SUPPORTING INFORMATION

Homogeneous dual-parametric coupled assay for simultaneous nucleotide exchange and KRAS/RAF-RBD interaction monitoring

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Experimental section

Materials and instrumentation

N-terminally HIS-tagged human G(i)α (Uniprot #P63096) and N-terminally HIS-tagged *B. pertussis* Tohama I strain PtxS1 (UniProt #P04977), were produced in *E. coli* and purified as described.[1] Alexa Fluor 680 NHS (A37567) was purchased from Thermo Fisher Scientific (MA, USA). The nonadentate europium-chelate-9d, {2,2',2",2'"-{[4'-(4'"-isothiocyanatophenyl)-2,2',6',2"-terpyridine-6,6"-diyl]bis(methylene-nitrilo)}tetrakis(acetate)}europium(III), used for Eu^{3+}-GTP, Eu^{3+}-GDP, and γ-GTP-Eu^{3+} conjugations, and the soluble quencher molecule, MT2, were obtained from QRET Technologies (Turku, Finland) and used according to manufacturer's instruction.[2,3] Streptavidin (SA) was purchased from BioSpa (Milan, Italy). NAP-5 gel filtration columns, IMAC (immobilized metal affinity chromatography) columns (Ni Sepharose High Performance nickel-charged resin), Superdex 75 resin, and syringe filters were from GE Healthcare (IL, USA). Black Corning 384 well low volume assay plates (4513) were used in all assays. The GTP nonhydrolyzable analog, 5'-guanylyl imidodiphosphate (GMPPNP), was purchased from Jena Bioscience (Jena, Germany). All other reagents, including analytical-grade solvents, malachite green (MG), O-phosphorylethanolamine, N,N'-dicyclohexylcarbodiimide (DCC), tert-butanol, morpholine, pyridine, protease inhibitor cocktail (P8849), Amicon concentrator columns, GTP, and GDP were acquired from Sigma-Aldrich (MO, USA). Cloning and production of KRAS proteins are described below in sections 1.2-1.4.

The Eu^{3+}-GTP, Eu^{3+}-GDP, and γ-GTP-Eu^{3+} purifications were carried out using reversed-phase adsorption chromatography, Dionex ultimate 3000 LC system from Thermo Fischer Scientific, Dionex, and Ascentis RP-amide C18 column from Sigma-Aldrich, Supelco Analytical. Time-resolved fluorescence (TRF) emission signals were measured at 615 nm, using 340 nm excitation wavelength (600 µs delay and 400 µs decay), and TR-FRET signals were monitored at 730 nm using 340 nm excitation wavelength (75 µs delay and 400 µs decay). All measurements were performed using a standard microtiter plate reader developed by Labrox Ltd (Turku, Finland) or Spark 20M from Tecan Life Sciences (Männedorf, Switzerland). Cell lysis was performed with Microfluidizer M-110EH from Microfluidics Corp. (MA, USA). KRAS and related proteins were all purified using NGC medium-pressure chromatography system from BioRad Laboratories Inc. (CA, USA). Protein concentrations and labeling degrees were determined by measuring A_{280} and A_{680} using Nanodrop 2000C spectrophotometer from Thermo Scientific.

KRAS and related protein cloning and production

Cloning. DNA constructs for the expression of G-Hs.KRAS4b (KRAS, 1-169) and Hs.RAF1(RAF-RBD, 52-131) were previously described.[4] A Gateway Entry clone for human SOS1catalytic domain (SOS^{cat}, 564-

1048) with an upstream tobacco etch virus (TEV) protease site (ENLYFQG) was generated using a PCR amplicon from Addgene #70601 flanked by Gateway recombination sites. A Gateway Entry clone of LF-Cpe.TpeL was generated by overlap extension PCR of a fragment containing amino acids 34-287 of *B. anthracis* lethal toxin (LT, Genbank KHG60250.1) and amino acids 4-567 of *Clostridium perfringens* TpeL (Genbank ACF49258.1) with a 6 amino acid (GGSGGL) linker between the two sequences. A TEV protease site (ENLYFQG) was placed upstream of the LT sequence, and Gateway recombination sites were introduced at 5' and 3' ends. The sequences of DARPins K27 and K55 published previously were synthesized as *E. coli* optimized versions (ATUM Inc., CA, USA) and transferred to Gateway Entry clones.[5] All final Entry clones were fully sequence verified prior to subcloning by Gateway LR recombination. DARPins were subcloned into pDest-521, a Gateway-adapted T7 promoter vector for *E. coli* expression, while SOS^{cat} (564-1048) and TpeL were subcloned into pDest-566 (Addgene 11517), which contains an N-terminal His6-MBP (maltose-binding protein) tag for fusion protein production in *E. coli*.[6]

Protein production. RAF-RBD (52-131) was expressed using the auto-induction media protocol and all other proteins were expressed using the Dynamite media protocol as described in previously.[6] The resulting cell pellets were stored at -80°C. Frozen cell pellets were thawed and resuspended in 20 mM HEPES, pH 7.3, 300 mM NaCl, 1 mM TCEP, and 1:200 (v/v) protease inhibitor cocktail. KRAS (1-169) and SOS^{cat} (564-1048) buffers were amended with 5 mM $MgCl_2$. Cells were resuspended in 10 ml of buffer per 1000 optical density (OD) units. OD was measured at cell harvest at A_{600}. Homogenized cells were lysed by passing twice through a Microfluidizer at 9,000 psi. Lysates were clarified by centrifugation at 7,900 x *g* (RCF average) for 90 min at 4°C. Clarified lysates were filtered through 0.45 μM Whatman PES syringe filters. All proteins, KRAS (1-169), RAF -RBD (52-131), and SOS^{cat} (564-1048) were purified in a similar manner using NGC medium-pressure chromatography systems. Clarified lysates were thawed, adjusted to 35 mM imidazole, and loaded at 3 ml/min onto IMAC columns equilibrated in IMAC equilibration buffer (EB) of 20 mM HEPES, pH 7.3, 300 mM NaCl, 1 mM TCEP, 35 mM imidazole, and 1:1000 protease inhibitor cocktail (5 mM $MgCl_2$ added for KRAS (1-169) and SOS^{cat} (564-1048)). The columns were washed to baseline with EB and proteins eluted with a 20 column-volume (CV) gradient from 35 mM to 500 mM imidazole in EB. Elution fractions were analyzed by SDS-PAGE and Coomassie-staining. Positive fractions were pooled and His6-TEV protease added at a 1:10 molar ratio. The digest proceeded while dialyzing to 20 mM HEPES, pH 7.3, 300 mM NaCl, and 1 mM TCEP (5 mM $MgCl_2$ added for KRAS (1-169) and SOS^{cat} (564-1048)) for two hours at room temperature and thereafter overnight at 4°C. The digested samples were processed by a second round of IMAC similar to the first round except that the equilibration and wash buffers did not contain imidazole. Column flow through and column wash were collected as fractions and the columns were developed with a 5 CV gradient to 50 mM imidazole. After

analysis of fractions by SDS-PAGE and Coomassie-staining, appropriate fractions (the target proteins elute either in the flow through or at ~10-20 mM imidazole) were pooled and concentrated in 10K MWCO centrifugation units. Pooled proteins were exchanged into final buffers by size exclusion chromatography (SEC) using appropriately sized columns packed with Superdex 75 resin. Final buffers were as follows: 20 mM HEPES, pH 7.3, 150 mM NaCl, and 1 mM TCEP (RAF-RBD (52-131)); 20 mM HEPES, pH 7.3, 150 mM NaCl, 1 mM TCEP, and 5 mM $MgCl_2$ (KRAS (1-169)); 20 mM HEPES, pH 7.3, 300 mM NaCl, 1 mM TCEP, and 5 mM $MgCl_2$ (SOS^{cat} (564-1048)). After analysis of fractions from the SEC by SDS-PAGE and Coomassie-staining, appropriate fractions were pooled, concentrated in 10K MWCO Amicon centrifugation units (3K MWCO for RAF-RBD (52-131)), filtered with a 0.22 μM syringe filter (low protein binding), assayed for protein concentration, dispensed as 0.25 ml aliquots, and snap frozen in liquid nitrogen.

