Inhibiting the RAS oncogenic protein has largely been through targeting the switch regions that interact with signalling effector proteins. Here, we report designed ankyrin repeat proteins (DARPins) macromolecules that specifically inhibit the KRAS isoform by binding to an allosteric site encompassing the region around KRAS-specific residue histidine 95 at the helix α3/loop 7/helix α4 interface. We show that these DARPins specifically inhibit KRAS/effector interactions and the dependent downstream signalling pathways in cancer cells. Binding by the DARPins at that region influences KRAS/effector interactions in different ways, including KRAS nucleotide exchange and inhibiting KRAS dimerization at the plasma membrane. These results highlight the importance of targeting the α3/loop 7/α4 interface, a previously untargeted site in RAS, for specifically inhibiting KRAS function.
Members of the RAS family of oncogenic proteins are frequently mutated in human cancers. In particular, KRAS mutations are the most prominent ones, representing around 86% of all RAS mutations\(^4\). KRAS mutants are major drivers of cancers, such as colorectal, lung or pancreatic cancers\(^3\). Isolation of selective KRAS inhibitors that block its function is therefore an important goal\(^1\). Nonetheless, selectively targeting KRAS is challenging, as RAS isoforms are highly similar in primary sequence with 82–90% amino acid sequence identity\(^5\).

Most current inhibitors target all RAS isoforms via their conserved effector lobe (defined as amino acid 1–86) by inhibiting RAS/effector interactions\(^4\–7\) or RAS nucleotide exchange\(^8\–9\). We identified such pan-RAS inhibitors in a previous study with the anti-RAS designed ankyrin repeat proteins (DARPins) K55 (RAS/effector interactions inhibitor) and K27 (RAS nucleotide exchange inhibitor)\(^8\). As an alternative, targeting RAS via its allosteric lobe (amino acids 87–166)\(^10\) is a possible way to inhibit its function in cells\(^11\–13\). The α3–α4 and α4–α5 interface in the allosteric lobe are potential dimerisation sites for RAS\(^14\–17\) and preventing KRAS dimerisation impacts the mitogen-activated protein kinase (MAPK) signalling pathway\(^18\). Recent studies have shown that dimerisation is a potential targetable feature of KRAS function\(^11\–13\). Notably, a monobody that targets both HRAS and KRAS on the α4–α5 site, disrupts RAS dimerisation, blocks RAF activation\(^12\) and inhibits tumour formation in vivo\(^13\). Nevertheless, none of these inhibitors are KRAS selective.

Specifically targeting directly mutant KRAS has been achieved with small molecules covalently binding the G12C mutant KRAS\(^19\–21\). This approach targets the G12C mutation that represents around 12% of KRAS mutations in cancers (Cosmic database v86, https://cosmic-blog.sanger.ac.uk/), and is only present in a subset of cancers, such as non-small cell lung cancer\(^22\). Therefore, alternative strategies are needed to inhibit the most frequent mutations of KRAS accounting for 88% of KRAS mutant cancers.

We report here the characterisation of two potent DARPins that selectively bind KRAS on a site of the allosteric lobe, encompassing histidine residue 95. The DARPin binding inhibits KRAS nucleotide exchange and KRAS dimerisation, thus impairing mutant KRAS–effector interactions and the downstream signalling pathways. These findings reveal a unique strategy to selectively inhibit KRAS.

**Results**

**Isolation of anti-KRAS-specific DARPins.** We performed a phage display selection of a diverse DARPin library\(^6\), followed by immunoassays with KRAS\(^{G12V}\) to isolate hits. We have identified two DARPins (designated K13 and K19) that bound to KRAS\(^{G12V}\). Biochemical analysis of the DARPins show K13 and K19 interact with KRAS independently of the nucleotide-bound state of the GTPase, and have K\(_d\)s around 30 and 10 nM, respectively (Supplementary Fig. 1a). The nucleotide and protein sequences of DARPins K13 and K19 are shown in Supplementary Fig. 1b, c and highlight a conserved amino acid sequence in the repeat regions with only six amino acids difference.

