Current small-molecule inhibitors of KRAS(G12C) bind irreversibly in the switch-II pocket (SII-P), exploiting the strong nucleophilicity of the acquired cysteine as well as the preponderance of the GDP-bound form of this mutant. Nevertheless, many oncogenic KRAS mutants lack these two features, and it remains unknown whether targeting the SII-P is a practical therapeutic approach for KRAS mutants beyond G12C. Here we use NMR spectroscopy and a cellular KRAS engagement assay to address this question by examining a collection of SII-P ligands from the literature and from our own laboratory. We show that the SII-Ps of many KRAS hotspot (G12, G13, Q61) mutants are accessible using noncovalent ligands, and that this accessibility is not necessarily coupled to the GDP state of KRAS. The results we describe here emphasize the SII-P as a privileged drug-binding site on KRAS and unveil new therapeutic opportunities in RAS-driven cancer.
Cell-free analysis of binding to RAS proteins was performed via protein-observed NMR spectroscopy (Fig. 1b,c). We expressed uniformly 15N-labeled KRAS 1–169, KRAS(G12C) 1–169, KRAS(G12D) 1–169 and HRAS 1–166 proteins and acquired a series of 1H–15N HSQC NMR spectra (Supplementary Note 1). The addition of either MRTX849 or MRTX1257 (200 \(\mu\)M) to the GDP-loaded state of either KRAS or KRAS(G12D) protein (100 \(\mu\)M) resulted in the formation of a new complex with strong chemical shift perturbations (CSPs) from the peaks of the unbound protein (Fig. 1b,c and Supplementary Note 1, spectra 2, 3, 6 and 7). The same CSPs were observed in samples containing substoichiometrically ligated proteins (100 \(\mu\)M protein and 30 \(\mu\)M ligand; Supplementary Note 1, spectrum 25), indicating that these ligands tightly bind the proteins with \(k_{off}\) values smaller than the frequency separation between peaks of bound and unbound proteins (<80 Hz). Although the lack of chemical exchange poses a challenge to assigning most peaks of the protein–ligand complexes to their respective residues, some key residues of the covalent KRAS(G12C)–MRTX849 and the noncovalent KRAS(G12D)–MRTX849 complexes could be reassigned from three-dimensional 1H–15N–1H NOESY-HSQC spectra. Similarities in perturbations of these residues (excepting glycine 13) support a similar binding conformation between the noncovalent (G12D) and covalent (G12C) complexes of MRTX849 (Fig. 1c, Supplementary Fig. 1 and Supplementary Note 1, spectra 26 and 27).

By contrast, no effects were observed on the spectra of KRAS or KRAS(G12D) proteins containing the nonhydrolyzable GTP analog GPPNHP (GNP) with 200 \(\mu\)M of either MRTX849 or MRTX1257 (Fig. 1b and Supplementary Note 1, spectra 14, 15, 18 and 19). Furthermore, only concentration-dependent peak broadening and weak CSPs were observed from the addition of either molecule to GDP-loaded HRAS 1–166 under the same conditions (Supplementary Note 1, spectra 10 and 11), suggesting only weak occupancy of the HRAS SII-P even at the highest concentration tested (100 \(\mu\)M protein and 300 \(\mu\)M ligand). The results of these HSQC NMR experiments show that MRTX849 and MRTX1257 bind KRAS proteins with high selectivity for the inactive GDP-loaded state and for the K-isoform over HRAS.
A series of similar ¹H–¹³N HSQC NMR experiments provided some evidence for weak binding of AMG510 (Fig. 1a) to the SII-P of GDP-loaded KRAS and HRAS proteins (Fig. 1b and Supplementary Note 1, spectra 1, 5 and 9). Peaks corresponding to residues in the SII-P broadened and exhibited weak (generally less than line-widths) CSPs in the presence of 200µM of AMG510. These experiments suggest that the reversible affinity of AMG510 to RAS proteins is probably too weak to be relevant to in-cell experiments conducted at lower concentrations, and that AMG510 must rely on the irreversible reaction at the mutant cysteine 12 for its inhibitory activity, which is consistent with previously published data.

Recently, compounds reported to target KRAS(G12D) were disclosed in patent applications by multiple groups²²–²⁵. We selected and synthesized an example from Mirati Therapeutics patent filings (MRTX-EX185, 6) with structural features similar to MRTX849/1257 (Fig. 1a). We found that MRTX-EX185 bound GDP-loaded KRAS and KRAS(G12D) by HSQC NMR spectroscopy (Fig. 1b and Supplementary Note 1, spectra 4, 8 and 28), but in stark contrast to MRTX849/1257, MRTX-EX185 also bound the active GNP state of these proteins (Supplementary Note 1, spectra 16 and 20). Furthermore, MRTX-EX185 also bound GDP-loaded HRAS (Supplementary Note 1, spectrum 12). In each of these five cases, identical CSPs were observed in samples containing either 100µM protein and 200µM ligand or containing 100µM protein and 30µM ligand (substoichiometric), indicating that the $k_{gb}$ values for these complexes are small (Supplementary Note 1, spectrum 25). When a substoichiometric amount of ligand (50µM) was added to a sample containing both GDP- and GNP-loaded KRAS proteins (100µM each), the GDP–KRAS–MRTX-EX185 complex formed exclusively, and the same experiment with KRAS(G12D) yielded the same result (Supplementary Fig. 2 and Supplementary Note 1, spectra 29 and 30). These results suggest that the relative affinity of MRTX-EX185 to the GTP state over the GDP state of KRAS proteins is greater than the noise limit of the spectra (more than ten for most peaks).

