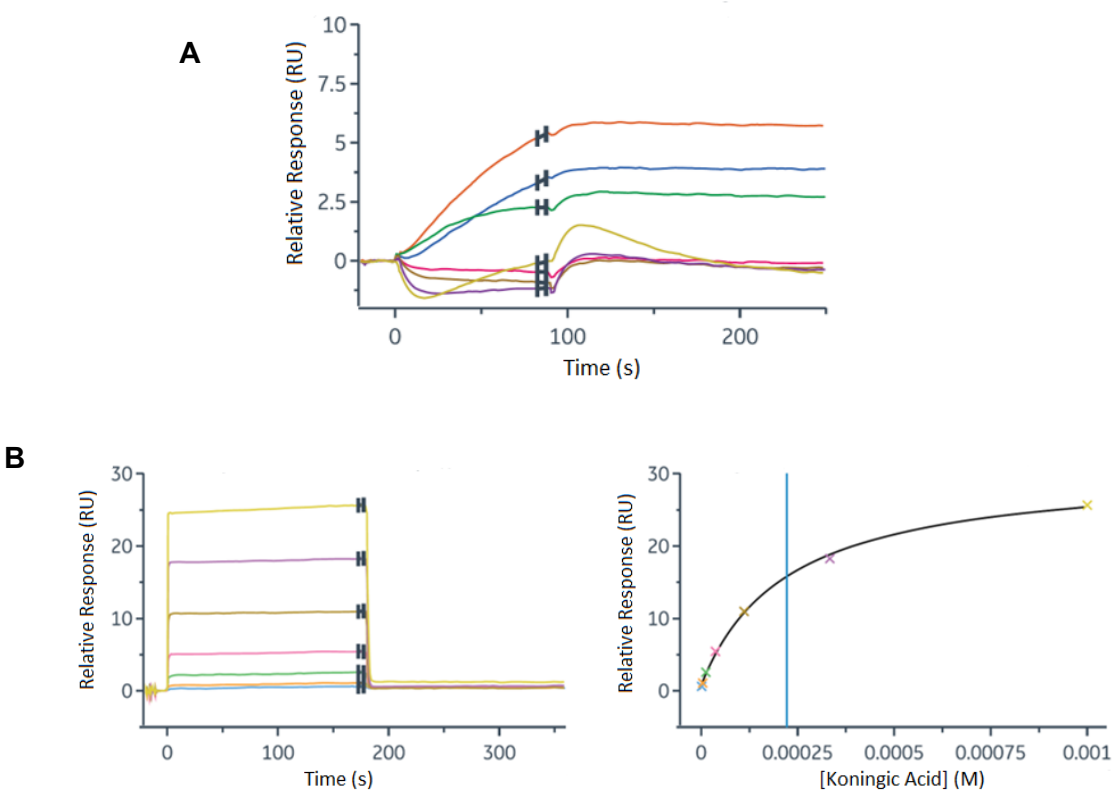
**Supplemental Table 5. KA binding does not affect GAPDH quaternary structure.** Analytical size exclusion chromatography shows that thermal destabilization of GAPDH that is observed upon covalent binding of KA is not due to disruption of the biological tetramer.



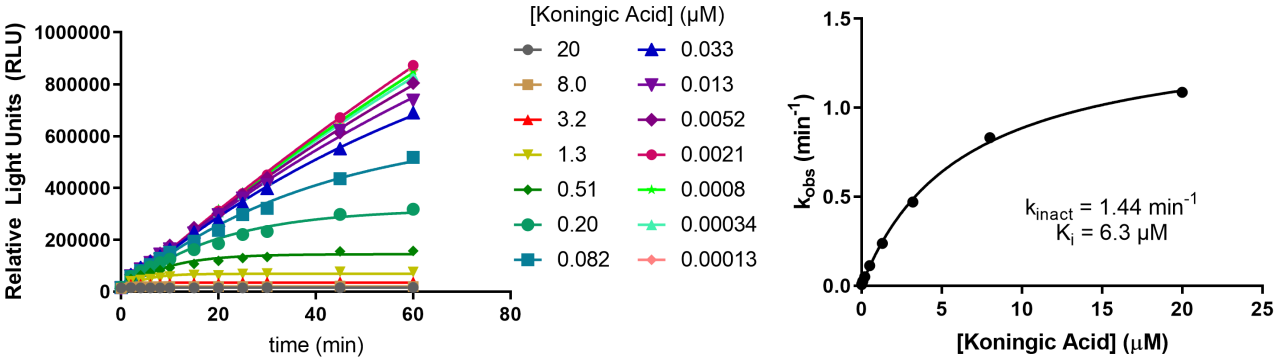

**Supplemental Figure 1. SEC6 is a fluorescent analog of SEC1 that maintains affinity to GAPDH.** (A) Chemical structure of a fluorescent, truncated analog of SEC1 (B) In-gel fluorescence evaluation of HeLa lysates labeled with SEC6 (100 μM, 1h).