DARPins K27 and K55 were purified using similar protocol as for RAF-RBD (52-131), with exceptions. Specifically, the TEV protease digestion and second IMAC were eliminated. TpeL was purified using the protocol for RAF-RBD (52-131), with exceptions. Specifically, the final protein pool was formed from the positive fractions from the second IMAC step, resulting in a final buffer of 20 mM HEPES, pH 7.3, 300 mM NaCl, 1 mM TCEP, and ~ 25 mM imidazole.

Nucleotide exchange. KRAS (1-169) was exchanged to replace the GDP that is bound to the purified protein with the GTP nonhydrolyzable analog, GMPPNP, essentially as described in previously, with minor exceptions.[4] Specifically, the initial buffer exchange step was omitted. The observed KRAS loading efficiency with GMPPNP was 92%, based on the HPLC analysis.

Quality of the used proteins

Protein quality was analyzed using several methods, not discussed here in details. Shortly, SEC was used to analyze monomeric behavior of the produced proteins, gel electrophoresis (GE) for protein size and purity determination, and mass spectrometry (MS) to find potential modifications and for batch-to-batch variation analysis. SDS-PAGE data for the KRAS and related proteins is presented in Figure S1, and SEC and SDS-PAGE data for the G(i)α in Figure S2.

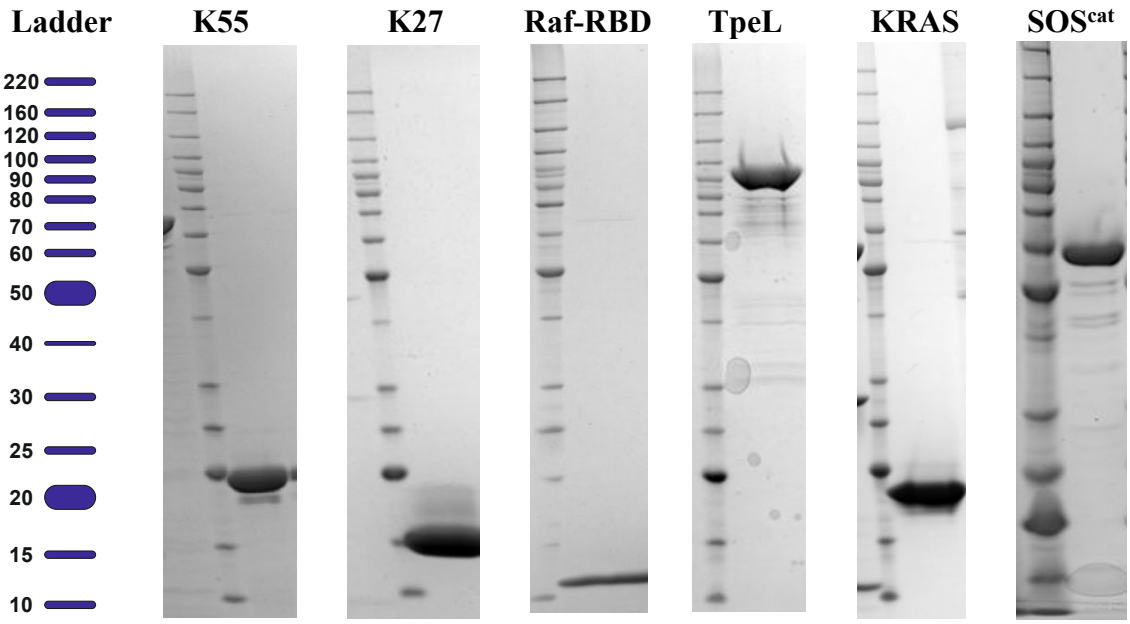

Figure S1. Quality of the KRAS and related proteins. Based on the SEC data, all proteins were found monomeric. Proteins were also analyzed with SDS-PAGE gel using Coomassie Blue staining. For GE, 5 mg of all proteins except Raf-RBD (1 mg) was loaded, and high purity was observed. Mass observed in GE, corresponded well with the MS data, observed (predicted) mass for K55, K27, Raf-RBD (52-131), TpeL, KRAS (1-169), and SOS^{cat} were 20453 (20406), 19496/19544 (19496), 9090 (9091), 96282 (96284), 19302 (19303), and 56921 (56922) Da, respectively. In case of DARPins K55 and K27, the mass difference corresponds Met (-131 Da) removal and gluconylation of the His.tag (+178 Da).

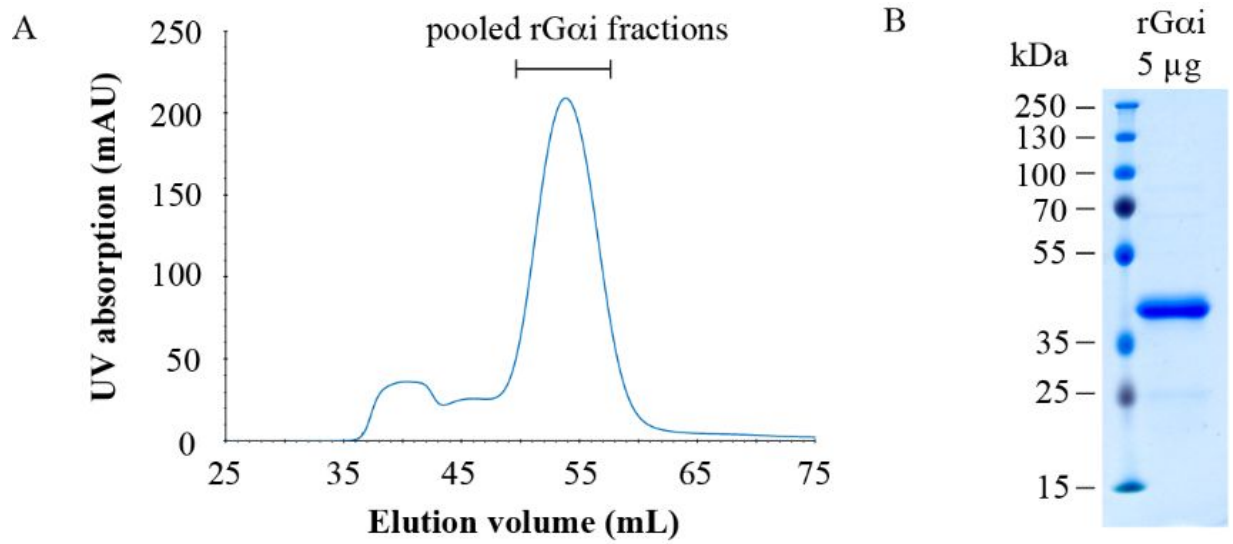

Figure S2. Purification of the recombinant human Gai (rGai). A) A representative chromatogram of the SEC based purification of rGai. The HIS-tagged rGai was purified with metal affinity chromatography prior to loading into the SEC-column, which was used as a secondary purification method and to analyze the monomeric behavior of the protein. B) The single band in PageBlue-stained SDS-PAGE gel of the pooled peak fractions of rGai (5 µg) matched with the theoretical size of the HIS-tagged rGai (42.4 kDa).