These cell-free NMR experiments show that MRTX849 and MRTX1257 engage KRAS proteins even in the absence of a nucleo-philic mutant cysteine 12. However, this engagement is selective for the inactive GDP-loaded state of the protein. The more recently disclosed MRTX-EX185, by contrast, engages both nucleotide states—albeit with preference for the inactive GDP-loaded protein—and might present an opportunity to inhibit even constitutively active (GTP-loaded) KRAS hotspot mutants. However, these NMR experiments require high concentrations of proteins and do not quantify the potency of these tightly binding compounds. Furthermore, in vitro binding assays may not be representative of the in-cell vulnerability of a regulated, effector-bound and membrane-localized protein such as KRAS.

Observing reversible KRAS SII-P occupancy in cells with BRET.

With our NMR results supporting the potential of KRAS and its hotspot mutants to be vulnerable to noncovalent SII-P occupancy, we asked whether these SII-P ligands engage KRAS in cells. We first assessed the antiproliferative effects of MRTX849 in a number of G12C and non-G12C KRAS mutant and KRAS WT cell lines (Supplementary Fig. 3a). Although MRTX849 inhibited the growth of SW-1990 (KRAS(G12D)) and HCT-116 (KRAS(G13D)) at micromolar concentrations, it also had the same effect on HEK293 (KRAS WT, RAS-independent) and A375 (BRAF V600E, RAS-independent), suggesting the antiproliferative effects may originate from RAS-independent toxicity (Supplementary Fig. 3a). We also measured the ability of MRTX849 to inhibit extracellular signal-related kinase (ERK) phosphorylation in a similar panel of cell lines (Supplementary Fig. 3b). We corroborated the strong potency of MRTX849 in KRAS(G12C)-driven cell lineages. However, in non-G12C driven cell lineages, the nonspecific cytotoxic effects were observed over the same concentration range as the inhibitory effects on ERK phosphorylation (Supplementary Fig. 3b), thus preventing a clear confirmation of cellular target engagement.

The interference from off-target toxic effects in these assays precluded the analysis of target engagement and prompted us to develop new approaches to determine ligand–RAS interaction in cells. To more directly query biological engagement of KRAS and HRAS with small-molecule target engagement in cells, a BRET reporter system was developed. We synthesized a pan-RAS BRET probe (7) by conjugating a fluorophore to a derivative of the reversible SI/II-P inhibitor BI-2852 (ref. ⁷) (Fig. 2a). Recognizing the multimeric and membrane-localized nature of RAS³⁸–³⁹, we sought to generate a BRET signal conditionally within membrane-associated RAS complexes⁴⁰. We configured a luminescent complementation-based system (NanoBiT) that was dependent upon RAS lipidation as the BRET donor (Fig. 2b,c and Supplementary Figs. 4 and 5). Luminescent imaging confirmed the membrane localization of RAS dimers in live cells (Supplementary Fig. 4). Furthermore, homodimeric NanoBiT-RAS was functionally validated using an intra-cellular CRAF-Ras binding domain (RBD)-HaloTag interaction assay (Supplementary Fig. 5a–d) and was competent to activate phospho-ERK (p-ERK) in cells (Supplementary Fig. 5e). Removal of critical lipidation residues (C185S or removal of the hyper variable region) resulted in a dramatic decrease in luminescence, supporting the need for membrane anchoring for the RAS multimerization (Fig. 2c and Supplementary Fig. 5f). Titration of BI-2852 did not impact the RAS dimerization NanoBiT signal in live cells, supporting that RAS is constitutively oligomerized in this assay system (Supplementary Fig. 5g). When cells expressing the BRET donor complexes were treated with the SI/II-P RAS BRET probe 7, we observed a strong BRET signal that was readily competed to instrument background by unmodified BI-2852 in cells (Fig. 2d,e and Supplementary Fig. 6). Moreover, BI-2852 had no effect on the BRET or luminescence in an irrelevant target engagement assay (Supplementary Fig. 6l), confirming specificity in the competitive effects observed in the RAS assays. Hill coefficients for BI-2852 across all RAS variants studied ranged from 1.3 to 3.3 with a mean of 1.9±0.7, consistent with cooperative binding of two BI-2852 molecules to a dimeric RAS, as proposed previously⁴¹. Competitive engagement results with a tert-butylxycarbonyl-protected precursor to the RAS BRET probe (8) indicated that the linker functionalization of the BI-2852 derivative resulted in a decrease in affinity to RAS (Supplementary Fig. 7), but that affinity was still sufficient to yield a strong BRET signal between dimeric RAS species and the RAS BRET probe in live cells.

