

Appendix 2A

Materials and Experimental Procedures

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Materials

Unless otherwise stated all chemicals were used as received.

Barcode Microfabrication and Validation

Chrome masks of the custom barcode design were purchased from University of California, Los Angeles, Nanoelectronics Research Facility, and a Karl Süss MA/BA6 mask aligner (SÜSS MicroTec AG) was used for UV exposure. Silicon wafers (Wafernet Inc.), SU8-2025, and SU8 developer (Microchem Corp) were used for the barcode mold fabrication. Anhydrous dimethylsulfoxide (DMSO), sodium dodecyl sulfate (SDS), and bis(sulfosuccinimidyl)suberate (BS3) used in barcode fabrication were purchased from American Type Culture Collection (ATCC), Sigma Aldrich, and ThermoFischer Scientific respectively. The Sylgard 184 elastomer, and poly-L-lysine coated glass slides used in DNA barcode microfabrication were purchased from Dow Corning and ThermoFischer Scientific respectively. The poly-L-lysine (PLL) solution (0.1% (w/w)) used for barcode fabrication was purchased from Sigma Aldrich. All ssDNA used for barcode fabrication and barcode validations were purchased from either Bioneer Inc. or IDT Inc.

Protein Expression, Purification, and Refolding

The Bacto Tryptone (Tryptone) and Bacto yeast (yeast) for the preparation of LB broth media were purchased from Becton, Dickinson, and Company. The ampicillin sodium salt, chloramphenicol, and isopropyl β -D-1-thiogalactopyranoside (1,6-IPTG, dioxane free) used for protein expression from *E. coli* were purchased from Sigma Aldrich. The one-shot B21(D3) *E. coli* cells and PQE80 vector (His₆-tagged human KRas Isoform 4B (residues 1-169)) used for expression of KRas protein were purchased from Life

Technologies and Qiagen respectively. Lysozyme (L6876), DNase I (10104159001), and RNase A (R6513-10MG) used for lysing cells containing cysteine-modified streptavidin (SAC) were purchased from Sigma-Aldrich. Cells containing KRas protein were lysed using a constant pressure cell disruptor (Constant Systems Ltd., Scotland, UK). Surfactants Triton X-100 and polysorbate 20 (Tween20) were purchased from Sigma Aldrich. The 20x phosphate buffered saline with 0.05% Tween 20 (PBST) and phosphate buffered saline (PBS) used for protein purification and immunofluorescent assays (IFAs) were purchased from Cell Signaling Technology and Corning respectively. The sodium bicarbonate (NaHCO_3), ammonium acetate (NH_4OAc), sodium acetate (NaOAc), sodium chloride (NaCl), imidazole, tris(hydroxymethyl)aminomethane (Tris), tris(hydroxymethyl)aminomethane hydrochloride salt ($\text{Tris}\cdot\text{HCl}$), guanidinium chloride ($\text{Guan}\cdot\text{HCl}$), magnesium chloride pentahydrate ($\text{MgCl}_2\cdot 5\text{H}_2\text{O}$), and beta-mercaptoethanol (βME) used in protein purification and IFA assays were purchased from Sigma Aldrich. The 2-imminobiotin agarose resin, Superdex 75 (10/300) increase column, and Ni-NTA superflow cartridge used for fast protein liquid chromatography (FPLC) purification were purchased from Sigma Aldrich, GE Healthcare Life Sciences, and Qiagen respectively. The Amicon Ultra-15 and Ultra-4 centrifugal filters used to concentrate protein samples were purchased from EMD Millipore.

SAC-DNA Conjugation and Validation

The tris-(2-carboxyethyl)phosphine hydrochloride (TCEP), anhydrous N,N-dimethylformamide (DMF), N-succinimidyl-4-formyl benzaldehyde (S-4FB) and maleimide 6-hydrazino-nicotinamide (MHPH) used for the conjugation of ssDNA to cysteine-modified streptavidin (SAC) were purchased from Sigma Aldrich and Solulink.

The biotin-A₂₀-Cy3 (Biotin*) probe used to test the biotin binding ability of the SAC-DNA set and used as a biotinylated blank for IFA assays was purchased from IDT Inc. The complementary ssDNA' used for conjugation to SAC were purchased from Bioneer Inc.