Alexa680-RBD and Alexa680-SA preparation

Alexa Fluor 680 NHS Ester was used in streptavidin and RAF-RBD conjugations to prepare SA-Alexa680 and RBD-Alexa680. SA (0.5 mg) and RAF-RBD (0.1 mg) were labeled with two-fold molar excess of Alexa680 in 100 µL or 175 µL, respectively. Light protected three hour SA-Alexa680 and 30 min RBD-Alexa reactions were performed in 50 mM carbonate buffer pH 8.3 (RT). SA-Alexa680 and RBD-Alexa680 conjugates were separated for the free Alexa680-label using 50 mM HEPES (pH 7) and NAP-5 columns according to the manufacturer instructions. The labeling degree for SA-Alexa680 and RBD-Alexa680 were determined based on the monitored absorbance at 280 and 680 nm, and the observed labeling degrees were 1.6 and 1.4, respectively.

γ-GTP-Eu^{3+} synthesis

The synthesis of γ-GTP-Eu^{3+} in depicted in Figure S3. GDP (44.3 mg, 0.1 mmol) was dissolved in 10 ml of tert-butanol/water (1:1). Morpholine (44 mg, 0.5 mmol) was added and the solution was then heated to reflux. DCC (103 mg, 0.5 mmol) dissolved in 10 ml tert-butanol was added to the above refluxing GDP solution. The reaction mixture was kept to reflux for 4 hours and the reaction mixture is then cooled to RT. The precipitate was removed by filtration, and all volatiles were evaporated in vacuum. 20 ml of water was added and the solution was extracted three times with ether (15 ml). The aqueous phase is then evaporated to dryness in vacuum. MALDI-TOF MS (negative mode) found 511.02, required 511.07 for $C_{14}H_{21}N_6O_{11}P_2$. The obtained GDP-morpholidated (22.9 mg, 0.045 mmol) was coevaporated twice with dry pyridine (15 ml). The residue was dissolved in pyridine and O-phosphorylethanolamine (63 mg, 0.45 mmol) was added to the solution. The reaction mixture was stirred overnight at RT. The solvent was removed in vacuum and the pure product was obtained by reverse phase HPLC purification. QTOF (Bruker) MS (negative mode) found 565.03, calcd for $C_{12}H_{20}N_6O_{14}P_3$-: 565.03 (Figure S4). The obtained GTP-ethanolamine was reacted with excess of ITC-activated {2,2',2",2'"-{[4'-(4'"-isothiocyanatophenyl)-2,2',6',2"-terpyridine-6,6"-diyl]bis(methylene-nitrilo)}tetrakis(acetate)}europium(III) in aqueous solution at pH 8.0. The final pure product was obtained by reverse phase HPLC purification (Figure S5).

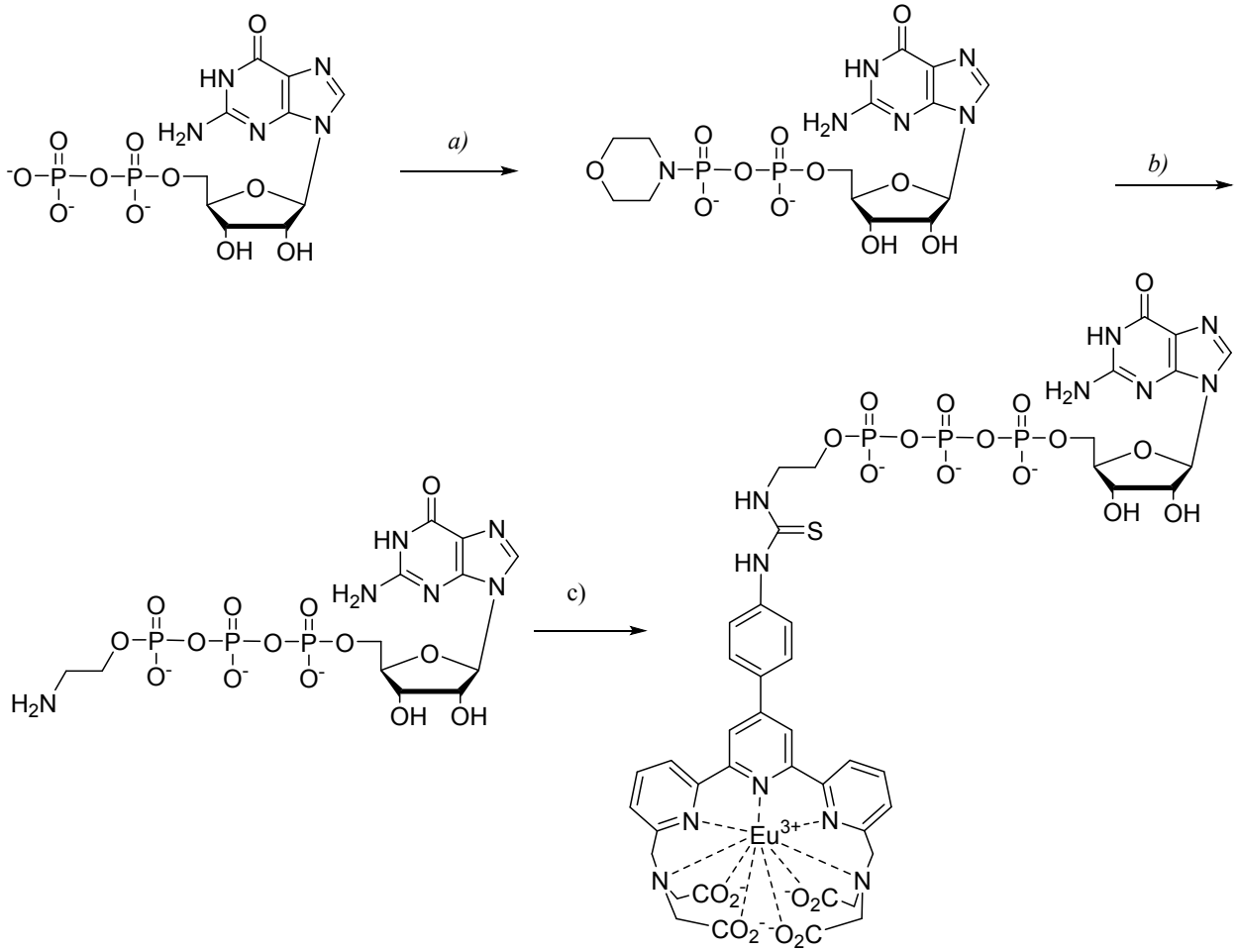

Figure S3. The synthesis of γ-GTP-Eu^{3+}. a). morpholine, DCC, t-BuOH/water, reflux, 4h; b). O-phosphorylethannolamine, pyridine; c). Terpyridine europium chelate with ITC activation, $NaHCO_3$, pH 8.0.