To determine whether the RAS BRET probe binds with a mechanism similar to that of BI-2852, we introduced a mutation (D54R) that is expected to abolish probe engagement on the basis of structural analysis (Supplementary Fig. 8a). As expected, mutation of D54 to R in KRAS resulted in complete loss of BRET with the RAS BRET probe (Supplementary Fig. 8c) without effecting dimerization (Supplementary Fig. 8b). KRAS(D54R) dimers were still competent to engage CRAF-RBD effectors in cells, as evidenced by its interaction with CRAF-RBD-HaloTag (Supplementary Fig. 8d). Although CRAF-RBD was able to interact with KRAS(D54R) dimers, BI-2852 was unable to inhibit the interaction, further validating this mutation as a negative control (Supplementary Fig. 8d). Overexpression of full-length CRAF also attenuated the BRET observed between KRAS and the RAS BRET probe (Supplementary Fig. 8e), further supporting that the probe binds with a mechanism similar to BI-2852 and is competitive with effector interactions.

To evaluate the sensitivity of the SI/II-P BRET probe to allosteric target engagement within the SII-P, live HEK293 cells expressing NanoBiT–KRAS(G12C) were challenged with SII-P ligands AMG510 or ARS-1620 in the presence of the RAS BRET probe (Fig. 3a). Time- and dose-dependent competition was observed
between AMG510 or ARS-1620 and the RAS BRET probe (Fig. 3b and Supplementary Fig. 9a,b). Unlike for BI-2852, AMG510 and ARS-1620 produced Hill coefficients closer to unity (0.8 and 1.1, respectively), consistent with a lack in cooperativity in the binding to the SII-P of KRAS(G12C). At a 2 h timepoint, BRET results with both AMG510 and ARS-1620 closely matched the potency of endogenous target engagement and p-ERK inhibition at identical timepoints in a number of G12C driven lineages (MIA PaCa-2, NCI-H358), corroborating the accuracy of the BRET method as a proxy for engagement in an endogenous cellular setting (Fig. 3C and Supplementary Fig. 9b). AMG510 demonstrated exquisite engagement selectivity for KRAS(G12C) compared with KRAS WT, other KRAS hotspot mutants, and HRAS WT (Supplementary Fig. 9c), consistent with previous reports for functional selectivity between KRAS(G12C) and non-G12C driven cancer cell lines. Like BI-2852, AMG510 did not impact the luminescence produced by NanoBiT–KRAS(G12C) (Supplementary Fig. 9d). Additional SII-P inhibitors were evaluated at KRAS(G12C) complexes, including MRTX849 and MRTX1257 (Supplementary Fig. 9e). Each produced BRET target engagement results that agreed closely with published cellular potency at KRAS(G12C) lineages. MRTX849/1257 were the most potent KRAS(G12C) inhibitors in the analysis, in close agreement with previous studies. Together the results for engagement of KRAS(G12C) with SII-P ligands support the potential of the BRET target engagement system to report on KRAS in its endogenous cellular setting, and that this system can be used to accurately query engagement across oncogenic KRAS mutants in live cells.
Although no engagement of MRTX849/1257 was observed for AMG510 (Fig. 5a), and 5b, Supplementary Table 1 and Supplementary Fig. 10b, a wide spectrum of engagement was observed (Figs. 4a,b and 5b, Supplementary Table 1 and Supplementary Fig. 10b–g). Although no engagement of MRTX849/1257 was observed for KRAS G12V and Q61R, modest engagement was observed for the remaining KRAS hotspot mutants in the single-digit micromolar range (IC_{50} ranging from 1 to 5 μM). Among the KRAS hotspot mutants excluding KRAS(G12C), the most potent engagement was observed for G13D, Q61H and Q61L (Supplementary Fig. 10d–f).

WT HRAS as well as two oncogenic HRAS mutants (G12C and G12V) were also evaluated for SII-P vulnerability using the BRET assay. No engagement was observed for WT HRAS with AMG510, MRTX849 or MRTX1257 (Supplementary Figs. 9c and 10h). Although HRAS(G12V) was also not vulnerable to SII-P engagement (Supplementary Fig. 10i), HRAS(G12C) showed vulnerability to both AMG510 and MRTX849 (Supplementary Fig. 10j). AMG510 demonstrated similar intracellular affinity towards HRAS(G12C) compared with KRAS(G12C), but MRTX849 demonstrated affinity for HRAS(G12C) that was three orders of magnitude weaker than that observed for KRAS(G12C), suggesting that the MRTX849 scaffold preferentially engages the K-isoform of RAS, which is consistent with our NMR spectroscopy results.

We next sought to accurately assess the contribution to SII-P engagement from noncovalent ligand–protein interactions. Because of the potential differences in the steric and electrostatic environments for the SII-P among the RAS variants, we synthesized derivatives of MRTX849 lacking the covalent acrylamide warhead and positioning groups with varied steric and electronic properties proximal to residue 12 (9–14, Fig. 5a), and we evaluated these compounds with the BRET target engagement assay (Fig. 5b, Supplementary Table 1 and Supplementary Fig. 11a). For most RAS variants, the saturated amide and sulfonamide derivatives (9–14) demonstrated comparable rank order vulnerability with those of MRTX849 and MRTX1257. Among non-G12C variants, WT KRAS remained the most vulnerable of all RAS isoforms to reversible engagement, followed closely by hotspot KRAS mutants G13D, Q61H and Q61L. KRAS(G12V), KRAS(Q61R) and WT HRAS showed weak to no engagement across all saturated amides, similar to the results observed with MRTX849/1257. Engagement of KRAS(G12C) by most of the saturated amide derivatives was impaired in the absence of the covalent mechanism, with the exception of the sulfonamide (14), which demonstrated modest affinity (3.0 μM, Fig. 4c).