The X-ray structure data of K13 and K19 in complex with KRAS\(^{G12V}\) show these DARPins bind to the allosteric lobe of KRAS, at the interface between helix α3/loop 7/helix α4 (Fig. 1a, b; Supplementary Table 1). The crystal structures show that when DARPins K13 or K19 bind to KRAS, a structural change appears in the KRAS molecule on the effector lobe, especially on the switch 1 loop (Supplementary Figs. 2a, b). However, the exact conformation of the switch 1 loop in the K13- and K19-bound states differ somewhat. This difference is most likely due to their different crystal-packing environments (Supplementary Fig. 2c, d). NMR chemical shift perturbation HSQC and hydrogen deuterium exchange with mass spectrometry (HDX-MS) data support the observed binding interface in solution of K19 in the allosteric lobe (Fig. 2a–c and Supplementary Figs. 3–5) and control DARPin K27 in the effector lobe (previously shown to interact with the switch regions of KRAS, NRAS and HRAS-GDP\(^8\)) (Supplementary Figs. 4–6). After K19 binding to KRAS, a small but significant increase in the dynamic mobility of the switch 2 loop is shown by the increase in de-protection observed by HDX-MS (Supplementary Figs. 3–4), and some small perturbations of the effector lobe HSQC resonances are observed in a few residues in the switch 2 region (Fig. 2a–c). Our data suggest that the conformational change observed by X-ray crystallography on the switch regions is most likely due to the crystal-packing effect, because the switch regions are flexible by nature.

DARPins K13 and K19 are KRAS selective binders. K13 and K19 interact with the allosteric lobe where the amino acids between the RAS isoforms are less conserved than the effector

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**Fig. 1** DARPins K13 and K19 bind KRAS to a novel allosteric site. a X-ray crystal structures of KRAS\(^{G12V}\)-GDP-bound to DARPin K13 (PDB 6H46) and KRAS\(^{G12V}\)-GDP-bound to DARPin K19 (PDB 6H47) with the indication of RAS switch regions, helix 3 (α3), helix 4 (α4) and loop 7 (L7) regions. b Expanded view of the interaction between the DARPin K13 and KRAS around histidine residue 95 of KRAS.
lobe. Thus, we assessed whether K13 and K19 could bind to all RAS family isoforms. While the switch region-binding DARPin K27 binds to K, N and HRAS in cell-based bioluminescent resonance energy transfer 2 (BRET2) assay, K13 and K19 show only strong binding with KRAS (Fig. 3a, b). In addition, K13 and K19 interact equally with the constitutively activated KRAS$^{S17N}$, the constitutively activated mutant KRAS$^{G12D}$ and wild-type KRAS, unlike K27, which does not contact the KRAS$^{S17N}$ mutant (Fig. 3a, b). Indeed, K13 and K19 bind KRAS away from the switch regions, and are not affected by the S17N mutation while K27 binding is expected to be affected as the flexibility of the switch regions is modified on that mutant. These selectivity findings were further supported using a co-immunoprecipitation assay with cells co-expressing 3xFLAG-tagged wild-type RAS and GFP-tagged DARPin. KRAS, NRAS and HRAS proteins were immunoprecipitated on beads, and the presence of DARPin–GFP bound to RAS proteins was assessed. The DARPin K27 was captured by all three RAS isoforms, but K13 and K19 were only captured by KRAS, while the negative control DARPin E3.58 was not co-immunoprecipitated by any RAS proteins (Fig. 3c).