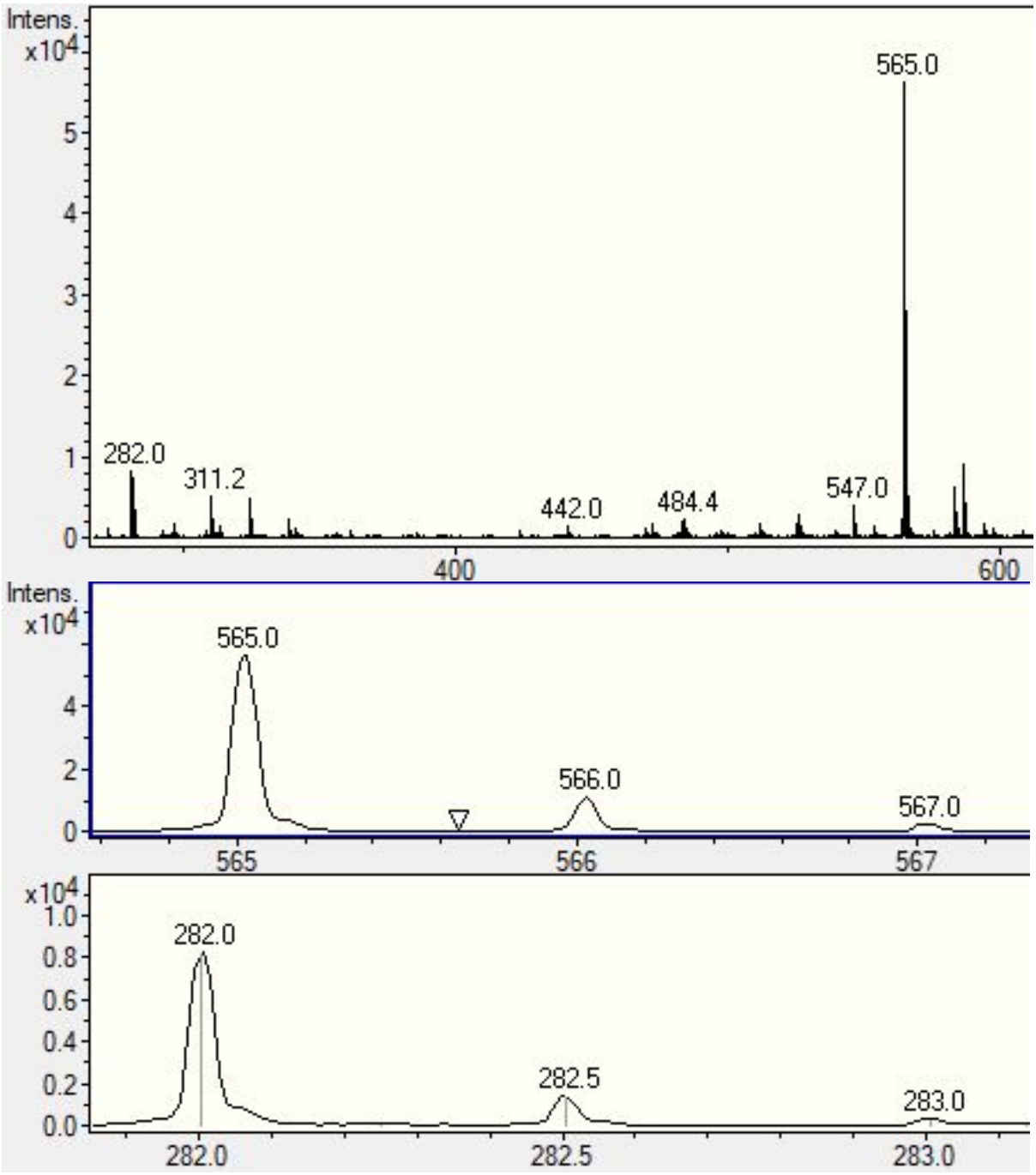

Figure S4. Quality control of the produced unlabeled γ-GTP. Mass spectra of γ-GTP diluted into 50:50 acetonitrile/H_2O measured with negative mode. The product ion (M-) was identified as the main component, with m/z = 565.0298 (zoomed spectra, middle) and secondary product ion (M2-), with m/z = 282.0067 (zoomed spectra, lowest).

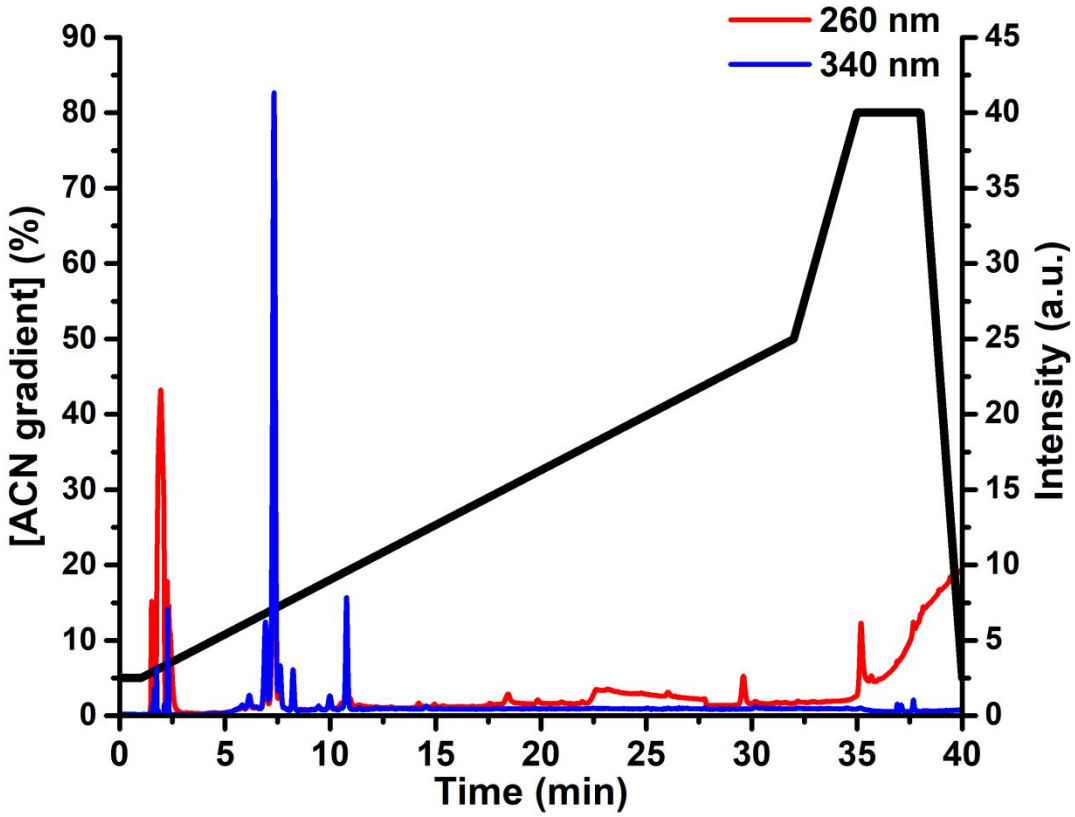

Figure S5. The analytical HPLC purification of γ-GTP-Eu^{3+}. The γ-GTP-Eu^{3+} labeling was purified using linear acetonitrile (ACN) gradient from 5 to 50% (black). Based on the analytical run, we observed three main peaks using measurement wavelengths of 260 nm (red) and 340 nm (blue). Peaks with the retention times of 2, 7, and 11 min are expected to refer non-labeled γ-GTP, γ-GTP-Eu^{3+}, and free Eu^{3+}-chelate. The peak observed at the time point of 7 min showed the highest intensity at 340 nm, and it was confirmed to bind Gα. This peak was detected in final purifications and it was used in all Gα assays.

KRAS concentration optimization for dual-parametric detection

KRAS concentration (0-600 nM) was optimized for dual-parametric format using two assay protocols in 15 µL final volume in assay buffer containing 20 mM HEPES (pH 7.5), 1 mM $MgCl_2$, 10 mM NaCl, 0.01% Triton X-100, and 0.005% γ-globulins. The first protocol is based on the use of single detection solution and in the second protocol the addition of Eu^{3+}-GTP (25 nM) and malachite green (MG, 24 µM) was separated. In both assays, KRAS (4 µL) was added in plate with or without 10 µM GDP (2 µL). The Eu^{3+}-GTP was added with (protocol 1, 4 µL) or without (protocol 2, 2 µL) quencher (MG). Signals were

monitored at 615 nm and 730 nm, and thereafter 2 µL of Son of Sevenless catalytical domain (SOS^{cat}, 0 or 10 nM) was added and signal monitoring was repeated after 5 and 15 min. RBD-Alexa680 was added in 2 µL volume, finalizing the 15 µL reaction used in protocol 1. Signal monitoring was performed at 615 nm and 730 nm (5 and 15 min), and after MG (2 µL) addition (protocol 2) the signal monitoring was again repeated.