Within the saturated amide series, 9 and 10 containing acetamide and propionamide moieties, respectively, were generally well tolerated. The methylsulfonamide derivative 14 was also well tolerated, in most cases demonstrating comparable engagement potency with the 9 and 10, except in the case of KRAS(G12C) where it was found to be moderately selective compared with other derivatives. The electron-deficient trifluoroacetamide 13 demonstrated right-shifted moderate to weak potency in all cases compared with 9 and 10, suggesting the importance of polar interactions with the amide carbonyl. The bulky iso-butyramide 11 demonstrated the weakest engagement potency among all of the amides across all RAS isoforms. Compound 11 also caused an increase in the BRET signal for some RAS variants (Supplementary Fig. 11a), which was probably related to cytotoxicity (Supplementary Fig. 11b,c). Posing a ring constraint to the branched isopropyl group (that is the cyclopropyl carboxamide presented in 12) improved the potency compared with 11, but still demonstrated only moderate to weak potency in most cases. These saturated amides elicited cytotoxic effects in a RAS-independent cell line at similar concentrations (Supplementary Fig. 11b,c); however, our BRET system still permitted the direct measurement of SII-P engagement without prohibitive interference from off-target toxicity.

MRTX-EX185 engages KRAS mutants and drives antiproliferation. Because our NMR results demonstrated the unique capability of MRTX-EX185 to bind to both the GDP state and the GTP state of KRAS(G12D) in a cell-free system, we next evaluated this compound...
KRAS(Y96D) engagement was not observed with MRTX849, inhibitors including MRTX849 (ref. 11) (Supplementary Fig. 12a).

Previously reported mutation conferring resistance to described SII-P also assayed engagement of KRAS(Y96D), which contains a previously reported mutation conferring resistance to described SII-P inhibitors including MRTX849 (ref. 11) (Supplementary Fig. 12a).

KRAS(Y96D) engagement was not observed with MRTX849, 10 or MRTX-EX185 by the BRET-based assay. This finding, in conjunction with the finding that all of the MRTX chemotypes in this study show weak to no binding to HRAS variants (in which residue 95 is a glutamine), is consistent with binding to the SII-P in a similar pose.

MRTX-EX185 was also evaluated for inhibition of KRAS(G12D):effector interactions in cells using a NanoBiT protein–protein interaction assay. MRTX-EX185 demonstrated time- and dose-dependent inhibition of the KRAS(G12D):CRAF(RBD) interaction (Supplementary Fig. 12b), providing support for functional disruption of MAPK signaling. The protracted inhibition of CRAF-RBD interactions may be due to the need for overexpression of the RBD, which would be expected to stabilize KRAS–GTP. Thus, evaluation of KRAS(G12D):CRAF interactions with endogenous proteins may be warranted to more accurately query the kinetics of pathway inhibition. p-ERK and cell viability analysis in SW-1990 cells confirmed that engagement with MRTX-EX185 translated into inhibition of mitogenic signaling and an antiproliferative effect in a G12D-driven lineage (Fig. 6b,c), with antiproliferative potency (70 nM) in close agreement with the BRET readout. Unlike the MRTX849 derivatives, nonspecific cytotoxicity did not confound the antiproliferative results, because MRTX-EX185 did not inhibit proliferation in a panel of control cell lines (Supplementary Fig. 12c).

Taken together, these results along with the NMR findings indicate that GTP-state compatibility may support the superior SII-P engagement for KRAS(G12D) in cells. We therefore attempted to extend the utility of MRTX-EX185 to additional KRAS hotspot mutants. MRTX-EX185 engaged numerous KRAS Q61, G12 and G13 mutant proteins (Figs. 5b and 6a, Supplementary Table 1 and Supplementary Fig. 12d). Notably, MRTX-EX185 engaged KRAS(G12V) in cells, which is the most GTP-biased G12 allele described. Although the potency against individual mutants may require tailored chemical optimization, and engagement of WT KRAS may constrain the therapeutic window, our observation that a SII-P ligand can engage several GTP hydrolysis-deficient KRAS mutants signifies exciting opportunities to drug these KRAS mutants through this pocket.

**Discussion**

Here we report subfamily-wide engagement of KRAS hotspot mutants with the preclinical inhibitor MRTX849 and structurally related molecules. This presents evidence of intracellular SII-P vulnerability across the prevalent oncogenic KRAS mutants including KRAS(G12D). To characterize target engagement across RAS species, we combined in vitro and intracellular biophysical approaches. NMR spectroscopy provided a defined system to observe reversible, noncovalent binding and to determine the impact of nucleotide status on KRAS vulnerability. However, cell-free methods are incapable of simulating the intracellular architecture where target engagement would naturally occur. To query engagement in cells, we developed a SI/II-P BRET probe that was competent to detect a variety of intracellular engagement mechanisms including ligands selective for either SI/II-P or SII-P. Mutually exclusive binding between the BRET probe and the SII-P ligands enabled a systematic evaluation of SI-P engagement across KRAS hotspot mutants. This mutual exclusivity is consistent with the dynamic nature of RAS effector occupancy in cells. It is probable that RAS occupancy in cells is conformationally selective, and on the basis of its small size, RAS may disfavor co-occupancy of multiple ligands or effector proteins. The BRET method reported here conditionally measures engagement at membrane-localized RAS complexes in cells. Target engagement results with known SII-P covalent inhibitors matched both engagement and MAPK inhibition within endogenous G12C driven lineages, supporting the accuracy of the engineered BRET method.