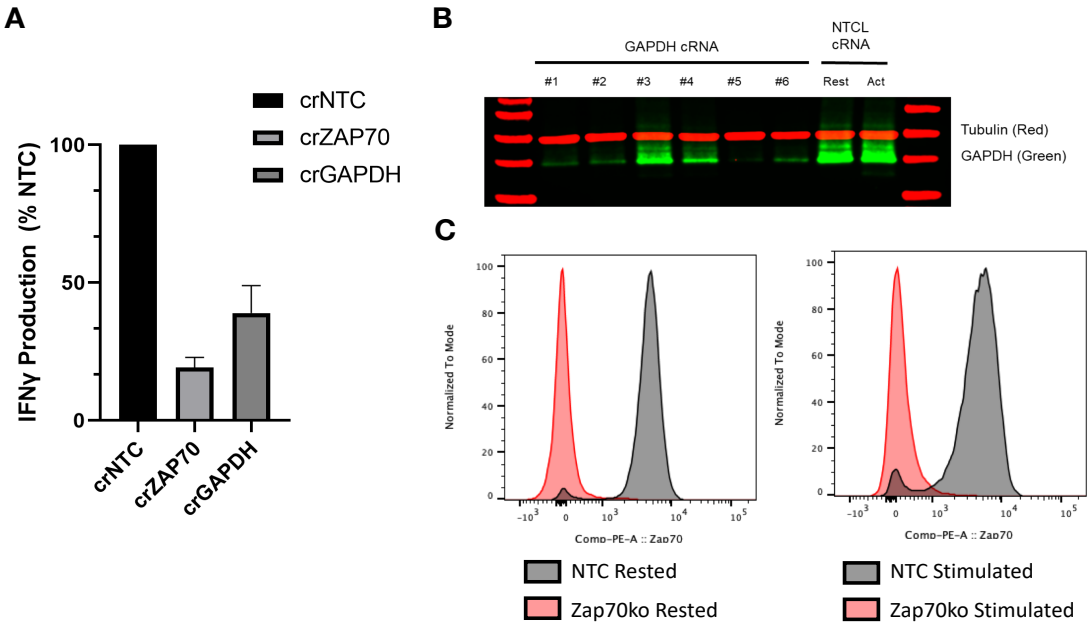

**Supplemental Figure 2. KA is a potent and selective GAPDH inhibitor in HeLa cells.** (A) HeLa cells were incubated with increasing concentrations of KA and lysates were labeled with SEC1 (100 μM, 1h). Protein labeling was evaluated by in-gel fluorescence. Error bars represent the standard deviation from three replicates. (B) Heavy:light $log_2R$ for all identified cysteines in vehicle (heavy) and 10 μM KA (light) treated HeLa cells (n=3, s.d. <25%).




**Supplemental Figure 3. KA binds irreversibly to wild-type GAPDH and reversibly to a catalytically-dead mutant (C152S).** (A) Representative SPR sensorgram for wild-type protein showing covalent, irreversible binding upon addition of KA. (B) Multi-cycle kinetic analysis of KA binding to GAPDH C152S. Binding to the protein was fast and reversible, $K_D$ = 230 ± 6 μM. N-terminal avi-tagged GAPDH was immobilized on a streptavidin-coated chip and varying concentrations of KA (3-fold, 7-point dilution series at a starting concentration of 1000 μM) were injected in the presence of saturating concentrations of $NAD^+$ (100 μM). Standard deviation was calculated based on two independent measurements of KA binding to GAPDH.




**Supplemental Figure 4. KA is a potent irreversible inactivator of GAPDH.** KA is a covalent inhibitor of GAPDH and inactivates the enzyme at a maximum potential rate ($k_{inact}$) of 1.44 $min^{-1}$ with a binding constant ($K_i$) of 6.3 μM. Enzyme activity was measured over time at varying concentrations of KA (14-point, 2.5-fold dilution series, top concentration of 20 μM) under balanced substrate conditions (24 μM $NAD^+$, 74 μM G3P, 6 mM $Na_2HAsO_4$). The pseudo-first order rate constant $k_{obs}$ was calculated from the progress curve with the following equation: $[P] = (v_i/k_{obs})[1-\exp(-k_{obs}*t)]$. For determination of $k_{inact}$ and $K_i$, $k_{obs}$ was plotted versus KA concentration and fit with the equation $k_{obs} = (k_{inact}*[KA])/(K_i + [KA])$.




**Supplemental Figure 5. INFγ production decreases upon GAPDH knockout.** CRISPR (cr) knockout of GAPDH results in the reduction of IFNγ production in CD4+ T cells. All values are means ± S.E. (error bars) from six different donors replicates (n=6). NTC = non-targeting control

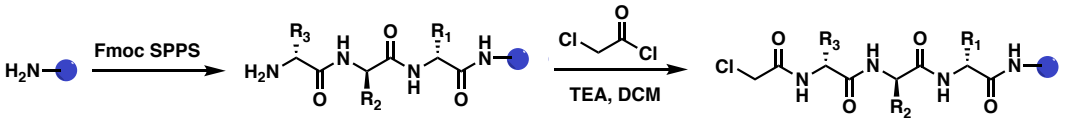
## Materials and Methods

All chemical and biological materials were purchased from Sigma Aldrich or Fisher Scientific unless otherwise noted. Fmoc-protected amino acids, PyBOP, and Rink Amide MBHA resin were purchased from Novabiochem (San Diego, CA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals. IA-light and IA-heavy were synthesized in-house according to Abo, M. *et al.*[1] PC biotin azide was purchased from Click Chemistry Tools and TAMRA azide was purchased from Lumiprobe. Anti-GAPDH (cat. 2118S), anti-myc (cat. 2278S) and rabbit anti-IgG secondary antibody (cat. 7074S) were purchased from Cell Signaling Technologies. Anti-ACTIVE MAPK (cat. V8031) was purchased from Promega.