In Situ Library Screen and Hit Bead Sequencing

The mouse anti-biotin-alkaline phosphatase conjugated antibody (ab) (#A6561), goat anti-rabbit-alkaline phosphatase conjugated ab (#A8025), and rabbit anti-Ras ab (CST #3965) used for the combined anti screen/pre-clear and the subsequent product/target screens were purchased from Sigma Aldrich and Cell Signaling Technology respectively. The combined 5-bromo-4-chloro-3-indoyl phosphate (BCIP)/ nitro blue tetrazolium (NBT) (#S3771) used to develop hits during the library screens was purchased from Promega. The concentrated hydrochloric acid used to quench the BCIP/NBT development was purchased from Sigma Aldrich. Sequencing of bead hits occurred via Edman degradation sequencing on a Procise Protein Sequencer (Applied Biosystems, California).

Peptide Synthesis and Purification

Fmoc-protected amino acids were purchased from Anaspec, AAPTec, Bachem, ChemPep, and Sigma-Aldrich. Biotin NovaTag™ resin was obtained from EMD Chemicals, Inc. and used for the synthesis of biotinylated peptides and epitopes used for the screens using standard Fmoc/'Bu coupling and cleavage protocols. The peptide one-bead-one compound (OBOC) library was prepared on Tentagel Resin purchased from RAPP Polymere. The Fmoc-protected propionic acid polyethylene glycol (PEG_n) linkers were purchased from ChemPep Inc. The L-ascorbic acid and copper (I) iodide (CuI) used for

click reactions were purchased from Sigma Aldrich. The N-methyl pyrrolidine (NMP), 1-[Bis (dimethylamino) methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU), and *N,N'*-diisopropylethylamine (DIPEA) used in peptide synthesis were bought from EMD Chemicals, Inc., ChemPep, and Sigma-Aldrich respectively. Piperidine, trifluoroacetic acid (TFA), and triethylsilane (TESH) were purchased from Sigma-Aldrich. The diethyl ether used to precipitate crude peptide was purchased from JT Baker. The Omnisolv grade acetonitrile (MeCN) used for peptide purification was purchased from EMD Millipore. Unless otherwise stated, peptide preparation was performed using a Titan 357 Automatic Peptide Synthesizer (AAPPTec, Louisville, KY) or a Liberty 1 Automated Peptide Synthesizer (CEM, North Carolina). Mass analysis was performed using a Voyager De-Pro matrix assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS) (Applied Biosystems, California). The crude peptides were dissolved in either DMSO (Sigma Aldrich) or (1:1) MeCN/doubly distilled water (MQ H₂O) w/ 0.1% TFA before purification by a gradient of 0% to 50% acetonitrile in MQ H₂O with 0.01% (v/v) TFA using a RP-HPLC (Beckman Coulter System Gold 126 Solvent Module and 168 Detector) using a C18 reversed phase semi-preparative column (Phenomenex Luna 10 μm, 250 × 10 mm). The concentration of peptides and epitopes was determined using a Nanodrop 2000 Spectrophotomer (ThermoFischer Scientific Inc., Massachusetts).

B-RAP Immunofluorescent Assays and Multi-Well Enzyme-Linked Immunosorbent Assays

The Bovine Serum Albumin (BSA, Biotin free A1933-25G) used in the IFAs and multi-well enzyme-linked Immunosorbent assays (ELISAs) was purchased from Sigma-Aldrich.

The non-fat dry milk powder used in the enzyme-linked Immunosorbent assays (ELISAs) was purchased from Best Value. The rabbit anti-Ras (CST #3965), Goat anti-rabbit IgG HRP-linked (CST #7074), goat anti-rabbit HRP-linked (CST #7074), and goat anti-rabbit-Alexafluor 647 conjugated (ab150079) were purchased from Cell Signaling Technologies and Abcam respectively. The ELISAs were run on either 96-well clear Pierce Neutravidin Plates (#15129) or Pierce Neutravidin Coated Plates (#15127) purchased from ThermoFischer Scientific. The TMB Microwell Peroxidase Substrate System (#50-76-00) that was used to develop ELISAs was purchased from KPL. The sulfuric acid ($\text{H}_2\text{SO}_{4(\text{aq})}$) used to quench the enzymatic amplification reaction in the ELISAs was purchased from JT Baker. The 96-well ELISA plates were read using a Flexstation 3 plate reader (Molecular Devices LLC, Sunnyvale, CA). All barcode slides were scanned using an Axon GenePix 4400A (Molecular Devices LLC, Sunnyvale, CA).