K13 and K19 bind KRAS by interacting with the H95 of KRAS. We evaluated which residue(s) are involved in this KRAS selectivity of K13 and K19. Analysis of the amino acids engaged in the interaction between the DARPins and KRAS, showed that K13 and K19 make extensive interactions with the histidine 95 and the glutamic acid 107, which are residues only present in KRAS (Supplementary Fig. 7a). Therefore, we introduced mutations on the positions 95 and 107 of wild-type (WT) KRAS$^{WT}$ substituting

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**Fig. 2** HSQC NMR chemical shift changes in KRAS after DARPin K19 binding. a Comparison between $^1$H, $^{15}$N TROSY spectra for apo KRAS$^{G12V}$ at 100 μM (black) overlaid with K19-bound KRAS$^{G12V}$ at 89 μM (green). DARPin concentrations were in slight excess. An expanded region is shown on the right. b Amino acids undergoing chemical shifts upon DARPin K19 binding on KRAS$^{G12V}$-GDP are shown in blue, residues not experiencing shifts are shown in turquoise in the KRAS$^{G12V}$ sequence (unassigned residues are not highlighted). The P-loop is highlighted in yellow, the switch 1 in dark yellow and the switch 2 in purple. c Ribbon representation of (b) with amino acids undergoing chemical shifts in blue and residues not experiencing shifts in turquoise in the KRAS$^{G12V}$-GDP structure (PDB 4TQ9) after DARPin K19 binding (unassigned residues are in grey). L7: loop 7, $\alpha$3: helix 3, $\alpha$4: helix 4.
HIS95 with leucine or glutamine (found at residue 95 in NRAS and HRAS, respectively) and substituting GLU107 with aspartic acid (found at residue 107 in both NRAS and HRAS). While the E107D mutation did not affect K13, K19 or K27 DARPins binding with KRAS (Fig. 3d), H95Q and H95L mutations drastically decrease K13 and K19 binding on KRAS WT but do not affect K27 binding as shown by BRET (Fig. 3d) or co-immunoprecipitation (Supplementary Fig. 7b). Supplementary Table 2 summarises the effects of RAS mutations on the binding of the DARPins. Finally, we assessed the residues of the DARPins important for binding to KRAS by mutating two tryptophans found in the first repeat of both K13 and K19 to glycine residues.

Fig. 3 K13 and K19 are KRAS selective binders. a BRET donor saturation assay between KRAS G12D or KRAS S17N (donors) and the DARPins (acceptors). b BRET donor saturation assay between KRAS WT, NRAS WT, NRAS Q61H, HRAS WT or HRAS G12V (donors) and the DARPins (acceptors). c Co-immunoprecipitation of 3xFLAG-KRAS WT, −NRAS WT and −HRAS WT with the DARPin-GFP2 fusions in 10% foetal bovine serum. IP Immunoprecipitation, WCE whole-cell extract. d BRET donor saturation assay between KRAS WT, KRAS H95Q, KRAS H95L or KRAS E107D (donors) and the DARPins (acceptors). Note that most of the donor saturation curves with K27 as acceptor are overlapping in panels b and d. Each experiment was performed three times (a–d). Error bars are mean ± SD of biological repeats. c Source data are provided as a Source Data file.
K13 and K19 inhibit KRAS function by at least two mechanisms. While K13 and K19 bind to the allosteric lobe of KRAS and interfere with effector interactions, it suggested new mechanism (s) by which DARPins inhibit KRAS function. To this end, we tested these DARPins in biochemical assays, where K13 and K19 were compared with the known RAS nucleotide exchange inhibitor K27 and known RAS/CRAF RBD interaction inhibitor K55. In vitro analyses show that DARPins K13 and K19 inhibit the biochemically coupled KRAS assay based on the inhibition of nucleotide exchange or KRAS–CRAF RBD interaction (Fig. 6a). As does K27 but, unlike K55, K13 and K19 do not prevent KRAS/CRAF RBD interaction in vitro (Fig. 6b). Nonetheless, the binding of the DARPins to KRAS is nucleotide independent (Fig. 3a; Supplementary Fig. 1a), and the binding occurs on a surface remote from the switch regions (Fig. 1). However, SOS not only contacts RAS near the switch 1 and 2 regions but also makes additional interactions with the residues 95–105 of the helix α3 [29]. These contacts would cause a clash between K13 and SOScat for KRAS binding (Fig. 6d) and indicate why K13 and K19 inhibit the nucleotide exchange of KRAS.