Expanding beyond inhibition of KRAS(G12C), the BRET system enabled us to observe engagement of WT KRAS and of the majority of KRAS hotspot mutants including G12D. As measured in the BRET system, the rank order vulnerability of KRAS hotspot mutants...
to SII-P engagement with MRTX849/1257 and related noncovalent inhibitors (9–14) did not fully correlate with reported rates of intrinsic hydrolysis using purified RAS proteins12. Specifically, the G13D, Q61H and Q61L mutants reportedly have among the lowest intrinsic hydrolysis rates of the hotspot mutants evaluated here, as determined in cell-free systems. Accordingly, these alleles should be among the least vulnerable to SII-P target engagement by GDP-state-specific inhibitors, even when considering the potential for steric and conformational effects to confer differential affinity. However, MRTX849/1257 and the noncovalent inhibitors 9, 10 and 14 engaged these mutants nearly as potently as they did WT protein, and more potently than they engaged the G12D and G12V mutants. In the case of G13D, this result may be explained by the high nucleotide exchange rate measured for this mutant12,13,26. In the cases of the Q61 mutants, earlier reports have noted similar discrepancies; a GDP-state-specific degrader was able to target KRAS(Q61H) in cells34, and KRAS(Q61L) was observed to possess a higher hydrolysis rate in a cellular context than in cell-free systems13. These earlier reports and our in-cell BRET data suggest that the nucleotide states of RAS proteins in a cellular setting may deviate from those quantified in a biochemically defined system, emphasizing the need for direct measurements of target engagement in cells when evaluating RAS-targeted inhibitors. Another factor that we cannot rule out is the potential contribution of RAS proteins in extracellular fractions to the behavior of the cellular BRET assay. RAS proteins in extracellular fractions could potentially contribute to this cellular BRET assay, which may have different properties compared with intracellular RAS proteins.

Because MRTX849/1257 demonstrate SII-P engagement across KRAS hotspot mutants, this chemotype may serve as the basis for development of allele-specific KRAS inhibitors beyond G12C. However, the GDP-state bias might limit the efficacy of this chemotype against a wider array of KRAS hotspot mutants that may predominantly reside in a GTP state in vivo. For example, KRAS(G12D) was less vulnerable than WT, and KRAS(G12V) and Q61R were largely inaccessible to MRTX849/1257. Thus, less subtle chemical modifications will probably be necessary to target these oncogenes, and engaging both nucleotide states of KRAS may be required. At the time of preparing this manuscript, structures of new KRAS(G12D) inhibitors were disclosed that were structurally similar to the MRTX849 chemotype12–15. We found that one such example, MRTX-EX185 (ref. 25), can bind GPPPNH-loaded KRAS and KRAS(G12D) 1–169 by NMR and engage KRAS(G12D) in cells by our BRET-based assay with <100 nM affinity. The increased affinity to KRAS(G12D) translated into potent inhibition of RAF effector interactions as well as potent antiproliferative effect. Although detailed analyses of this new chemotype’s binding mode have not yet been published, its ability to also access the active nucleotide state of KRAS SII-P is probably a key contributor to its increased engagement potency against KRAS(G12D) in cells. An expanded evaluation of MRTX-EX185 is warranted to determine the kinetics of inhibition and pathway inactivation in a KRAS G12D setting. Among the KRAS hotspot mutants, KRAS(G12V) is expected to be even more heavily biased towards the GTP state compared with G12D13. Consistent with GTP-state accessibility, MRTX-EX185 engaged KRAS(G12V) in cells with submicromolar affinity. Together our target engagement and NMR spectroscopy results support a broad opportunity to target KRAS SII-P in a manner decoupled from nucleotide status.

We have shown that KRAS hotspot mutants offer wider opportunities for SII-P engagement than previously understood; in particular, some proteins bearing activating mutations may be more accessible to GDP-state inhibition in some cellular contexts than predicted solely on the basis of biochemical GTP hydrolysis rates. Furthermore, recently disclosed chemotypes capable of directly binding the active GTP-loaded state present even wider opportunities for SII-P engagement across KRAS hotspot mutants. Thus, our work highlights the importance of methods to directly assay target engagement in cells to compliment phenotypic assays and in vitro biochemical assays. Methods to query target occupancy...
These capabilities should aid in the evaluation and optimization of new and improved medicines for RAS-driven cancers and prevalent RASopathies.

Online content
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Methods
Cell transfections and BRET measurements. HEK293 cells (ATCC), HeLa cells (ATCC), A375 cells (ATCC), HCT-116 cells (ATCC), NCI-H358 cells (ATCC), NCI-H464 cells (ATCC), MIA PaCa-2 cells (ATCC) and SW-1990 cells (ATCC) were cultured in DMEM (Gibco) with 10% FBS (Sigma-Aldrich) + 75% CO₂ incubation in a humidified, 37 °C/5% CO₂ incubator. H1975 cells (ATCC) were cultured in RPMI 1640 (Gibco) + 10% FBS, with incubation in a humidified, 37 °C/5% CO₂ incubator.