Measuring the Functional Effect of the Allosteric Ligands on KRas Protein GTPase

Activity

The intrinsic GTPase activity of WT KRas protein was measured using the GTPase-Glo Assay Kit (#V7681) from Promega Corporation (Madison, WI) on opaque white 96-well plates (#6005290) from Perkin Elmer Life Sciences (Waltham, MA). Luminescence was recorded on the Flexstation 3 plate reader used for multi-well ELISAs.

Procedures

DNA Barcode Chip Patterning and Validation

The DNA barcode chips were prepared by micro channel-guided flow patterning as described in **References 27** and **28** of the main text. A PDMS slab having the micro-channels was made by soft lithography on a silicon wafer. Its mold was designed as

Figure S1 and prepared with SU8 2025 negative photoresist. The fabricated mold contained microfluidic circuits of 20 parallel channels with 50 μm width and ~ 40 μm height. Sylgard® 184 PDMS pre-polymer and curing agents were mixed in a 10:1 ratio, degassed, $\sim 60\text{g}$ of the mixture poured onto the mold, and baked for two hours at 80 °C for curing. The cured PDMS slab was peeled off the mold, cut into individual microfluidic molds, and the inlet and outlet holes of the microfluidic circuits were punched with the sizes of two mm and 0.5 mm respectively. The number of the inlets and outlets punched out were determined by the number of single stranded DNAs (ssDNAs) used in the assay, and fifteen orthogonal ssDNAs (**B-Q**, Table S2) were used in this study. The slab was then aligned with a PLL glass slide, and bonding occurred with baking at 80 °C for two hours. After cooling briefly, the inlet wells were loaded with 3 μL of a PLL solution (0.1% (m/m) in H_2O), and the PLL solution was flowed and dried by 13.8 kPa nitrogen gas blowing through the solution-loading device overnight. The next day, C6 amine-modified DNA solutions (300 μM in (3:2 (v/v)) PBS/DMSO) were individually mixed (1:1) with a 2 mM BS3 cross-linker solution in PBS. Each freshly prepared mixture was flown through a channel under 13.8-20.6 kPa of nitrogen gas using the solution-loading device for 1 hour, and then only the assembled PDMS slab and the bonded PLL slide were incubated at room temperature for 2 hours in a humidified chamber. After incubation, the PDMS slab was removed, and the DNA patterned PLL slides were washed both a 0.02% aqueous SDS solution and doubly distilled water (MQ H_2O) (3x), and spun dry.

Barcode Validation

To validate the DNA barcode chips, a 5'-modified Cy3-labeled complementary ssDNA cocktail was prepared in 1% BSA in PBS (50nM each ssDNA). The validation occurred over two rounds (B, D, F, H, K, N, P, M then C, E, G, I, L, O, Q) in order to check for channel leaks and crossover. A 120 μL aliquot of the validation solution was applied to a small region at the bottom edge of the DNA barcode before incubating at 37 °C for one hour. After incubation, this region was washed with 1% BSA in PBS followed by PBS (2x), and the slide was spun dry before being scanned by Axon GenePix 4400A (532 nm, PMT 450, Power 15% (23W)) (Figure S2).

Expression of Cysteine-Modified Streptavidin (SAC) Protein

The SAC protein was expressed using a modification of the procedure reported by Sano and Cantor.¹ A 100 mL starter culture of autoclaved LB media (10.0 g Tryptone, 5.00 g yeast, 10.0 g NaCl per L H₂O) was prepared by inoculating with 50 μL of 100 mg/mL of ampicillin (final concentration 50 $\mu\text{g}/\text{mL}$) and 100 μL of 34 mg/mL chloramphenicol (final concentration 34 $\mu\text{g}/\text{mL}$) followed by a sterile pipet scraping of a 50% (v/v) glycerol stock containing transformed *E. coli* BL21(D3) cells. The starter culture incubated overnight at 37 °C and 250 RPM before adding 10.0 mL of starter culture aliquots to six 2800mL Fernbach-Style Culture Flasks containing 1.00 L autoclaved LB media with 500 μL of 100 mg/mL of ampicillin (final concentration 50 $\mu\text{g}/\text{mL}$), 1000 μL of 34 mg/mL chloramphenicol (final concentration 34 $\mu\text{g}/\text{mL}$), and 1000 μL of 40% (w/w) autoclaved glucose (final concentration 0.4% (w/w)). The flasks were left to culture at 37.0 °C, 250 RPM until $A_{680} = 0.500$, and induction was triggered with 1000 μL of a 400 mM 1,6-IPTG solution (final concentration 400 μM). Expression continued at 37.0 °C, 250 RPM for four hours before spinning down the cells at 6000 RPM, 5 minutes at 4 °C. The cells