KRAS enzymatic glycosylation

KRAS glycosylation was performed using TpeL toxin (0-10 nM) and two UDP-sugars (UDP-GlcNAc or UDP-Glu). KRAS (200 nM) loaded with GDP or GMPPNP (nonhydrolysable GTP analog) was incubated with TpeL and UDP-sugars (25 µM) in 6 µL. Reaction was performed using glycosylation buffer containing 20 mM HEPES (pH 7.5), 1 mM $MgCl_2$, 0.4 mM $MnCl_2$, 0.01% Triton X-100, and 0.005% γ-globulins. After 30 min incubation, Eu^{3+}-GTP (25 nM), MG (15 µM), and SOS^{cat} (10 nM) were added in 6 µL of buffer without $MnCl_2$ but additional 10 mM NaCl. Nucleotide exchange was monitored at 615 nm and 730 nm during 20 min of incubation. Finally, RBD-Alexa680 (25 nM) was added and signal monitoring was continued for 60 min in 15 µL final volume.

KRAS (200 nM) glycosylation was also performed after Eu^{3+}-GTP or Eu^{3+}-GDP (25 nM) exchange, using TpeL toxin (0-50 nM) and UDP-GlcNAc (25 µM). Nucleotide exchange reaction (15 min) was performed in the presence of MG (15 µM) and 10 nM SOS^{cat} at 5 µL volume, using assay buffer without $MnCl_2$. TpeL and UDP-GlcNAc were both added in 4 µL of glycosylation buffer and incubation was continued for 20 min. Finally, RBD-Alexa680 (25 nM) was added and signals at 615 nm and 730 nm were monitored after 15 min in 15 µL final volume.

Glycosylation was also studied with the previously introduced nucleotide exchange assay.[7,8] Briefly, GMPPNP- or GDP-loaded KRAS was incubated with TpeL (0-100 nM) and 25 µM UDP-sugar (UDP-GlcNAc or UDP-Glu) in a 6 µL volume. After 20 min reaction in glycosylation buffer, 6 µL of detection solution (10 nM Eu^{3+}-GTP and 1.2 µM MT2) were added in buffer where $MnCl_2$ was replaced with 10 mM NaCl. After TRF-signal monitoring, SOS^{cat} (200 nM) was added and TRF-signal monitoring from the 15 µL reaction was repeated multiple times during 60 min incubation.

G(i)α loading test with γ-GTP-Eu^{3+}

Before the enzymatic assay, new synthetized γ-GTP-Eu^{3+} (Figure S3-S5) was tested in GTP titration (0-25 μM) using constant 20 nM G(i)α and six γ-GTP-Eu^{3+} (1-50 nM) concentration with individually fixed MT2 concentration (2-4 μM) (Figure S6). G(i)α was first incubated for 30 min in a EDTA containing assay buffer (20 mM HEPES (pH 7.5), 5 mM EDTA, 5 mM NaCl, 0.01% Triton X-100, 0.005% γ-globulins). GTP and γ-GTP-Eu^{3+} in complex with MT2 were added in $MgCl_2$ containing assay buffer (20 mM HEPES (pH 7.5), 20 mM $MgCl_2$, 5 mM NaCl, 0.01% Triton X-100, and 0.005% γ-globulins) to reach the final 15 μL volume. TRF-signals at 615 nm were monitored after 30 min.

Data analysis

In all assays, the signal-to-background ratio (S/B) was calculated as μ_{max}/μ_{min} and coefficient of variation (CV%) $(\sigma/\mu)*100$. The K_d value was calculated using the linearized Cheng–Prusoff equation, $EC_{50} = (([K_i]/K_d) \times [C]) + K_i$, in which C is the Eu^{3+}-GTP concentration.[7] The K_i value in the assay was calculated using the Cheng–Prusoff equation, $EC_{50}/(1 + [C]/K_d)$. In all formulas μ is the mean value, and σ is the standard deviation (SD). Data were analyzed using Origin 8 software and the half maximal inhibitory concentration (IC_{50}) and the half maximal effective concentration (EC_{50}) values were obtained using standard sigmoidal fitting functions. Data were analyzed using Origin 8 software (OriginLab, Northampton, MA).

Results

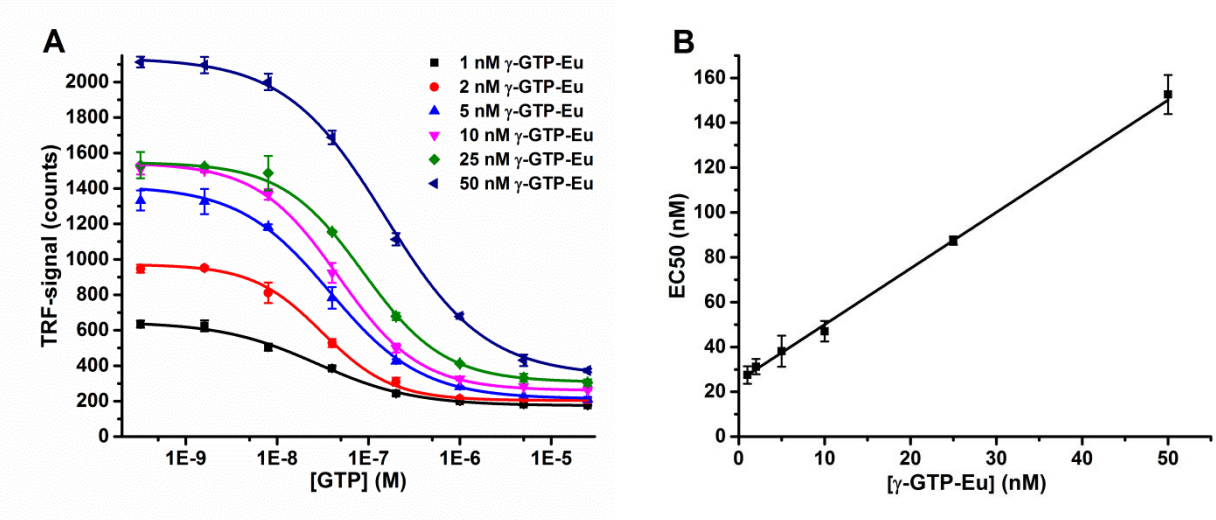

Figure S6. Binding affinity of the synthetized γ-GTP-Eu^{3+} to G(i)α. A) γ-GTP-Eu^{3+} binding to G(i)α was estimated by performing GTP titration (0-25 μM) with constant G(i)α concentration (20 nM) and six different γ-GTP-Eu^{3+} concentrations (1-50 nM). MT2 quencher concentration was individually fixed for different γ-GTP-Eu^{3+} concentrations (2-4 μM). B) The affinity estimation was performed using linearized Cheng-Prusoff equation.[9] Observed EC_{50} values from each individual assays with different γ-GTP-Eu^{3+} concentrations were blotted against the γ-GTP-Eu^{3+} concentrations, determining the K_d value at Y-axis crossing point. The observed K_d value for γ-GTP-Eu^{3+} binding to G(i)α was 25 ± 1 nM. Data represent mean ± SD (n=3).