In addition, we investigated whether the DARPins could interfere with KRAS dimerisation, as it has been previously described with a different allosteric inhibitor, the monobody NS1 [12]. Indeed, the α3–α4 and α4–α5 regions are defined as the potential dimer interfaces of KRAS [14–17] and K13 and K19 interact with the α3–α4 site of KRAS (Supplementary Fig. 7a).
Consequently, the binding of K13/K19 with KRAS is expected to perturb its dimerisation. We tested this hypothesis by BRET experiments involving the dimerisation of mutant K-, N- and H-RAS. K13 and K19 essentially inhibit KRASG12D dimerisation, while NRAS Q61H and HRAS G12V dimers formation is less affected by the DARPins (Fig. 7a–c).

It has been previously reported that the CAAX motif of RAS is mandatory for its dimerisation. Therefore, we investigated RAS dimerisation using KRASG12V deleted of its last 22 amino acids (a KRASG12V166 mutant missing the whole hypervariable region, HVR, including the CAAX motif) that should remove its ability to form RAS dimers. First, we showed that KRASG12V166 was soluble and expressed in the cytoplasm of cells (Supplementary Fig. 10a) and was not able to form dimers in cells by BRET donor saturation assays (Supplementary Fig. 10b). We then performed a BRET competition assay with K13 and K19 against KRASG12V FL or KRASG12V166 interaction with CRAF RBD showing that removal of the HVR from KRASG12V only reduced K13/K19 inhibition of KRASG12V166/CRAF RBD interaction (Supplementary Fig. 10c). These results show that RAS dimerisation in cells is not the only mechanism of inhibition of KRAS by the DARPins, as these retain the ability to inhibit KRASG12V166/CRAF RBD interaction.

**Fig. 5** K13/K19 inhibitory potency in cells. a Western blot analyses of the activation state of the RAF/MEK/ERK and PI3K/AKT pathways in cancer cell lines bearing or not a mutated RAS upon overexpression of the DARPin-mCherry fusions. E3.5 is a negative and DARPin K27 is a positive control. b Quantifications of pERK/ERK, pMEK/MEK and pAKT/AKT signals from (a). These signals were normalised to the negative control DARPin E3.5. Statistical analyses in b were performed using a one-way ANOVA followed by Dunnett’s post-hoc tests (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001). Each experiment was performed at least four times (a–b). Error bars are mean ± SEM of biological repeats (b). a, b Source data are provided as a Source Data file.
Fig. 6 K13 and K19 inhibit KRAS nucleotide exchange. a KRAS biochemical coupled assay for inhibition of nucleotide exchange or KRAS/CRAF RBD interaction by DARPin K13, K19 or negative control DARPin E3.5. KRAS<sup>G12V</sup> loaded with GDP was incubated for 15 min with a dilution series of DARPins K13, K19, K27 or E3.5. SOS and GTP<sub>γS</sub> were added to allow nucleotide exchange, followed by CRAF RBD. The KRAS<sup>G12V,GTPγS</sup>/CRAF RBD complex was quantitated by the FRET signal. b KRAS/CRAF RBD inhibition assay testing DARPin K13, K19, K55 (a known KRAS/CRAF RBD inhibitor) and E3.5. The FRET signal was measured for the interaction between CRAF RBD and KRAS<sup>G12V</sup> loaded with GTP<sub>γS</sub> and inhibition of the signal monitored at varying concentrations of DARPins K13, K19, K55 and E3.5. Error bars represent the mean ± SD (n = 3). c MANT dGDP and MANT GTP nucleotide exchange and release assays. SOS-mediated exchange of MANT-labelled dGDP or GTP on KRAS<sup>G12V</sup> was studied over time upon addition of 10 μM concentrations of DARPins. Included controls were previously published RAS-binding DARPin K55 (non-inhibitor of nucleotide exchange) and K27 (inhibitor of nucleotide exchange). For release assays, DARPins were added after equilibration of MANT nucleotides. In these assays, the binding of MANT-labelled nucleotides to KRAS<sup>G12V</sup> results in an increase in fluorescence, detected by measuring light emission at a wavelength of 450 nm. Error bars represent the mean ± SD (n = 8). d Superimposition of K19/KRAS<sup>G12V</sup>·GDP (PDB 6H47) and SOScat structures (PDB 1BKD)
Discussion