were resuspended in 50 mL of a 10 mM Tris, 1 mM EDTA, 130 mM NaCl buffer at pH=8.0 and spun down (2x). The cells were then flash frozen in N_{2(l)} and stored at -80.0 °C until needed.

Isolation of SAC Inclusion Bodies from e. Coli Cells

The cell pellet was thawed in ice before resuspending in two 50-mL falcon tubes with 40 mL of TEX buffer (30mM Tris, 2mM EDTA, 0.1% TritonX). Each tube was charged with 40 mg fresh lysozyme powder (Final concentration 1.0 mg/mL), vortexed until mixed, and allowed to lyse for 30min while tumbling at RT. The solution was very viscous after lysis. The DNA and RNA were degraded by adding 400 µL of 10 mg/mL DNase and 10 mg/mL RNase in TE Buffer (10 mM Tris, 130 mM NaCl, 1 mM EDTA) (final concentration 10 µg/mL), 960 µL of 500 mM MgCl₂ (final concentration 12 mM), and 40 µL of 1 M MnCl₂ (final concentration 1 mM) to each tube of cell lysate, and the solution was allowed to digest for 30 minutes while tumbling at RT. After digestion, the solution was spun down at 7800 RPM, RT for 10 minutes. The resulting inclusion body (IB) pellets were both washed in 40 mL TEX buffer and spun down at 7800 RPM, 5 minutes at RT. Pellets were washed with 40 mL buffer minus Triton X again before spinning down at 7800 RPM, 5 minutes at RT once more. Each pellet was taken up in 10 mM Tris and spun down at 7800RPM, 10 minutes at RT, aliquotted, and stored at -80.0 °C until needed. If the final pellet is light brown then some DNA is still present. This will be removed at the beginning of the refolding procedure.

Refolding and Purification of SAC Protein

The procedure described here is a modification of the procedure developed by Sano and Cantor.¹

*****After the initial denaturing keep all solutions at 4 °C*****

An IB aliquot was dissolved in 1000 μ L denaturing buffer (6 M Guanidine • HCl at pH=1.5 with 10 mM β ME), vortexed, spun down at 13,000 RPM, 2 min at RT, and filtered using a 0.45 μ M low-protein binding filter. **The resulting solution should be clear and nearly colorless.** The A_{280} was measured on a Nanodrop2000 spectrophotometer, and the concentration of denatured SAC monomer was calculated.² The denatured SAC solution was diluted to 1000 μ L in denaturing buffer and added dropwise to a rapidly stirring solution of refolding buffer (50 mM NH_4OAc , 150 mM NaCl, and 10 mM β ME at pH=6.0) (Final [denatured SAC] \sim 4 μ M). The stir rate was then decreased to about half of its original value, and the solution was covered by aluminum foil to refold overnight. After sterile filtration with a 0.45 μ m low-protein binding filter the resulting solution was concentrated to 10-15 mL using Amicon Millipore filters (10,000-30,000 MWCO) before dialyzing the refolded SAC protein in buffer A (50 mM NaHCO_3 , 500 mM NaCl, 10 mM β ME at pH=11.0) until the solution had a pH of \sim 11 (about 2 hours). The crude protein was then diluted (1:1) with buffer A, mixed with 2 mL of 2-iminobiotin agarose resin, and allowed to incubate with tumbling in the cold room for one hour. After incubation the supernatant was eluted (3x) before eluting with buffer A until the A_{280} went to baseline. Pure SAC was eluted with buffer B (50 mM NaOAc, 50 mM NaCl at pH=4.0) until the A_{280} went to baseline again. Fractions with pure SAC were pooled and dialyzed against a PBS solution (PBS, 10 mM β ME, pH=7.5) overnight. The SAC was concentrated to \sim 1 mg/mL final concentration, divided into 100 μ L aliquots, and stored at -80 °C.