For Ras cellular BRET measurements, a luciferase donor signal was produced at multicentric Ras probes in a NanoBiT approach. Amino-terminal (N-terminal) large BiT (LgBiT) or small BiT (SmBiT) Ras fusions were encoded in pNB3K and pNB4K (respectively) expression vectors (Promega), including flexible 15-residue linkers (GSGGGGGGSGGGGSGGGGSGGGGGGGGGSGGGGGGGGGGGGGGG) between the tag and each Ras isoform. All KRAS open reading frames (ORFs) were based upon KRAS4B (UniProt P01116-2) and all HRAS ORFs were based upon UniProt isoform 1 (P01112-1). All Ras ORFs were full-length unless otherwise noted. HEK293 cells were transfected with SmBiT–Ras and LgBiT–Ras fusion constructs using FuGENE HD (Promega) according to the manufacturer’s protocol. Briefly, SmBiT–Ras and LgBiT–Ras constructs were diluted together into Transfection Carrier DNA (Promega) at a mass ratio of 1:1 in Opti-MEM (Gibco), after which FuGENE HD was added at a ratio of 1:3 (µg of DNA: µl of FuGENE HD). For example, for a 1 ml size transfection complex, 1 µg each of the SmBiT–Ras and LgBiT–Ras DNA solutions were combined with 8 µg of Transfection Carrier DNA in 1 ml of Opti-MEM. One part (vol) of FuGENE HD complexes thus formed were combined with 20 parts (vol) of HEK293 cells suspended at a density of 2 × 10⁶ cells/ml in cell culture medium. All chemical inhibitors were prepared as concentrated stock solutions in DMSO (Sigma–Aldrich) and diluted in Opti-MEM (unless otherwise noted) to prepare working stocks. Cells were equilibrated with the Ras BRET probe and test compound before BRET measurements, with an equilibration time of 2 h unless otherwise noted. Ras BRET probe was prepared first at a stock concentration of 100 nM in DMSO, after which the 100X stock was diluted to a working concentration of 20X in BRET probe dilution buffer (12.5 mM HEPES, 31.25% PEG-400, pH 7.5). For Ras BRET probe dose response measurements, the Ras BRET probe was added to the cells in an eight-point, two-fold dilution series starting at a final concentration of 2 µM. For target engagement analysis, the Ras BRET probe was added to the cells at a final concentration of 1 µM. To measure BRET with the Ras BRET probe, NanoBiT Target Engagement substrate (Promega) was added according to the manufacturer’s recommended protocol, and filtered luminescence was measured on a GloMax Discover luminometer equipped with 450 nm BP filter (donor) and 600 nm LP filter (acceptor), using 0.5 s integration time. Unlike the BRET probe, the acceptor count was not subtracted from the donor count. Raw BRET ratios were calculated by dividing the acceptor counts by the donor counts. Milli-BRET units (mBU) were calculated by multiplying the raw BRET values by 1000. When normalized BRET was used, mBU values were normalized using equation (1):

\[
\text{Normalized BRET (µBU)} = \frac{[A-C]}{(B-C) - C} \times 100
\]

Where A = mBRET in the presence of test compound and BRET probe, B = mBRET in the presence of vehicle and BRET probe and C = mBRET in the presence of a saturating 20-µM dose of BI-2852. Apparent BRET probe affinity values (half-maximum effective concentration (EC₅₀)) were determined using the sigmoidal dose response (variable slope) equation available in GraphPad Prism (equation 2):

\[
Y = \frac{\text{Bottom} + (\text{Top} - \text{Bottom}) \times \text{Fraction}}{1 + 10^{\frac{(\log_{10}(EC_{50}) - X)}{\text{Hill Slope}}}}
\]

In some cases, the Ras BRET probe was not saturable up to the solubility limit of the BRET probe, so the EC₅₀ value of the BRET probe is reported as >1 µM. For determination of test compound potency, competitive displacement data were plotted with GraphPad Prism software and data were fit to equation (2) to determine the IC₅₀ value.

To measure the interaction of KRAS NanoBiT dimers with CRD-CRD-RBD-HaloTag, cells were transfected as described above, except that DNA encoding untagged full-length CRAF (UniProt P00409-1) was substituted for the Transfection Carrier DNA. To measure the interaction of KRAS NanoBiT dimers with CRD-CRD-RBD-HaloTag, cells were transfected as described above, except that DNA encoding untagged full-length CRAF (UniProt P00409-1) was substituted for the Transfection Carrier DNA.

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integration time. The ratio of the acceptor and donor emission signals for each individual well was calculated by

\[ TR\ \text{FRET} = \frac{[\text{Signal} \ 665\ nm]}{[\text{Signal} \ 620\ nm]} \times 10,000 \]

Total ERK data were acquired via the same procedure using the Total ERK cellular kit (Cisbio) on the same cell lysates. For each reporter (p-ERK or total-ERK), the TR FRET ratio was normalized to the respective DMSO control. The ratio of p-ERK over total-ERK was calculated and fit to equation (2) above.