### I. Fmoc-solid-phase peptide synthesis (SPPS)

General Procedure

Peptides were synthesized by manual solid-phase synthesis on Rink Amide MBHA Resin using fluoren-9-ylmethoxycarbonyl (Fmoc) as the protecting group for all the $\alpha$-amino functionalities. An OBzl side-chain protecting group was used on Fmoc-phosphoserine and a Trt side chain protecting group was used on Fmoc-serine. Amino acid coupling was performed using benzotriazole-1-yl-oxy-tris-pyrrolidinophosphoniumhexafluoro-phosphate (PyBOP) as the coupling reagent. The chloroacetamide electrophile was added to each peptide following Scheme 1. Full length peptides were cleaved from the resin for 2 hr using a cleavage cocktail containing 90% trifluoroacetic acid (TFA), 5% dichloromethane (DCM), 2.5% triisopropylsilane (TIS) and 2.5% $H_2O$. All peptides were purified by preparative reverse-phased HPLC on a Waters 1525 HPLC with a Waters 2489 UV/Vis Detector. Preparative HPLC was performed using a Waters XBridge Prep C18 Column (10 x 250 mm, 5µm) and a water-acetonitrile mobile phase with a gradient moving from Buffer A (95% water, 5% acetonitrile, 0.1% TFA) to Buffer B (5% water, 95% acetonitrile, 0.1% TFA). The mobile phase was run at a flow rate of 5mL/min for 35 min and peptides were detected with UV detection at 220 nm and 280 nm. Mass spectrometry data for each peptide was collected using an Agilent 6230 LC TOF mass spectrometer with an Agilent Zorbax C18 column (2.1 x 150 mm, 5 µm). Peptides were eluted with a water-acetonitrile gradient moving from 5% to 95% acetonitrile (0.1% Formic acid) over 30 minutes with a flow rate of 200 µL/min and UV and MS Detection.




Scheme S1. Synthesis of peptide probes bearing chloroacetamide electrophile (SEC1-5)

Synthesis of SEC1

Rink Amide MBHA resin was used with the following amino acid residues Fmoc-Phe-OH, Fmoc-Pra-OH, Fmoc-Pro-OH, and Fmoc-Ser(PO(OBzl)OH)-OH under standard synthesis conditions. After the final coupling and deprotection step, the resin was dried by washing with dichloromethane (DCM). The resin was swelled in dry N,N-Dimethylformamide and chloroacetyl chloride (3 eq) followed by triethylamine (3 eq) were added to the vessel. The reaction vessel was capped and rocked for 16 hrs. The solvent was removed and the resin was washed with DMF (3 x 3 mL) and DCM (5 x 3 mL). The peptide was cleaved as described above and purified by HPLC to give the pure peptide SEC1. HPLC $t_R$ = 17.85 min; ($C_{33}H_{40}ClN_6O_{10}P$) m/z calc'd 746.2232 ; obs'd 747.2024

Synthesis of NJP14

The standard procedure outlined for SEC1 was used except the Fmoc-Ser(PO(OBzl)OH)-OH was replaced with Fmoc-Ser(Trt)-OH. The peptide was cleaved using the conditions described above and purified by HPLC to give the pure peptide NJP14.HPLC $t_R$ = 19.19 min; ($C_{33}H_{39}ClN_6O_7$) m/z calc'd 666.2569; obs'd 667.2581

Synthesis of SEC2

The standard procedure outlined for SEC1 was used except the first Phe residue was replaced with Fmoc-Ala-OH. The peptide was cleaved using the conditions described above and purified by HPLC to give the pure peptide SEC2. HPLC $t_R$ = 15.37 min; ($C_{27}H_{36}ClN_6O_{10}P$) m/z calc'd 670.1919; obs'd 671.1998

Synthesis of SEC3

The standard procedure outlined for SEC1 was used except the second Phe residue was replaced with Fmoc-Ala-OH. The peptide was cleaved using the conditions described above and purified by HPLC to give the pure peptide SEC3. HPLC $t_R$ = 15.97; ($C_{27}H_{36}ClN_6O_{10}P$) m/z calc'd 670.1919; obs'd 671.1879

Synthesis of SEC4

The standard procedure outlined for SEC1 was used except the Fmoc-Pro-OH residue was replaced with Fmoc-Ala-OH. The peptide was cleaved using the conditions described above and purified by HPLC to give the pure peptide SEC4. HPLC $t_R$ = 17.62 min; ($C_{31}H_{38}ClN_6O_{10}P$) m/z calc'd 720.2076; obs'd 721.2089

Synthesis of SEC5

The standard procedure outlined for SEC1 was used except the Fmoc-Ser(PO(OBzl)OH)-OH was replaced with Fmoc-Ala-OH. The peptide was cleaved using the conditions described above and purified by HPLC to give the pure peptide SEC5. HPLC $t_R$ = 19.80 min; ($C_{33}H_{39}ClN_6O_6$) m/z calc'd 650.2620; obs'd 651.2577