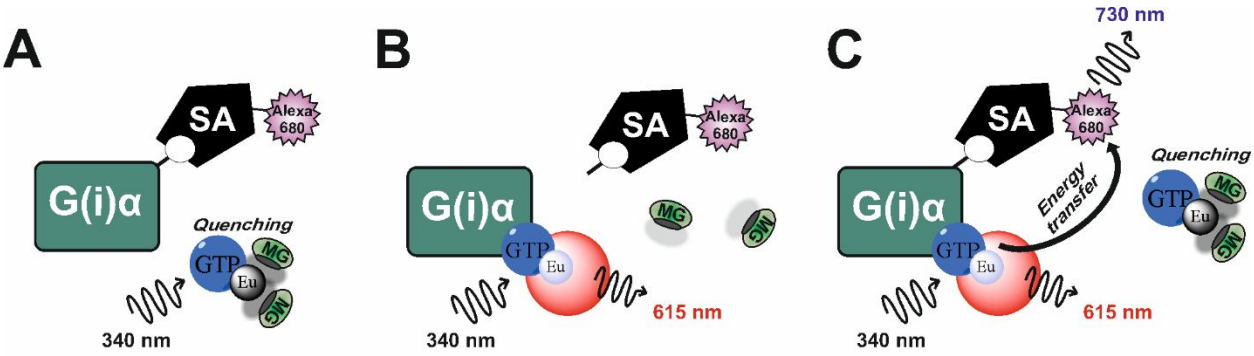

Figure S7. Principle of the dual-parametric G(i)α subunit ADP-ribosylation assay. In all assays, γ-GTP-Eu^{3+} is used directly or as an energy transfer donor, and SA-Alexa680 bound to biotin-NAD^+ serves as an energy transfer acceptor. A) In the assay with G(i)α subunit, the ADP-ribosylation (PTX + biotin-NAD^+) can occur independently of the nucleotide, but no TR-FRET signal is monitored if γ-GTP-Eu^{3+} is not bound to G(i)α (EDTA/$MgCl_2$ exchange). In solution, γ-GTP-Eu^{3+} signal is quenched in the presence of malachite green (MG). B) Before nucleotide loading, G(i)α can be emptied using EDTA to chelate $MgCl_2$. When γ-GTP-Eu^{3+} is added with excess of $MgCl_2$, γ-GTP-Eu^{3+} is associated to G(i)α and the loading can be monitored from the increased TRF-signal (615 nm) in the presence of MG. G(i)α subunit bound γ-GTP-Eu^{3+} is protected from MG and high TRF-signal is monitored. C) By performing both G(i)α ADP-ribosylation and γ-GTP-Eu^{3+} loading with EDTA/$MgCl_2$ exchange, increased direct TRF-signal at 615 nm and TR-FRET signal at 730 nm can be monitored after reaction is excited at 340 nm. TRF-signal at 615 nm confirms the proper G(i)α loading with γ-GTP-Eu^{3+} and enables efficient energy transfer from Eu^{3+} donor to Alexa680 acceptor needed to observe information about G(i)α ADP-ribosylation status at 730 nm.

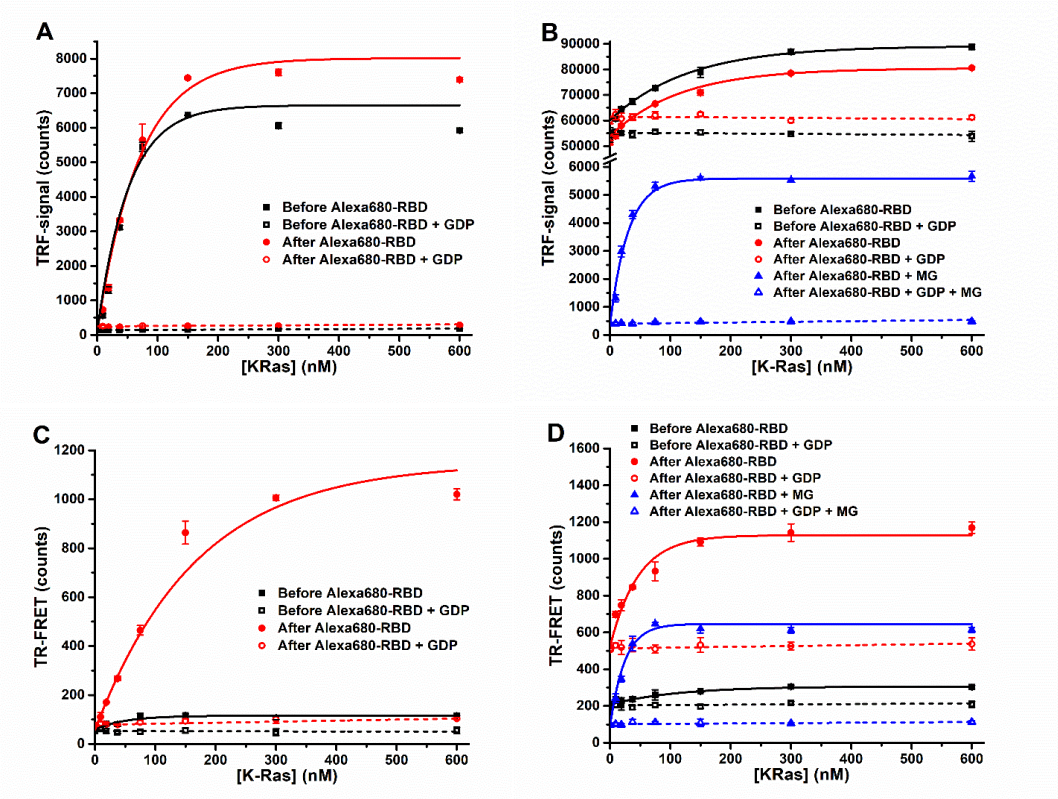

Figure S8. Coupled nucleotide association and RAS/RAF-RBD interaction monitoring. A) Nucleotide association was monitored (ex. 340/em. 615 nm) in KRAS titration, before (black) and after (red) RBD-Alexa680 addition, using constant concentration of premixed detection complex (Protocol 1: Eu^{3+}-GTP/MG) and SOS^{cat} (10 nM) in the presence (solid) or absence (dashed) of GDP (10 µM). With or without RBD-Alexa680 the maximal S/B ratios were 29 and 42 and in both cases it was monitored at 150 nM KRAS. B) Eu^{3+}-GTP (25 nM) association, with 10 nM SOS^{cat}, was also monitored in multistep KRAS titration (Protocol 2), before (black) and after (red) RBD-Alexa680 addition and after MG (24 µM) addition (blue) in a reaction with (solid) or without (dashed) 10 µM GDP. Maximal S/B ratios monitored at 600 nM KRAS and before/after RBD-Alexa680 were 1.7 and 1.3, respectively. After MG addition and S/B ratio monitored was 11.9 monitored at 150 nM KRAS. C) KRAS/RAF-RBD interaction was also monitored (ex. 340/em. 730 nm) in KRAS titration with SOS^{cat} using the Protocol 1 (Eu^{3+}-GTP/MG). Measurement was performed before (black) and after (red) RBD-Alexa680 addition and with (solid) or without (dashed) GDP. With or without RBD-Alexa680 the maximal S/B ratio was 2.2 and 10.1 and at 300 nM KRAS. D) Similarly, KRAS/RAF-RBD interaction was monitored in multistep KRAS titration with SOS^{cat} and Eu^{3+}-GTP (Protocol 2). Signals were monitored before (black) and after (red) RBD-Alexa680 addition and after MG addition (blue) in reaction with (solid) or without (dashed) GDP. Maximal S/B ratios monitored at 600 nM KRAS and before/after RBD-Alexa680 were 1.5 and 2.2, respectively. After MG addition and S/B ratio monitored was 5.9 monitored at 75 nM KRAS. Data represent mean ± SD (n=3).