Preparation of SAC-DNA Conjugates

For each planned SAC-DNA conjugation, two Zeba columns were prepared (3 x 300 μ L of 5 mM TCEP in PBS, 3.9k RPM, 1 min at RT). Each 100 μ L aliquot of SAC was desalted in two separate Zeba columns to remove the β ME (3.9k RPM, 2 min at RT). After transferring to Eppendorf tubes, 6 μ L anhydrous DMF was added followed by 6 μ L MHPH (100 mM in anhydrous DMF). Separate Eppendorf tubes were charged with 80 μ L of 500 μ M of conjugation ssDNA in PBS followed by 15 μ L anhydrous DMF and 20 μ L S-4FB (100 mM in anhydrous DMF). The SAC and DNA solutions were vortexed gently, briefly spun down, and left to react at RT in the dark for four hours. For each conjugation in progress, four Zeba columns were buffer exchanged with citrate buffer (150 mM NaCl, 50 mM sodium citrate, pH=6.0) (3 x 300 μ L citrate buffer, 3.9k RPM, 1 min at RT). The SAC and DNA solutions were desalted separately (2 x 3.9k RPM, 2 min, at RT) before combining each SAC protein aliquot with a unique ssDNA solution. The solutions were vortexed gently, briefly spun down, and left to react in the dark at RT overnight. The reactions were quenched by placing at 4 °C. Each SAC-DNA conjugate was purified by FPLC using a Superdex75 Increase column (isocratic in PBS, 0.5 mL/min, 0.5 mL fractions, 75 minutes). Fractions containing pure SAC-DNA were pooled and concentrated using Amicon Ultra-4 Centrifugal filters (30k MWCO): 3900 RPM, 30 minutes at 4 °C. The concentrated SAC-DNA proteins were quantified³ using a Nanodrop2000 spectrophotometer in the ssDNA nucleic acid mode (using two for the average number of ssDNA strands conjugated as previously established) and stored at 4 °C until needed.

Biotin Binding Test of SAC-DNA Conjugates

Buffers used:

Wash buffer: PBS + 0.05% Tween20 (PBST)

Blocking Buffer: PBS + 1% BSA

***Wash steps used 50 μL /well**

***Incubation steps used 30 μL /well**

****After loading the Biotin* probe, change pipette tips every time that you aspirate or add solution to a well to prevent cross contamination****

A prefabricated PDMS template was aligned onto the DNA barcode and the microchip slide was taped into a 10 cm petri dish. The wells were washed with PBST before loading blocking buffer and placing the platform into a 37 °C incubator for one hour. A cocktail containing 50 nM of each SAC-DNA in PBS was prepared and added to the pre-blocked wells. The SAC-DNA conjugates were allowed to hybridize to the DNA barcode at 37 °C for one hour before washing the wells with PBST (3x). Each well was loaded with 50 nM, 100 nM, 150 nM, 200 nM, 300 nM, or 400 nM Biotin* in PBS (Figure S5), and the platform was left to shake covered at RT for one hour. The wells were washed with PBST (3x) before peeling off the PDMS slab and dipping the barcode into PBS, (1:1) PBS/MQ H₂O, MQ H₂O (2x). The barcode was then spun dry and read on the Genepix (532 nm, PMT 450, Power 15% (23 W)).

WT KRas Protein Expression and Purification

The KRas protein was expressed and purified using a modification of the procedure reported by Kuriyan.⁴ A starter culture of 100 mL of autoclaved LB media was inoculated with 100 μL of 100 mg/mL of ampicillin (final concentration 100 $\mu\text{g}/\text{mL}$) followed by a scraping of a 25% (v/v) glycerol stock containing transformed *E. coli* BL21(D3) cells. The starter culture was left in an incubator at 37.0 °C, 250 RPM overnight before adding 10.0