Synthesis of SEC6

The standard procedure outlined for SEC6 was used except the Fmoc-Phe-OH residues were not added. The peptide was cleaved using the conditions described above and purified by HPLC to give the pure peptide. Following purification, the peptide (1eq) and

rhodamine-azide (1 eq) were dissolved in a 80% mixture of MeOH and water. Sodium ascorbate (200 mg/mL) and Copper (II) sulfate (85 mg/mL) were added to the solution and the sample was stirred overnight. The solvent was evaporated to leave the crude pink solid. The fluorescent peptide was purified by HPLC to give the pure peptide SEC6. HPLC $t_R$ = 18.29 min; ($C_{43}H_{50}ClN_{10}O_{12}P$) m/z calc'd 964.3036; obs'd 965.2888

## II. Cell culture and protein expression

Cell culture

All cell lines maintained at 37°C under 5% $CO_2$. HeLa and HEK293T were grown in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic (Pen/Strep; 25 µg/mL Amphotecerin B, 10,000 units/mL Penicillin, 10,000 µg/mL Streptomycin). MCF10A and MCF10CA1a cells were grown in DMEM supplemented with 5% horse serum (HS), 1% Pen/Strep, EGF (20 ng/mL), Hydrocortisone (0.5 mg/mL), Cholera toxin (100 ng/mL) and Insulin (10 µg/mL). Jurkat cells were grown in RPMI 1640 supplemented with 10% FBS and 1% Pen/Strep.

Preparation of lysates

The cells were grown to 100% confluence before being harvested by scraping and pelleted. Cells were washed with Dulbecco's phosphate buffered saline (DPBS) and the pellet was resuspended in an appropriate volume of DPBS. Cell suspensions were lysed by sonication using an ultrasonic tip sonicator (Cole Palmer). Whole cell lysates were separated into soluble and insoluble lysate fractions by centrifugation at 4°C (16,000 x g, 15 min). The insoluble fraction was discarded and the protein concentration of the soluble fraction was determined using the DC Protein Assay Kit (Bio-Rad).

Overexpression of GAPDH in HEK293T cells

A pcDNA3.1-myc/His plasmid containing the ORF of GAPDH was used for transfection and mutagenesis. The GAPDH C152S mutant was generated following the Quikchange procedure (Stratagene) using the primers: forward: 5'-GCTAAGCAGTTGGTGGTGCTG GAGGCATTGCTGATGATCTTG-3' and reverse: 5'- CAAGATCATCAGCAATGCCTC CAGCACCACCAACTGCTTAGC-3'. The mutant was sequenced and found to contain only the desired mutation. HEK293T cells were grown as described above. Transfection was performed in a 10cm plate of ~70% confluent. Serum-free media (600 µL) and Lipofectamine 3000 transfection reagent (40 µL) were combined. Plasmids for WT GAPDH and C152 mutant GAPDH (8.8 µg) were diluted in serum-free media (600 µL). Diluted plasmids and lipofectamine were combined in a 1:1 ratio and incubated at RT for 5 minutes. The plasmid solution was added to the plate of HEK293T cells. Cells were incubated for 48 hrs at 37°C under 5% $CO_2$. For the mock transfection, water was added in place of the plasmid. Lysates were prepared as described above and expression was evaluated by western blot and in gel fluorescence.

GAPDH Protein Expression/Purification

Full-length human GAPDH (https://www.uniprot.org/uniprot/P04406) was cloned into the pET21b E. coli expression vector with an N-terminal, TEV protease-cleavable 6xHis tag.

Recombinant protein was expressed in E. coli and purified initially from cell lysate by Ni-affinity chromatography. The His tag was removed from GAPDH by incubating with His-tagged TEV protease, which was then removed by passage over a second Ni-NTA column. The flow through containing tag-free GAPDH was further purified by size exclusion chromatography over a HiLoad 16/600 Superdex™ 75 pg column into a buffer composed of 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM DTT. Peak fractions containing GAPDH were pooled and concentrated to approximately 13 mg/ml using a Millipore Amicon Ultra Centrifugal Filter (15mL, 30K MWCO). Purified protein was stored at -80 °C.

## III. Peptide probe labeling, fluorescent gel analysis, and western blotting

SEC1-5/NJP14 probe labeling

HeLa cell lysates (50 µL, 2 mg/mL) were incubated with the peptide probe (SEC1-5/NJP14, 100 µM) at room temperature (RT) for 1 hr. Labeled lysates underwent click chemistry and in-gel fluorescence analysis, TAMRA azide (Rh-$N_3$), Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (1 mM, 50X fresh stock in water), Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA ligand) (100 µM, 17X stock in DMSO:t-Butanol 1:4), and Copper(II) sulfate (1 mM, 50X stock in water) were added to the lysates. The samples were incubated at room temperature for 1 hr.

In-gel fluorescence analysis of SEC6

HeLa cell lysates (50 µL, 2 mg/mL) in DPBS were treated with SEC6 (100 µM) at RT for 1 hr. Labeled lysates were then subjected in-gel fluorescence analysis as described above.