Induction of ERK phosphorylation by expression of KRAS constructs. The impact of expressing untagged or NanoBit-tagged KRAS variants on ERK phosphorylation was evaluated in HEK293 cells expressing an ERK1 substrate protein using AlphaLISA SureFire Ultra p-ERK1/2 (Thermo2/Tyr204) and AlphaLISA SureFire Total ERK 1/2 assays (PerkinElmer). The ERK1 substrate protein was NanoLuc-ERK1 (NanoLuc-MAPK3; Promega), which was coexpressed with the RAS vectors to create a uniform total-ERK level across various transfection conditions and minimize the potential for non-specific ERK isoform expression. Total ERK levels were measured as previously published. Moreover, the NanoLuc tag on the ERK1 substrate allowed total luminescence to be used as an independent method to ensure uniform expression across samples. HEK293 cells were transfected as described above for cellular RAS target engagement assays, except that the KRAS constructs, NanoLuc-ERK1 and Transfection Carrier DNA were combined at a mass ratio of 2:1:7, as indicated in the figures. Cells were plated directly into 96-well tissue culture-treated assay plates (Corning) at 2 x 10^5 cells per ml (100 µl total volume) and allowed to express overnight.

For p-ERK and total-ERK measurements, the medium was aspirated from the assay wells, after which 62 µl of AlphaLISA lysis buffer was added to the cells and the plates were incubated for 10 min at 30 °C. Cell lysates thus obtained were then analyzed using the AlphaLISA SureFire Ultra assays above according to the manufacturer’s instructions. Fluorescence emission measurements at 615 nm were recorded on a BMG CLARIOstar instrument using the AlphaLISA protocol. Raw fluorescence values from p-ERK measurements were normalized to the raw fluorescence values from the total-ERK measurements from the same lysate samples to produce the p-ERK/total-ERK ratio that was used for comparison. The p-ERK or total-ERK positive control lysates provided with the AlphaLISA SureFire Ultra kits were diluted 1:1 with lysis buffer before analysis.

Total luminescence was measured in the NanoLuc-ERK1 expressing cell samples as described above for cellular RAS target engagement measurements, except that the Nano-Glo protocol (unfiltered luminescence) was used on the GloMax Discover instrument.

Preparation of U-15N Ras proteins. The plasmids for bacterial expression of HRAS 1−166 (WT; His-TEV-N; pProEx; ampicillin resistance) and KRAS 1−169 (WT, G12C and G12D; His-TEV-N; pJ411; kanamycin resistance) have been previously published. BL21(DE3) competent cells were transformed with 1–2 ng of plasmid, and cultures were grown at 37 °C in M9 minimal media containing 1 g L−1 glucose, 1.5 g L−1 NaCl, 50 µg mL−1 ampicillin, and 50 µg mL−1 kanamycin.

1D 1H NMR spectra were recorded on a 800 MHz Bruker Avance spectrometer with 120 ms mixing time. NMR spectra were acquired under the same conditions. For the mixed samples containing untagged or NanoBit-tagged KRAS variants with their ligands, the signal-to-noise ratio was improved by averaging the signal-to-noise ratio. Some spectra were also acquired with 0.5, 10 and 200 mM dmso-d6, and/or with minor adjustments to pH and temperature for comparison to previously published assignments.

HSQC NMR buffer: 40 mM HEPES, 150 mM NaCl, 4 mM MgCl2, 7% D2O. Titrated to pH 7.4 with NaOH.

\[ H^+−\text{N−H NOESY-HSQC NMR sample preparation and acquisition.} \]

A 0.15-µmol sample of U−15N KRAS(G12C)−GDP−MRTX849 protein in storage buffer was diluted to 400 µl with NOESY NMR buffer on ice. The buffer was exchanged to the NOESY NMR buffer with a desalting column (5 µl HiTrap Desalting, Cytiva and AKTA FPLC, GE). The protein containing fractions were combined (1.5 ml), concentrated to 0.3 ml (10k MWCO Amicon Ultra-4, EMD), transferred to a 5-mm Shigemi NMR tube (BMS-3) and gently sparged with Ar before sealing with paraffilm (final concentration ~0.5 mM). 1H−15N HSQC spectra were acquired on an 800 MHz Bruker Avance spectrometer at 298 K. A second 2D HSQC spectrum was acquired after the 3D NOESY−HSQC experiment with identical parameters to confirm sample stability during the 28 h acquisition.

A 0.374-µmol sample of U−15N KRAS(G12D)−GDP in protein buffer was diluted with NOESY NMR buffer, and the buffer was exchanged as described above. The concentration of the resulting 1.5-ml solution was determined to be 0.20 ± 0.03 mM by a BCA assay (Pierce, ThermoFisher Scientific); and this solution was concentrated to 0.50 ml (0.60 mM). A 200 µl aliquot (0.12 µmol) of this solution was diluted to 294 µl with the same buffer, and MRTX849 or EX185 (6 µl, 20 mM in dmso-d6) was added. The samples were prepared as described above, and the same series of spectra were acquired. The remainder of the protein solution (100µl, 0.060 µmol) was diluted to 294 µl with the same buffer, dmso-d6 (6 µl) was added, and 1H and 2D fast −15N HSQC spectra were acquired from this sample for comparison.

NOESY NMR buffer: 20 mM sodium phosphate, 140 mM NaCl, 15 mM MgCl2, 10 mM EDTA, 3 mM Na2N3, 1 mM GDP, 1 mM DSS, 10% D2O. Titrated to pH 7.4 with NaOH.