mL starter culture aliquots to six 2800 mL Fernbach-Style Culture Flasks containing 1.00 L autoclaved LB media with 1000 μL of 100 mg/mL of ampicillin (final concentration 100 mg/mL). The flasks were left to culture at 37.0 $^{\circ}\text{C}$, 250 RPM until $A_{680} = 0.500\text{-}0.600$ and induction was triggered with 1000 μL of a 250 mM 1,6-IPTG solution (final concentration 250 μM). The cells were then left to express overnight at 18.0 $^{\circ}\text{C}$, 250 RPM before being spun down, resuspended in buffer A (20 mM Tris, 500 mM NaCl, 20 mM imidazole, 5 mM MgCl_2 , pH=8.0), flash frozen in $\text{N}_2(\text{l})$, and stored at -80.0 $^{\circ}\text{C}$ until needed. After thawing and douncing, the cells were lysed using a cell disruptor, the cell wall lysate spun down at 8000 RPM, 4 $^{\circ}\text{C}$ for 20 minutes, sterile filtered with a 0.45 μm low-protein binding filter, and purified using FPLC with a Ni-NTA superflow cartridge and a gradient of buffer A to buffer B (20 mM Tris, 300 mM NaCl, 250 mM imidazole, 5 mM MgCl_2 , pH=8.0). Fractions containing pure KRas were pooled and dialyzed against Tris buffered saline (TBS) (25 mM Tris, 150 mM NaCl, 10 mM MgCl_2 , pH=7.5) overnight. The resulting solution was concentrated using Amicon Ultra-15 centrifugal filters (10k MWCO), quantified,² separated into aliquots, flash frozen in $\text{N}_2(\text{l})$, and stored at -80.0 $^{\circ}\text{C}$ until needed.

In-Situ Library Click Screen Combined Pre-clear/Anti-Screen

The *in-situ* click dual SynEp library screen followed a procedure similar to the one outlined in **Reference 1** from the main text using 450 mg of Pra-capped one-bead-one-compound (OBOC) library. Blocking was performed overnight at 4 $^{\circ}\text{C}$ with blocking buffer (1% BSA and 0.1% Tween20 in TBS). After washing with blocking buffer (3 x 3 minutes) incubation with 25 μM of each scrambled SynEp in binding buffer (0.1% BSA and 0.1% Tween20 in TBS) occurred overnight at 4 $^{\circ}\text{C}$. The library was washed with

TBS (3 x 1 minute) then stripped with 7.5 M Gua•HCl (pH=2.0) at RT for one hour to remove any non-covalently bound scrambled SynEps. Ten rinses with TBS preceded another incubation with blocking buffer at RT for one hour. After five quick rinses of the library with blocking buffer the library was incubated with a cocktail of a (10,000:1) dilution of the mouse anti-biotin-alkaline phosphatase conjugated ab (#A6561), (1,000:1) dilution of the rabbit anti-Ras ab (CST #3965), and a (10,000:1) dilution of the goat anti-rabbit-alkaline phosphatase ab (#A8025) in binding buffer to perform the preclear and anti-screen in one assay. Wash the beads (5 x 3 minutes) with a high salt buffer (25 mM Tris•HCl, 10 mM MgCl₂, 700 mM NaCl, pH=7.4) and a low salt buffer (5 x 3 minutes) (25 mM Tris•HCl, pH=7.4). The developing buffer was prepared with 66 µL of BCIP (50 mg/mL in 70% DMF) in 10mL of developing buffer (100 mM Tris•HCl, 150 mM NaCl, 1 mM MgCl₂) and incubated with the library beads in a 20cm petri dish for ten minutes before adding 66 µL of NBT (50 mg/mL in 70% DMF) and incubating for an additional fourteen minutes. The beads were then washed 5x with TBS, and stored in 0.1 M HCl_(aq) in a 20 cm petri dish. Any beads that turned purple during the combined preclear/anti-clear were promiscuous binders and consequently were picked out using a 10-µL micropipette and discarded. After removing all of the sticky beads the remaining beads were washed with 7.5 M Guan•HCl (pH=2.0) for 30 minutes, rinsed with MQ H₂O (10x), and incubated in NMP overnight to remove any trace purple coloring. Final rinses with MQ H₂O (3x), TBS (7x) preceded an overnight incubation at 4 °C with blocking buffer.