Fluorescent gel analysis

SDS-PAGE loading buffer 2x (reducing, 50 µL) was added to the Rh-labeled samples and 15 µL of this solution was separated by SDS-PAGE at 100 volts (V) for 2.5 hours on a 12.5% polyacrylamide gel. Gels were visualized on a Bio-Rad ChemiDoc MP imaging system. After fluorescent visualization, gels underwent a typical procedure for Coomassie staining and destaining. Stained gels were visualized on a Bio-Rad ChemiDoc MP imaging system.

Western blotting

Cell lysates were prepared as described above. Normalized lysates were run on a 12.5% polyacrylamide gel at 150V. Proteins were transferred to a nitrocellulose membrane at 75V for 90 min. Membranes were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Tween20 (TBST) for 1 hr at RT, and then incubated with antibodies against GAPDH (1:1000), the myc epitope (1:1000), or ACTIVE MAPK (1:5000) overnight at 4°C. Membranes were washed 3 times with TBST and incubated with an HRP-linked secondary antibody (1:2000) for 1 hr at RT. Membranes were washed 3 times with TBST and incubated with SuperSignal West Pico PLUS chemiluinescent substrate. The membranes were imaged with the Bio-Rad ChemiDoc MP imaging system.

## IV. Mass spectrometry sample preparation and data analysis: Identification of SEC1 targets

Probe labeling, click chemistry and streptavidin enrichment

Jurkat cell lysates (500 µL, 2 mg/mL) in DPBS were treated with SEC1 (100 µM) or DMSO for 1 hr at RT and then treated lysates underwent click chemistry. PC biotin azide (200 µM, 100x stock in DMSO), Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (1 mM, 50X fresh stock in water), Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA ligand) (100 µM, 17X stock in DMSO:t-Butanol 1:4), and Copper(II) sulfate (1 mM, 50X stock in water) were added to the lysates. The probe -labeled samples were incubated at RT for 1 hr and then spun down for 10 min at 4°C. The pellets were washed with cold MeOH and resuspended by sonication with the tip sonicator. Samples were centrifuged and washed a second time with MeOH. The pellets were solubilized in DPBS containing 1.2% SDS via sonication and heating for 5 min at 85°C. The solubilized samples were diluted to a final concentration of 0.2% SDS in DPBS (5 mL) and incubated with 100uL of streptavidin-agarose beads for 16 hrs at 4°C with rotation. The solutions were incubated at RT for 3 hrs. The strep beads were washed with 0.2% SDS in DPBS (5 mL), DPBS (3 x 5 mL), and water (3 x 5 mL). The beads were pelleted by centrifugation (1400 x g, 3 min) between washes. Washed beads were carried through on-bead trypsin digestion.

On-bead trypsin digestion

Washed beads were resuspended in 6M urea/PBS (500 µL) containing 10mM dithiothreitol (DTT, 20X stock in water) and heated at 65°C for 20 min. 20 mM iodoacetamide (IA, 50X stock in water) was added to the samples and incubated at 37°C for 30 min. Following reduction and alkylation, the beads were pelleted by centrifugation (1400 x g, 3 min) and resuspended in 200µL of tetraethylammonium bicarbonate (TEAB, 100mM), $CaCl_2$ (1 mM, 100X stock in water), and trypsin (2 µg, Promega). Trypsin digestion was performed at 37°C overnight with shaking.

On-bead ReDiMe labeling

Post tryptic digestion, SEC1-labeled samples were labeled with 8 µL of light 20% formaldehyde and DMSO-treated samples were labeled with 8 µL of heavy 20% formaldehyde($^{13}CD_2O$). 8 µL of 0.6 M sodium cyanoborohydride was added to each sample and incubated at RT for 1 hr. Samples were placed on ice, and the reactions were quenched with 32 µL of 1% ammonium hydroxide. MS-grade formic acid (16 µL) was added to each sample and samples were combined pairwise (DMSO and SEC1-labeled) leaving the strep beads behind. Samples were desalted using Sep Pak C18 cartridges (Waters), eluted with 1.5 mL of Buffer B (20% water, 80% ACN, 0.1% formic acid), dried by speed-vac and resuspended in 25 µL of Buffer A (95% water, 5% ACN, 0.1% formic acid). Samples were stored at -20°C for future MS analysis.

LC/LC-MS/MS analysis

Mass spectrometry analysis was performed on a Thermo Fisher Orbitrap Exploris 240 mass spectrometer coupled to a Dionex Ultimate 3000 RSLnano UHPLC. Desalted peptide samples (5 µL) were loaded onto a 4 cm C18 trap column (Acclaim Pepmap 100)

and eluted onto a 15 cm C18 analytical column (Acclaim Pepmap RSLC) using a gradient of 0-100% Buffer B in Buffer A (Buffer A: 100% water, 0.1% formic acid; Buffer B: 20% water, 80% ACN, 0.1% formic acid). The flow rate through the column was set to 300 nL/min with a spray voltage of 2.1 kV. One full MS scan was followed by 20 data dependent scans of the $n^{th}$ most intense ions with dynamic exclusion enabled.