We reported here KRAS-selective inhibitors that bind to a previously untargeted site on the allosteric lobe of KRAS and prevent KRAS-mediated signalling. We have studied two DARPinS, sharing a conserved amino acid sequence, that bind on the same interface of KRAS, the helix α3/loop 7/helix α4 region. The tryptophan residues 35 and 37 of K13 and K19 contact the histidine 95 of KRAS, which is a residue only found in KRAS thereby conveying their KRAS selectivity. These DARPinS differ significantly from our previously isolated anti-RAS DARPinS that included a pan-RAS-GTP binder (K55) that inhibits RAS/effecter interactions and a pan-RAS-GDP binder (K27) that prevents RAS nucleotide exchange, both DARPinS targeting the switch regions of RAS. We have now characterised K13/K19 DARPinS that specifically contact KRAS (GTP and GDP bound) on an allosteric site remote from the switch regions and impede KRAS nucleotide exchange and dimerisation.

Even though K13/K19 bind to both mutant and WT KRAS, they only prevent RAS-dependent signalling in KRAS mutant cancer cells (HCT116) and do not affect RAS signalling in RASWT cancer cells (MC7-7) (Fig. 5). In a previous study, an antisense oligonucleotide (AZD4785) was described that selectively targets KRAS mRNA30, but both wild-type and mutant mRNAs AZD4785 downregulates both WT and mutant KRAS protein, but only inhibits the downstream MAPK and PI3K signalling pathways of mutant KRAS expressing cells and not of KRASWT expressing cancer cells30. Therefore, our data also suggest that inhibition of both WT and mutant KRAS do not affect the RAS downstream signalling pathways of RASWT cancer cells. This effect could be explained by compensatory and/or redundancy mechanisms of the other RAS isoforms31.

Although the crystal structures of K13 and K19 bound to KRAS show substantial conformational change on both switch regions, the NMR HSQC and HDX-MS only show a small conformational change on the switch 2 region of KRAS upon the DARPinS binding (Fig. 2 and Supplementary Figs. 3–6). In addition, K13/K19 do not prevent the interaction KRASG12V/CRAF RBD in vitro (Fig. 6b), indicating that the conformational change of the switch 2 is not the main driver of KRAS function inhibition in cells. The mechanism by which K13 and K19 inhibit KRAS function is complex. KRAS dimerisation occurs through the α3–α4 and/or α4–α5 interfaces that are dimerisation interfaces of KRAS14–17. However, only the α4–α5 has been functionally tested in cells11–13. We now show that targeting α3–α4 interface also leads to KRAS function inhibition. The mechanism underlying this inhibition could rely on inhibition of RAF activation as described with a monobody12. The DARPin binding may also promote a certain degree of membrane occlusion of the effect-binding region of KRAS and impede the binding of effectors on the switch regions32. Nevertheless, because the GEF SOS contacts the α3 region of RAS29 (as well as switch 1 and 2), K13 and K19 presumably also impede KRAS nucleotide exchange by engaging the α3–α4 site. Therefore, it adds another level of complexity in the interpretation of the mechanisms responsible for KRAS inhibition. We conclude that K13/K19 can affect KRAS function by at least two different mechanisms, including nucleotide exchange and RAS dimerisation inhibition.