In-Situ Library Click Screen Product/Target Screens

The pre-blocked library was washed with blocking buffer (3 x 5 minutes) before loading 25 µM of each SynEp in binding buffer and incubating at RT overnight. After

rinsing with TBS (3x) the library was incubated with 7.5 M Guan•HCl (pH=2.0) for one hour at RT before rinsing with TBS (10x). The library then underwent an additional one hour incubation with blocking buffer at RT before rinsing with blocking buffer (5x), and incubating with a (10,000:1) dilution of the mouse anti-biotin-alkaline phosphatase conjugated ab in binding buffer for one hour at RT. Development of the library followed the same procedure as the preclear/anti-screen, and the darkest beads were set aside for Edman degradation sequencing. The remaining ~50 light purple beads from the product screen were prepped following the same procedure after the preclear/anti-screen and screened again, using appropriately scaled amounts of reagents, against 25 μ M of the full-length KRas protein. After developing, additional beads were picked for a total of seven dark beads from the product/target screens of which five beads yielded readable sequences by Edman degradation sequencing.

Peptide Synthesis Protocols

All cyclic peptides and epitopes were prepared following the procedures outlined in **Reference 1**.

The peptides and epitopes were isolated using the following procedure. The resin was rinsed with DCM (5x) and dried under vacuum. A 20 mL scintillation vial was charged with a stir-bar, resin, and 3-5 mL cleavage solution (95% TFA, 2.5% TESH, 2.5% H₂O) and allowed to stir at room temperature for 2-2.5 hours. The solution was then filtered into 40 mL of cold diethyl ether, vortexed for 10 seconds, and stored at 4 °C overnight. The precipitated protein was centrifuged into a pellet at 4500 RPM for 10-15 minutes prior to decantation of the supernatant. The crude peptides were dissolved in either DMSO or (1:1) MeCN/H₂O w/ 0.1% TFA before HPLC purification, and lyophilization

of desired fractions. The resulting lyophilized powder was dissolved in DMSO, quantified,² and stored at 4 °C when not in use.

WT KRas Binding Curves Using the B-RAP Technology

Buffers used:

Wash buffer: PBS + 0.05% Tween20 (PBST)

Blocking Buffer: PBS + 1% BSA

Protein Incubation Buffer: Tris-buffered saline (TBS) + 0.05% Tween20 (TBST)

1° ab buffer: PBS + 5% BSA

2° ab buffer: PBS + 1% BSA

***Wash steps used 50 $\mu\text{L}/\text{well}$**

***Incubation steps used 30 $\mu\text{L}/\text{well}$**

****The plate must be covered during incubation steps to protect the fluorescent blank****

****After loading the KRas protein change tips every time that solution is aspirated or added to a well to prevent cross-contamination****

A pre-fabricated PDMS template was aligned onto the DNA barcode microchip, and the microchip was taped into a 10 cm petri dish. The wells on the platform were wet with 50 μL PBST before filling with blocking buffer and placing into a 37 °C incubator for 1hr. Concurrently, 40 μL 1% BSA in PBS solutions containing 750 nM of a SAC-DNA conjugate and 3.75 μM of one biotinylated PCC ligand or biotin-A₂₀-Cy3 blank were prepared for each SAC-DNA conjugate. The biotinylated ligands were allowed to complex with the SAC protein for one hour before pooling the SAC-DNA-ligand solutions (final [SAC-DNA-ligand conjugates] = 50 nM). The blocking buffer was

aspirated, and each well was loaded with the SAC-DNA-ligand conjugates cocktail for hybridization with the DNA barcode at 37 °C for one hour. The wells were washed with PBST (3x) before loading serially diluted solutions of KRas protein in protein buffer (0 to 400 µM). After shaking at RT for one hour, the wells were rinsed with PBST (5x), making sure to pipet up/down with the first addition of PBST. A (100:1) dilution of CST rabbit anti-Ras Ab (#39655) in 1° ab buffer was added to each well before shaking at RT for one hour. After rinsing the wells with PBST (3x), the wells were loaded with a (200:1) dilution of Abcam goat anti-rabbit-Alexafluor 647 linked ab (ab150079) in 2° ab buffer before shaking at RT for one hour. A final rinse of the wells with PBST (3x) preceded peeling off the PDMS slab from the barcode microchip and dipping the barcode into the following solutions: PBS, (1:1) PBS: MQ H₂O, MQ H₂O (2x). After being spun dry, the barcode was read on the Genepix (635 nm, PMT 600, PWR 40% (60 W); 532 nm, PMT 450, PWR 15% (23 W)). Data was extracted using $10^{\text{data blocks}/\text{barcode lane}}$, double background corrected using the ab-only well fluorescence and dummy ligand fluorescence in each well, and graphed in Graphpad (Sigmoidal 4PL mode with the Hill coefficient set=1). The peeled-off PDMS slab was rinsed under MQ H₂O and stored in MQ H₂O at RT until further use.