Data analysis

Protein identification and quantification of SEC1 targets was performed using the Sequest HT algorithm in Proteome Discoverer (PD; version 2.4; Thermo Fisher Scientific). The raw data was searched using a concatenated target/decoy non-redundant variant of the human UniProt database. Trypsin was used as the protease, and the maximum number of missed cleavages was set to 2. Peptide precursor mass tolerance was set to 10 ppm, and fragment mass tolerance was set to 0.02 Da. Acetylation and methionine-loss of the protein N-terminus or a combination of the two were set as dynamic modifications, along with methionine oxidation. In addition to a static modification on cysteine, static modifications of +28.031 (light) or +34.063 (heavy) on the N-terminus of the peptide and on lysine were included to account for dimethylation of primary amines. The false discovery rate (FDR) of peptide identification was set to 1%. To identify protein targets of SEC1, the resulting non-normalized light:heavy ratios were filtered for high protein FDR confidence with abundance ratios in every run and a molecular weight (MW) between 30 and 45 kDa as shown in Figure 1D. The full dataset can be found in Supplemental Table 1.

## V. Gel-based experiments

In gel-fluorescence: MCF10 cell lines

MCF10A and MCF10CA1a cell lysates (50 µL, 2 mg/mL) in DPBS were treated with 10µM KA or DMSO as a control. Lysates were incubated at RT for 1 hr and then labeled with SEC1 (100 µM) at RT for 1 hr. Labeled lysates were then subjected to click chemistry and in-gel fluorescence analysis, as well as western blotting analysis, as described above.

Competitive in-gel fluorescence: $H_2O_2$

HeLa cell lysates (50 µL, 2 mg/mL) in DPBS were treated with increasing concentrations of hydrogen peroxide (10, 100, 500 µM, 1 mM and 10 mM) or water as a control. Lysates were incubated at RT for 1 hr and then labeled with SEC1 (100 µM) at RT for 1 hr. Labeled lysates were then subjected to click chemistry and in-gel fluorescence analysis as described above.

Competitive in-gel fluorescence: GAPDH inhibitors

Jurkat cell lysates (50 µL, 2 mg/mL) in DPBS were treated with Iodoacetate (10 µM, 100X stock in water), Bromopyruvic acid (10 µM, 100X stock in DMSO), Koningic acid (10 µM, 100X stock in DMSO), DMF (10 µM, 50X stock in DMSO) or DMSO as a control. Lysates were incubated at RT for 1 hr and then labeled with SEC1 (100 µM) or IA-light (100 µM) at RT for 1 hr. Labeled lysates were then subjected to click chemistry and in-gel fluorescence analysis as described above.

Competitive in-gel fluorescence: Koningic acid

Jurkat and HeLa cell lysates (50 µL, 2 mg/mL) in DPBS were treated with increasing concentrations of Koningic acid (100, 500 nM, 1, 2, 4, 6, 8, 10 µM) or DMSO as a control. Lysates were incubated at RT for 1 hr and then labeled with SEC1 (100 µM) at RT for 1 hr. Labeled lysates were then subjected to click chemistry and in-gel fluorescence analysis as described above.

## VI. Biochemical assays

Mechanism of Inhibition Biochemical Assay

Koningic acid mechanism of inhibition was determined by varying each substrate separately and measuring GAPDH activity over time. One µL of KA in 100% DMSO was added to a 384-well polypropylene, clear microplate (Greiner, 781280) at varying concentrations (12-point, 3-fold dilution, top concentration of 120 µM). Ten µL of the varying substrate (8-point, 2-fold dilution; top concentration of 600 µM for D-glyceraldehyde 3-phosphate (G3P) and 200 µM for $NAD^+$) was dispensed into the plate. The reaction was initiated with 40 µL of enzyme and substrates (0.25 nM GAPDH, 6 mM sodium arsenate, and either 24 µM $NAD^+$ or 74 µM G3P). The reaction was quenched over the linear portion of the reaction curve with the addition of bromopyruvic acid at a final concentration of 1 mM. Enzymatic activity was measured by the generation of NADH product using the NAD/NADH-Glo detection kit (Promega). Addition of 5 µL reaction mix was combined with 5 µL of detection reagent and incubated for 45 minutes at room temperature in a 384-well polystyrene, white microplate (PerkinElmer ProxiPlate, 6008280). Total bioluminescent signal was measured using an Envision multimode plate reader (PerkinElmer). $NAD^+$ and G3P data were globally fit to an uncompetitive and competitive inhibition model (respectively) using Prism 9.2 (GraphPad).

Fractional Activity Biochemical Assay

The partition ratio was determined by measuring residual GAPDH activity at varying concentrations of KA following rapid dilution of enzyme-$NAD^+$-inhibitor complex. GAPDH (100x, 25 nM) was pre-incubated with $NAD^+$ (100x, 4.8 mM) and varying concentrations of inhibitor (100x, 7-point, 2-fold dilution, top concentration of 400 nM) for 60 minutes. The solution was then diluted in assay buffer (50 mM Bicine, pH 7.6, 0.004% Tween-20, and 0.005% bovine serum gelatin) 100-fold and enzymatic activity was measured by the generation of NADH product using the NAD/NADH-Glo detection kit (Promega). The assay was performed in a 384-well polystyrene, white microplate (PerkinElmer ProxiPlate, 6008280) under balanced conditions (74 µM G3P, 6 mM sodium arsenate, and 24 µM $NAD^+$) at 0.25 nM enzyme. The reaction was quenched over the linear portion of the reaction curve with the addition of bromopyruvic acid at a final concentration of 1 mM. Total bioluminescent signal was measured using an Envision multimode plate reader (PerkinElmer). The partition ratio (r) was calculated using a simple linear regression and is equal to x-axis point of intersection minus 1.