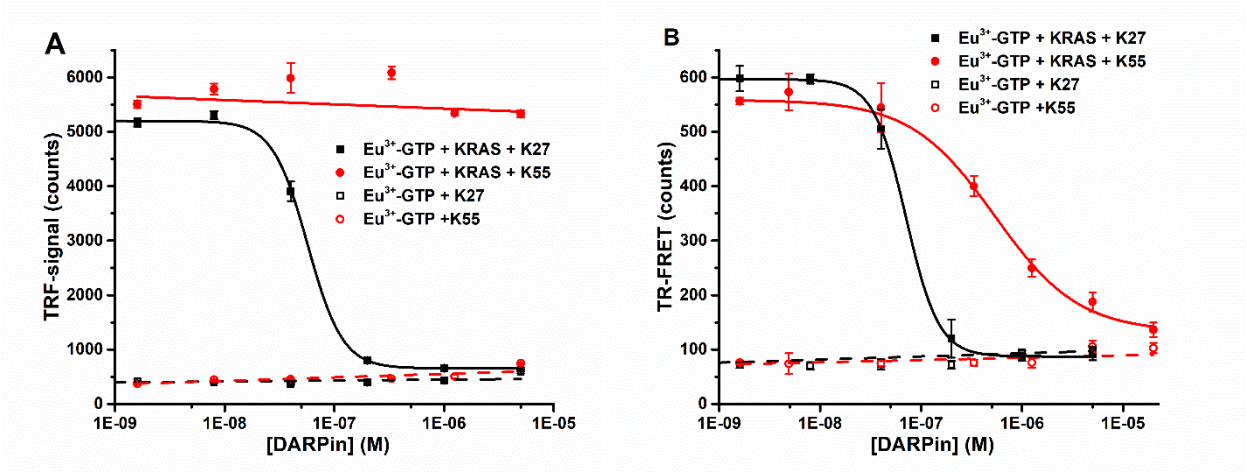

Figure S9. DARPins K27 and K55 titrations in coupled nucleotide association and KRAS/RAF-RBD interaction assay. A) DARPins (0-20 µM) were titrated using 200 nM GDP-loaded KRAS. Eu^{3+}-GTP (25 nM) association was induced with SOS^{cat} (10 nM) addition and TRF-signals were monitored at 340/615 nm after 15 min. K27 (black, solid) inhibited the nucleotide exchange reaction (IC_{50} = 57 ± 5 nM), as K55 (red, solid) with KRAS showed no inhibition. K27 (black, dashed) and K55 (red, dashed) reactions without KRAS showed no DARPin related effect, Eu^{3+}-GTP free in solution is quenched in the presence of MG (24 µM). B) After RBD-Alexa680 (25 nM) addition, reactions with KRAS together with K27 (black, solid) or K55 (red, solid) and reactions without KRAS but with K27 (black, dashed) or K55 (red, dashed) were monitored at 340/730 nm after 15 min incubation. Both K27 and K55 showed concentration dependent inhibition of RBD-Alexa680 interaction with Eu^{3+}-GTP loaded KRAS. Again, no signal were monitored without KRAS. The monitored IC_{50} values were 71 ± 8 nM and 536 ± 65 nM for K27 and K55 respectively. Data represent mean ± SD (n=3).

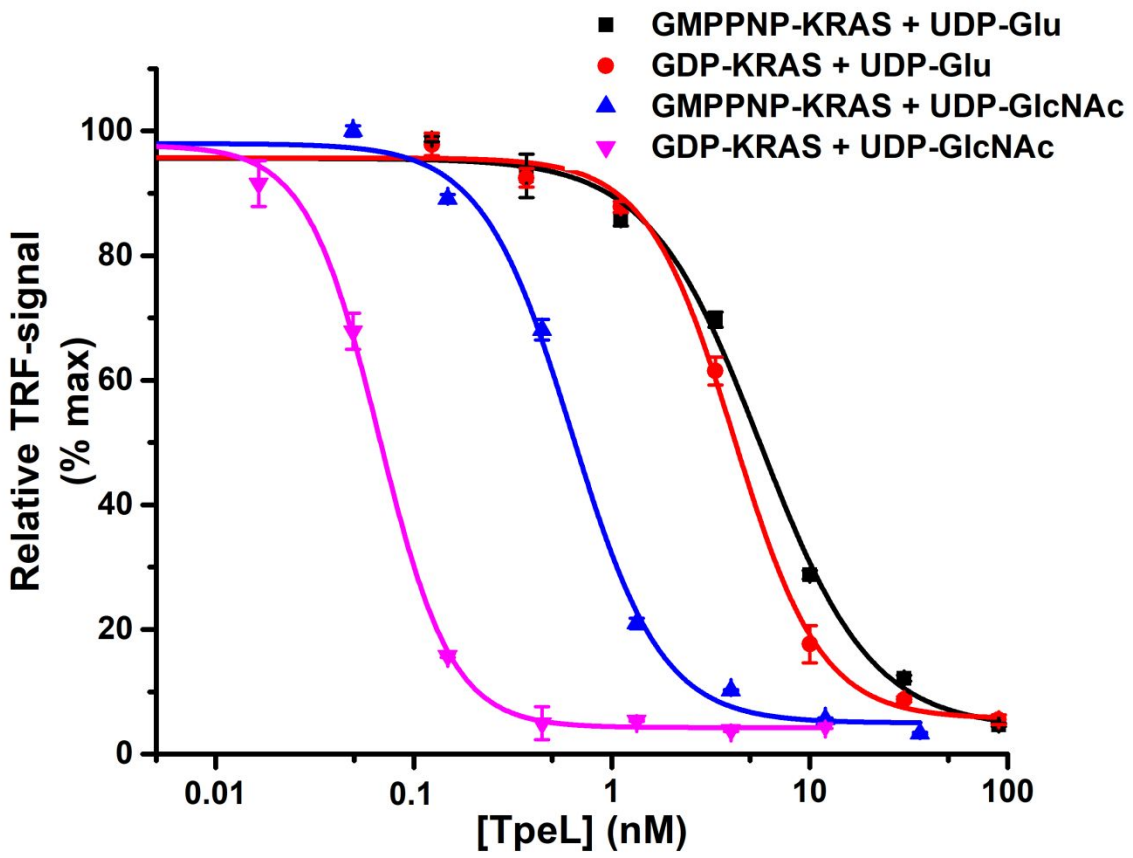

Figure S10. KRAS glycosylation monitoring using conventional QRET nucleotide exchange assays. TpeL (0-100 nM) catalyzed KRAS (200 nM) glycosylation was studied with two UDP-sugars (25 µM) and KRAS loaded with GDP or GMPPNP, GTP analog. Glycosylation was studied in Eu^{3+}-GTP (10 nM) association assay with SOS^{cat} (200 nM) and in the presence of MT2 (1.2 µM). With GDP-loaded KRAS, the preferred UDP-sugar for TpeL was UDP-GlcNAc (magenta) over UDP-Glu (blue). The monitored IC_{50} values for UDP-GlcNAc and UDP-Glu were 0.07 ± 0.01 nM and 4.1 ± 0.2 nM, respectively. Clear preference in nucleotide exchange blocking was not seen between UDP-GlcNAc (red) and UDP-Glu (black) when the assay was performed with GMPPNP-KRAS. The monitored IC_{50} values for UDP-GlcNAc and UDP-Glu were 0.63 ± 0.05 nM and 5.6 ± 0.5 nM, respectively. Data represent mean ± SD (n=3).