WT KRas Binding Curves using the Multi-Well ELISA Technology

Buffers used:

- Blocking Buffer: TBS + 5% milk + 0.05% Tween20
- Antibody (ab) Buffer: TBS, 5% BSA, 0.05% Tween20
- Binding Buffer: TBS, 0.1% BSA, 0.05% Tween20

***All steps were completed at room temperature**

***All wash steps used 200 μL solution/well**

***All incubations used 100 μL solution/well except for the 5% milk blocking step, which used 200 μL solution/well**

A 96-well Pierce Neutravidin Plate was washed with binding buffer (3 x 5 minutes at RT) before loading plate with a 1 μM solution of either blank (biotin-PEG₅-NHAc) (singly) or biotinylated PCC ligand (in triplicate). The plate incubated for two hours before washing with binding buffer (3 x 5 minutes). Blocking buffer was added to each well and the plate blocked for one hour before undergoing washing with binding buffer (3 x 5 minutes). Each well was loaded with either binding buffer or KRas solution (0 \rightarrow 300 μM), and the plate was incubated for thirty minutes. Plate washing with binding buffer (3 x 5 minutes) preceded incubating the plate with a (1000:1) dilution of 1^o antibody (ab) rabbit anti-Ras (CST #3965) in ab buffer for thirty minutes. The plate was washed with binding buffer (3 x 5 minutes), loaded with a (2000:1) dilution of 2^o ab goat anti-rabbit, HRP-linked ab (CST #7074) in ab buffer, and incubated for an additional thirty minutes. The plate was rinsed with binding buffer (3 x 5 minutes), TBS (1 x 5 minutes), loaded with a (1:1) mixture of TMB Peroxidase Solution and TMB Peroxidase Solution B, and developed with occasional agitation for 8-12 minutes. After quenching the enzymatic reaction with 1M H₂SO_{4(aq)} (100 μL) the plate was read at $\lambda = 450$ nm within ten minutes. The data was double background corrected using the ab-only absorbance and the dummy ligand absorbance, plotted using Prism GraphPad 7 (Sigmoidal 4PL mode with the Hill coefficient set=1), and an EC₅₀ value was calculated.

Testing Allosteric Ligands for Inhibition of Intrinsic KRas Protein GTPase Activity

The GTPase assays were run in triplicate on a multi-well plate using the GTPase Glo Assay kit from Promega with 10 μM KRas protein and varying concentrations of ligand (1 μM to 100 μM **L1a**, **L8** and 2.5 μM to 250 μM **L2**). All reagents were warmed to RT before use. A single opaque white 96-well plate was charged with 12.5 μL GTPase/GAP buffer (GTPase buffer w/ 1 mM DTT), 10 μM KRas in GTPase/GAP buffer, or 10 μM KRas protein with either **L1a**, **L2**, or **L8**. **Running the survey assays on the same plate**

allowed for direct comparison of the curves, but it necessitated the use of the first row/column on the multi-well plate which introduced some noise to the low ligand concentration points. A 12.5 μL aliquot of a 2x GTP solution (10 μM rGTP in GTPase Buffer) was then added to each well before allowing the plate to shake at RT for two hours (initial GTPase inhibition assay). The GTPase Glo reagent was reconstituted in the GTPase Glo Buffer (4 μL GTPase Glo (500x) reagent, 1996 μL GTPase Glo Buffer, 1 μL 10 mM ADP) immediately before adding 25 μL to each well. Shaking at RT for thirty minutes preceded the addition of 50 μL of the detection reagent to each well. The plate was covered and incubated for a total of ten minutes before reading the luminescence with a Flexstation 3 plate reader (All wavelengths mode, 500 ms integration time), graphed using Graphpad Prism 7 (Sigmoidal 4PL mode), and an IC_{50} value was calculated.

For the full GTPase inhibition curve for **L2** shown in Figure 4, the above procedure was followed with the change that the incubation with KRas occurred over or four hours rather than two.

References

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- (3) *The extinction coefficients for the ssDNA strands were calculated using the IDT oligo analyzer at <https://www.idtdna.com/calc/analyzer>.*
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