Jump Dilution Biochemical Assay

Compound reversibility was evaluated by measuring enzymatic activity of full-length GAPDH over time following the rapid dilution of the enzyme-inhibitor complex. GAPDH (100x, 25 nM) was pre-incubated with a saturating concentration of inhibitor (10x $IC_{50}$, 500 nM) and $NAD^+$ (100x, 2.4 mM) for 30 minutes. The solution was then diluted in assay buffer (50 mM Bicine, pH 7.6, 0.004% Tween-20, and 0.005% bovine serum gelatin) 100-fold and enzymatic activity was measured by the generation of NADH product using the NAD/NADH-Glo detection kit (Promega). The assay was performed in a 384-well polystyrene, white microplate (PerkinElmer ProxiPlate, 6008280) under balanced conditions (74 µM G3P, 6 mM sodium arsenate, and 24 µM $NAD^+$) at 0.25 nM enzyme. The reaction was quenched over the linear portion of the reaction curve with the addition of bromopyruvic acid at a final concentration of 1 mM. Total bioluminescent signal was measured using an Envision multimode plate reader (PerkinElmer).

Thermal Shift Assay

Aliquots of GAPDH were thawed from -80 °C and centrifuged at 12,000 RPM for 5 minutes at 4 °C. Clarified protein was diluted to 2.5 µM in the same buffer used for size exclusion (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM DTT). Stock solutions of cofactors and inhibitors were prepared at 50-fold the final desired assay concentration in DMSO. GAPDH protein was mixed with various inhibitors and/or cofactors and buffer, incubated on ice for 2 hours, and centrifuged at 12,000 RPM for 5 minutes at 4 °C. Finally, 24.5 µL of the clarified protein complexes were transferred to PCR tubes and 0.5 µL of 250X SYPRO® Orange was added. The final 25 µL reaction mixture contained 2 µM GAPDH, various specified concentrations of cofactor and/or inhibitor (or 2% DMSO for compound-free samples), and 5X SYPRO® Orange. Reaction mixtures were heated in a LightCycler® 480 II (Roche) from 20 °C to 95 °C in increments of 10 °C /60 s and fluorescence emitted after excitation at 480nm was recorded at each step. Melting temperatures ($T_m$) were derived from the inflection point of the fluorescence vs temperature curves of each melting reaction.

Surface Plasmon Resonance Assay

Full-length, N-terminal avi-tagged GAPDH was diluted to 20 µg/mL and immobilized on a streptavidin sensor chip at a flow rate of 10 µL/min for 480 seconds. An average surface density of 4.4 kRU was obtained. All experiments were performed using a Biacore 8K instrument (Cytiva). The characterization of KA-GAPDH interactions was conducted at 25 °C in 10 mM HEPES at pH 7.4, 150 mM NaCl, 0.05% P20, 100 µM $NAD^+$, and 2% DMSO. Compounds were injected in multi-cycle experiments for 90 s at a flow rate of 30 µL/min, followed by a dissociation phase up to 270 s. KA was tested at a top concentration of 1000 µM (7-point, 3-fold dilution series). Sensorgrams were double referenced (reference surface, blanks) prior to analysis. Kinetic parameters were determined as average values based on two replicate experimental series.

## VII. Mass Spectrometry Sample Preparation: Identification of Koningic acid targets

Probe labeling, click chemistry and streptavidin enrichment

Jurkat and HeLa cell lysates (500 µL, 2 mg/mL) in DPBS were treated with Koningic acid (1, 5, or 10 µM) or DMSO as a control for 1 hr at RT. DMSO-treated lysates were subsequently labeled with IA-heavy (100 µM) and Koningic acid-treated lysates were labeled with IA-light (100 µM). Following probe labeling, lysates underwent click chemistry. Diazobenzene biotin azide (azo-tag, 200 µM, 100x stock in DMSO), Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (1 mM, 50X fresh stock in water), Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA ligand) (100 µM, 17X stock in DMSO:t-Butanol 1:4), and Copper(II) sulfate (1 mM, 50X stock in water) were added to the lysates. The samples were incubated at RT for 1 hr and then spun down for 10 min at 4°C. The pellets were washed with cold MeOH and resuspended by sonication with the tip sonicator. Samples were centrifuged and washed a second time with MeOH. The pellets were solubilized in DPBS containing 1.2% SDS via sonication and heating for 5 min at 85°C. The solubilized samples were diluted to a final concentration of 0.2% SDS in DPBS (5 mL) and incubated with 100uL of streptavidin-agarose beads for 16 hrs at 4°C with rotation. The samples were warmed to room temperature, pelleted by centrifugation (1400 x g, 3 min) and the supernatant was discarded. The strep beads were washed with 0.2% SDS in DPBS (5 mL), DPBS (3 x 5 mL), and water (3 x 5 mL). The beads were pelleted by centrifugation (1400 x g, 3 min) between washes. Washed beads were carried through on-bead trypsin digestion.