None of the mechanisms above would explain why DARPinS K13 and K19 disrupt the binding of K27 to KRASWT in a BRET competition assay in cells (Supplementary Fig. 11a). This interaction should not rely on RAS dimerisation, since K27 interacts predominantly on the switch 1 of KRAS–GDP and no steric clash appears visible between K27 and K13 or K19 (Supplementary Fig. 11b, c). Therefore, the mechanism of inhibition of KRAS by K13 and K19 seems more complex than only the prevention of nucleotide exchange and KRAS dimerisation. While K27 only binds KRAS–GDP, K13/K19 also interact and lock down GTP-bound KRAS (Fig. 6c; Supplementary Fig. 1a). Hence, K13/K19 could inhibit the GTPase-activating proteins (GAP) binding on KRAS. The superimposition of the structures of two GAP proteins (p120 RAS GAP, PDB 1WQ1 and NF1, PDB 1NF1) with the structures of KRASG12V–GDP/K13 (Supplementary Fig. 12a, b) and KRASG12V–GDP/K19 (Supplementary Fig. 12c, d) shows that the amino terminal end of these DARPinS would overlap with the bound GAP proteins, suggesting that K13/K19 may be GAP inhibitors. Even though a putative GAP inhibitory mechanism could explain the inhibition of KRASWT/K27 interaction by K13/K19, it is not the mechanism that prevails in either mutant KRAS or KRASWT cancer cells. Indeed, RAS signalling is decreased in HCT116 cells (bearing both mutant and WT KRAS alleles), and is not increased in MCF-7 cells (homozygous for KRASWT) when K13/K19 are expressed (Fig. 5). Nevertheless, HT1080 cells (bearing NRASQ61K and KRASWT alleles) expressing K19 present an increased level of pERK and pMEK signals (Fig. 5). This result could be assigned to the inhibition of the GAP binding on KRASWT by K19. However, this effect is not observed in T24 cells (bearing HRASG12V and KRASWT alleles), suggesting this increase of signalling might be cell line dependent. Therefore, the GAP inhibitory effect on RAS downstream signalling could be balanced by the dimerisation inhibition of KRASWT and/or by compensatory mechanisms involving for instance the two other RAS isoforms33 and explaining why RAS signalling is not always affected in KRASWT cancer cells, such as MCF-7.

The therapeutic use of these inhibitors with an intracellular protein such as KRAS is currently limited, but this work has identified an allosteric site on KRAS and the different inhibitory mechanisms involved in targeting this interface. Hence, the α3/loop 7/α4 region could be targeted for inhibition via alternative approaches. In future studies, the DARPinS K13 and K19 could be used as surrogates to select small molecules targeting the α3/loop.

![Fig. 7 K13/K19 selectively prevent KRAS dimerisation. BRET competition assays with homodimerisation of (a) KRASG12V, (b) of NRASQ61H and (c) of HRASG12V using DARPinS K13 or K19 as competitors and DARPin E3.5 as non-binding negative control. Statistical analyses in a–c were performed using a one-way ANOVA followed by Dunnett’s post-hoc tests (**P < 0.01, ***P < 0.001, ****P < 0.0001). Each experiment was performed four times (a–c). Error bars are mean ± SD of biological repeats. a–c Source data are provided as a Source Data file](https://doi.org/10.1038/s41467-019-10419-2-2)
7a/e allosteric interface, especially around the KRAS selective residue H95. The surrogate method has been successfully used previously to isolate RAS-binding compounds using an anti-RAS intracellular single-domain antibody fragment. Furthermore, an in silico study showed a potential small molecule-binding site on that interface. This would be a promising way to isolate KRAS selective small inhibitors for the treatment of KRAS mutant cancers.