NMR data analysis. Spectra were analyzed with Bruker Topspin 4.0, CCPNMR Analysis v3 (ref. 39) and/or MestReNova v14.1. Chemical shifts were referenced to DSS at 0 ppm; 1H chemical shifts were referenced to 1H−15N HSQC sample preparation and acquisition.

\[ \delta = \frac{\text{Resonance} - \text{Reference}}{\text{Reference}} \times 10^6 \]

Quantification and statistical analysis. Data from multiple independent experiments (n) are presented as mean ± s.e.m. and data involving technical replicates are presented as mean ± s.d. as indicated in the figure captions. The number of experimental or technical replicates for each experiment is also number of experimental or technical replicates for each experiment is also described in each individual figure caption. Apparent affinity values were determined using the sigmoidal dose−response (variable slope) equation available in GraphPad Prism (v8). Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.
**Data availability**
Source data are provided with this paper. The authors declare that the data supporting the findings of this study are available within the article, the accompanying Source Data, the Supplementary Information and the Supplementary Data. Additional information, resources and reagents will be made available upon reasonable request; requests should be directed to and will be fulfilled by the lead contact M.B.R.

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**Author contributions**
D.M.P. performed and analyzed the data from protein NMR spectroscopy experiments. J.A.W., J.D.V. and C.R.C. designed and synthesized the BRET probe. M.R.T. designed the RAS expression constructs. M.B.R. and J.D.V. designed the in-cell RAS BRET assay system. C.A.Z. performed and analyzed the data from the BRET assays. Q.Z. synthesized the noncovalent SII-P inhibitors. J.D.V. and M.B.R. performed and analyzed the CellTiter-Glo antiproliferation experiments. B.F.B. and M.T.B. designed and performed the RAS-RAF interaction assay. Q.Z. and Z.Z. performed and analyzed the data from the ERK phosphorylation assays. K.M.S. and M.B.R. guided the study and supervised the research from their respective groups. J.D.V., D.M.P., Q.Z., Z.Z., K.M.S. and M.B.R. wrote the manuscript.

**Competing interests**
J.D.V., J.A.W., C.A.Z., M.R.T., M.T.B., B.F.B., C.R.C. and M.B.R. are employees of Promega Corporation, which holds patents related to the NanoBRET Target Engagement method. K.M.S. is an inventor on patents owned by University of California San Francisco covering KRAS targeting small molecules licensed to Araxes and Ersaca. K.M.S. has consulting agreements for the following companies, which involve monetary and/or stock compensation: Revolution Medicines, Black Diamond Therapeutics, BridGene Biosciences, Denali Therapeutics, Dice Molecules, eFFECTOR Therapeutics, Ersaca, Genentech/Roche, Janssen Pharmaceuticals, Kumquat Biosciences, Kura Oncology, Mitokinin, Type6 Therapeutics, Venthera, Wellospring Biosciences (Araxes Pharma), Turning Point, Ikena, Initial Therapeutics and BioTheryX.

**Additional information**
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Correspondence and requests for materials should be addressed to Matthew B. Robers or Kevan M. Shokat.
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection: No software was used for data collection.

Data analysis: GraphPad Prism v8 and v9 were used for the creation of plots, fitting of curves, and calculations of S.E.M. CCPNMR Analysis v3 was used to analyze 2D NMR spectra and create contour plots. Image J was used to process bioluminescence images.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
No sample size calculation was performed. Sample sizes were chosen on the basis of prior studies in NMR and BRET/Luminescence experiments that showed significant effects with similar sample sizes.

Data exclusions
No data were excluded from these analyses.

Replication
Replicated experiments were successful and support the conclusions drawn in this report. In the case of inhibitor potency information, the data generated in this study were reproducible by 2-3 independent scientists. All data in main figures were reproduced by 2-3 independent scientists. Bioluminescence imaging was replicated in 2 independent experiments by the same scientist. The data in supplementary figures 3A, 5F, 9(a, b, and e), 10(a-h), 11a, and 12(a) were reproduced by 2 independent scientists in 2-3 independent experiments. Experiments in supplementary figures 3, 5(b, c, d, e, and g), 6(b), 7b, 8(b-e), 9(c and d), 10(i and j), 11(b and c), and 12(b-d) were only performed once by single scientists. All attempts at replication were successful.

Randomization
No formal randomization method was used in this study to avoid mislabeling during inhibitor testing.

Blinding
Blinding was not relevant to this study because no bias could be made by the subject or the tester in the experiments performed.

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Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)
HEK-293 cells (ATCC, CAT# CRL-1573), HeLa cells (ATCC, CAT# CCL-2), A-375 cells (ATCC, CAT# CRL-1619), HCT-116 cells (ATCC, CAT# CCL-247), NCI-H522 cells (ATCC, CAT# CRL-5807), NCI-H667 cells (ATCC, CAT# CRL-5834), MiaPaCa-2 Cells (ATCC, CAT# CRL-1421), and SW-1990 cells [ATCC, CAT# CRL-2172] were cultured in DMEM (Gibco) + 10% FBS (Seradigm), with incubation in a humidified, 37°C/5% CO2 incubator. H1975 cells (ATCC, CAT# CRL-5908) were cultured in RPMI 1640 (GIBCO) + 10% FBS, with incubation in a humidified, 37°C/5% CO2 incubator. Cells were passed for at least two generations after cryorecovery before they were used for assays.

Authentication
We did not perform cell line authentication.

Mycoplasma contamination
All cell lines were tested mycoplasma negative using MycoAlert™ Mycoplasma Detection Kit (Lonza).

Commonly misidentified lines
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HEK293 and HeLa cells were used in this study.