On-bead trypsin digestion and diazobenzene cleavage

Washed beads were resuspended in 6M urea/PBS (500 µL) containing 10mM dithiothreitol (DTT, 20X stock in water) and heated at 65°C for 20 min. IA (20mM, 50X stock in water) was added to the samples and incubated at 37°C for 30 min. Following reduction and alkylation, the beads were pelleted by centrifugation (1400 x g, 3 min) and resuspended in 200µL of 2M urea/PBS, $CaCl_2$ (1 mM, 100X stock in water), and trypsin (2 µg, Promega). Trypsin digestion was performed at 37°C overnight with shaking. Beads were pelleted by centrifugation (1400 x g, 3 min) and sequentially washed with PBS (500 µL x 3) and $H_2O$ (500 µL x 3). Labeled peptides were eluted from the beads by sodium dithionite-mediated cleavage of the diazobenzene of the azo-tag. For this, beads were incubated with sodium dithionite (50µL, 50mM in PBS) for 1 h at RT. Beads were pelleted by centrifugation (1400 rcf x 3 min) and the supernatant was collected. The cleavage process was repeated twice more with 50 mM sodium dithionite (75 µL) and supernatants were combined with the previous. The beads were then washed twice with water (75 µL) and supernatants were collected and combined with previous. Formic acid (17.5 µL) was added to the combined supernatants and the samples were stored at -20°C for future MS analysis.

LC/LC-MS/MS analysis

Mass spectrometry analysis was performed on a Thermo Fisher LTQ Orbitrap Discovery mass spectrometer coupled to an Agilent 1200 series HPLC. IA-light and IA-heavy labeled peptides were pressure loaded onto a 250 µm fused-silica desalting column

packed with 4 cm of Aqua C18 reverse phase resin (Phenomenex) using a high-pressure injection cell (Next Advance). The peptides were separated on a 100 µm fused silica biphasic column with a 5 µm tip. The tip was packed with 10 cm of C18 resin and 3 cm Partisphere strong cation exchange resin (SCX, Whatman) and peptides were eluted using a gradient of 0-100% Buffer B in Buffer A (Buffer A: 95% water, 5% ACN, 0.1% formic acid; Buffer B: 20% water, 80% ACN, 0.1% formic acid). Five separate salt pushes (0%, 50%, 80%, 100%, 100%) were used to elute peptides from the SCX onto the C18 resin and into the mass spectrometer, as originally outlined in Weerapana *et al.*[2] The flow rate through the column was set to ~0.25 µL/min and the spray voltage was set to 2.75 kV. One full MS scan was followed by 8 data dependent scans of the $n^{th}$ most intense ions with dynamic exclusion enabled.

Data analysis

MS data from all samples were searched using the SEQUEST algorithm[3] against a concatenated target/decoy non-redundant variant of the human UniProt database. A static modification of +57.02146 on cysteine was included to account for alkylation by IA and differential modifications of +228.13749 (light) or +234.15762 (heavy) on cysteine were included to account for labeling of IA-light or IA-heavy appended to the azo-tag. SEQUEST output files were filtered using DTASelect 2.0[4,5] with -trypstat and -modstat options and a maximum false discovery rate of 5% applied. Additionally, peptides were required to be fully tryptic (-y 2), have a found modification (-m 0), a delta-CN score greater than 0.06 (- d 0.06), and only a single peptide was required per locus (-p 1). Quantification of heavy/light (H:L) ratios was performed using the CIMAGE quantification package as previously described.[6]

## VIII. Immune cell activation and inhibition

Jurkat cell activation

Jurkat cells (5mL, 2.0x$10^6$ cells/mL) were diluted in 45mL of fresh RPMI-1640. DMSO or PMA (50uL, 20ng/mL) was added to each sample. Activation was performed for 10 and 20 minutes. Following activation, cells were spun down (200 x g, 4min) and the pellets were washed in ice cold PBS (5 mL x 2). Cell pellets from each condition were lysed in ice-cold DPBS via sonication with a tip sonicator. For samples that were going to be used in an ACTIVE MAPK western blot, 1% phosphatase inhibitor cocktails 2 and 3 (Sigma Aldrich) was added to the DPBS prior to sonication. Whole cell lysates were separated into soluble and insoluble lysate fractions by centrifugation at 4°C (16,000 x g, 15 min). The insoluble fraction was discarded and the protein concentration of the soluble fraction was determined using the DC Protein Assay Kit (Bio-Rad).

Th1 Effector Cellular Assay

One µl of a varying concentration of KA (8 point, 3-fold dilution, starting concentration of 30 µM in neat DMSO) was added in triplicate to a 96-well sterile U-bottom plate (Falcon). The 0% inhibition control consisted of 1 µL neat DMSO, the 100% inhibition control of 1 µL 1 mM Ibrutinib (5 µM final concentration). One hundred microliters of CD4+ Th1 eff

cells (2 million cells/mL) was added to each well and incubated at 37 °C for 30 minutes. One hundred microliters of Immunocult+ (human CD3/CD28/CD2 T cell activator, StemCell Technologies) was then added to each well. The plate was allowed to incubate for 24 hours at 37 °C. The following day, the plate was spun down to pellet the cells and the supernatant transferred (1:50 dilution) into an ELISA plate (Meso Scale Discovery). Concentration of cytokine was determined following the manufacturer's instructions.

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