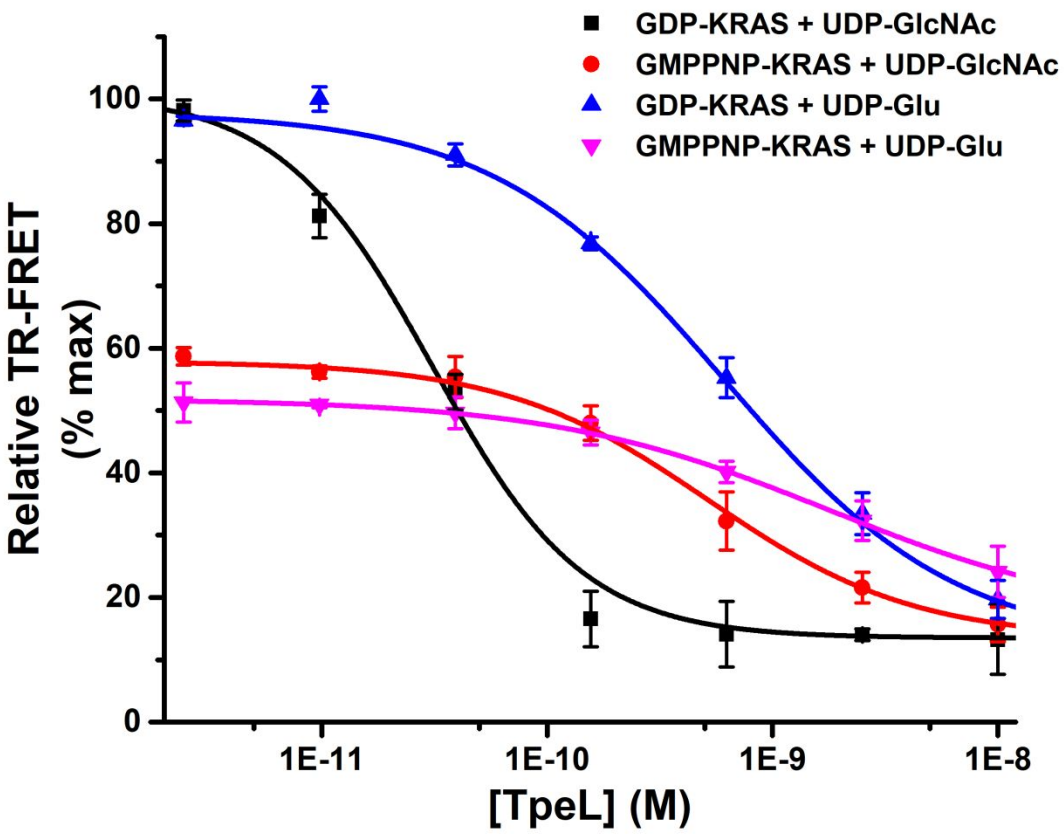

Figure S11. KRAS glycosylation monitoring using dual-parametric QTR-FRET assay. TpeL catalyzed KRAS glycosylation was studied with two UDP-sugars (25 µM) and KRAS (200 nM) loaded with GDP or GMPPNP. TpeL reaction (30 min) was first performed to glycosylate KRAS and thereafter Eu^{3+}-GTP (25 nM) was loaded using SOS^{cat} (10 nM) in the presence of MG (15 µM). TRF-signals after Eu^{3+}-GTP association were monitored at 615 nm and clear TpeL concentration dependent nucleotide exchange blocking was monitored as presented in Figure 5. After nucleotide exchange reaction, RBD-Alexa680 (25 nM) was added and KRAS/RAF-RBD interactions were monitored at 730 nm. Similarly as saw in TRF-signal monitoring, TR-FRET measurement confirmed the TpeL preference to UDP-GlcNAc and GDP-KRAS (black) preference over GMPPNP-KRAS (red). The monitored IC_{50} values were 0.03 ± 0.01 nM and 0.52 ± 0.11 nM, respectively. Also the TpeL reaction performed with UDP-Glu could block nucleotide exchange reaction and the followed KRAS/RAF-RBD interaction with both GDP-KRAS (blue) and GMPPNP-KRAS (magenta), but with significantly higher TpeL concentration. The monitored IC_{50} values were 0.61 ± 0.13 nM and 1.8 ± 0.1 nM, respectively. Also with the UDP-Glu, GDP-KRAS was preferred over GMPPNP-KRAS. Data represent mean ± SD (n=3).

Table S1. IC_{50} values for KRAS glycosylation by TpeL using GDP- or GMPPNP-KRAS and UDP-GlcNAc or UDP-Glu monitored with QTR-FRET or QRET.

KRAS	UDP-sugar	QTR-FRET		QRET	QTR-FRET
		TRF (615 nm)	TR-FRET (730 nm)	TRF (615 nm)	TR-FRET (730 nm)
GDP-KRAS	**UDP-GlcNAc**	0.03 ± 0.01 nM	0.03 ± 0.01 nM	0.07 ± 0.01 nM	
GDP-KRAS	**UDP-Glu**	0.91 ± 0.15 nM	0.61 ± 0.13 nM	4.1 ± 0.2 nM	
GMPPNP-KRAS	**UDP-GlcNAc**	0.44 ± 0.08 nM	0.52 ± 0.11 nM	0.63 ± 0.05 nM	*0.30 ± 0.02 nM
GMPPNP-KRAS	**UDP-Glu**	10.0 ± 7 nM	1.8 ± 0.1 nM	5.6 ± 0.5 nM	

* Eu^{3+}-GTP association followed by TpeL enzyme reaction and QTR-FRET detection

References

(1) Ashok, Y.; Miettinen, M.; de Oliveira, D.K.H.; Tamirat, M.Z.; Näreoja, K.; Tiwari, A.; Hottiger, M.O.; Johnson, M.S.; Lehtiö, L.; Pulliainen, A.T. Discovery of compounds inhibiting the ADP-ribosyltransferase activity of pertussis toxin. *ACS Infect Dis.* **2020**, in press.

(2) Kopra, K.; Ligabue, A.; Wang, Q.; Syrjänpää, M.; Blaževitš, O.; Veltel, S.; van Adrichem, A.J.; Hänninen, P.; Abankwa, D.; Härmä, H. A homogeneous quenching resonance energy transfer assay for the kinetic analysis of the GTPase nucleotide exchange reaction. *Anal Bioanal Chem.* **2014**, 406, 4147-4156.

(3) Kopra, K.; van Adrichem, A.J.; Salo-Ahen, O.M.H.; Peltonen, J.; Wennerberg, K.; Härmä, H. High-throughput dual screening method for Ras activities and inhibitors. *Anal Chem.* **2017**, 89, 4508-4516.

(4) Dharmaiah, S.; Tran, T.H.; Messing, S.; Agamasu, C.; Gillette, W.K.; Yan, W.; Waybright, T.; Alexander, P.; Esposito, D.; Nissley, D.V.; McCormick, F.; Stephen, A.G.; Simanshu, D.K. Structures of N-terminally processed KRAS provide insight into the role of N-acetylation. *Sci. Reports.* **2019**, 9, 10512.

(5) Guillard, S.; Kolasinska-Zwierz, P.; Debreczeni, J.; Breed, J.; Zhang, J.; Bery, N.; Marwood, R.; Tart, J.; Overman, R.; Stocki, P.; Mistry, B.; Phillips, C.; Rabbitts, T.; Jacksom, R.; Minter, R. Structural and functional characterization of a DARPin which inhibits Ras nucleotide exchange. *Nat Commun.* **2017**, 14, 16111.

(6) Taylor, T.; Denson, J.-P.; Esposito, D. Optimizing expression and solubility of proteins in *E. coli* using modified media and induction parameters. *Methods Mol. Biol.* **2017**, 1586, 65–82.

(7) Kopra, K.; Ligabue, A.; Wang, Q.; Syrjänpää, M.; Blaževitš, O.; Veltel, S.; van Adrichem, A.J.; Hänninen, P.; Abankwa, D.; Härmä, H. A homogeneous quenching resonance energy transfer assay for the kinetic analysis of the GTPase nucleotide exchange reaction. *Anal Bioanal Chem.* **2014**, 406, 4147-4156.

(8) Kopra, K.; van Adrichem, A.J.; Salo-Ahen, O.M.H.; Peltonen, J.; Wennerberg, K.; Härmä, H. High-throughput dual screening method for Ras activities and inhibitors. *Anal Chem.* **2017**, 89, 4508-4516.

(9) Newton, P.; Harrison, P.; Clulow, S. A novel method for determination of the affinity of protein: protein interactions in homogeneous assays. *J. Biomol. Screening.* **2008**, 13, 674-682.