**Methods**

Isolation of DARPin K13 and K19. DARPin were isolated from a phage display library by phasing selections on biotinylated KRASG12V (1–166) pre-bound to streptavidin magnetic beads, as previously described. DARPin K19 was isolated from a first round phage display output that was further selected against KRASG12V (1–166) by ribosome display and error prone mutagenesis, using methods described by Groves et al.

To convert a DARPin phage display library into a format compatible with ribosome display, primer DARPin RD1 (5’-AGACCCAACGTTTCCCTCTAGAGTTAATTTTGTTTAACTTTAAGAAGGAGATATAT-3’) was used in place of primer SDCAT-DP75. For characterisation, DARPin were sub-cloned to the PET16b vector and expressed cytoplasmically in BL21 (DE3) Escherichia coli (New England Biolabs). A list of the primers used in this study is shown in Supplementary Table 3. Following lysis in BugBuster plus Benzonase (EMD Millipore), DARpins were purified to homogeneity using nickel-chelate chromatography, followed by size exclusion chromatography to provide a monomeric protein in PBS (pH 6.5).

**KRAS expression and purification.** The human KRAS gene sequence (residues 1–166, Isolfilm 2B, P01116-2) containing the G12V substitution was cloned into a PET28a vector with a N-terminal His tag. KRASG12V was expressed in BL21 (DE3) Escherichia coli (New England Biolabs) and cell pellets resuspended in 50 mM HEPES (pH 7.4), 100 mM NaCl, 20 mM Imidazole, 2 mM TCEP and 2 mM MgCl2. Following sonication, the sample was centrifuged at 20,000 rpm and proteins purified from the supernatant by Ni-NTA chromatography on a 5-ml HiTrap column (GE Life Sciences, cat # 17-5248-02). Following elution in 50 mM HEPES (pH 7.4), 500 mM NaCl, 400 mM Imidazole, 2 mM TCEP and 2 mM MgCl2, samples were further purified by size exclusion chromatography on a Superdex 75 column. Final buffer composition was 50 mM HEPES (pH 7.4), 100 mM NaCl, 2 mM MgSO4. KRASG12V was biotinylated via an Avi tag using BirA enzyme and then exchanged with either GDP or GTP (Son of Sevenless). The GTPγS had a tendency to be hydrolysed to GDP on storage, so the levels were determined by electrospray mass spectroscopy.

**X-ray structure.** DARpins K13 and K19 were mixed with KRASG12V-GDP in equimolar ratio with a final concentration of 16 mg/mL and incubated on ice for 30 min prior to crystallisation screening at 277 and 293 K. Hits were observed in 3.1. Binding was monitored by 1H-15N 2D TROSY (transverse relaxation optimized spectroscopy) experiments and parameters from Bruker library. Uniformly 15-N-labelled spectra were collected at 298 K on a Bruker 800 MHz, Avance III spectrometer, using MSE data collected for the triplicate undeuterated control samples. The resultant peptide list was imported into DynamX where peptides were assigned in DynamX was performed using the standard parameters, but all peptide-level differences in deuterium uptake were excluded from analyses due to rapid back exchange. Significant differences were determined by performing a Student’s t test using the pooled standard deviation or peptide standard deviation, whichever was the greater. The p-values were adjusted for false discovery using the Benjamini-Hochberg procedure. Differences with p < 0.05 were considered significant.

**Peptide-level differences in deuteration uptake are calculated according to the Eq. (1):**

$$\Delta \text{uptake}_{i} = \text{uptake}_{i}^{\text{apo}} - \text{uptake}_{i}^{\text{rc}}$$

**Difference in deuteration uptake for peptide i in state of interest uptakeapo: Deuteration uptake for peptide i apo state**

**Deuteration uptake for peptide i in state of interest uptakerc:**

The mean residue-level differences were calculated using the Eq. (2):

$$\text{res}_{i} = \frac{1}{N_{\text{peps}}} \sum_{j=1}^{N_{\text{peps}}} \Delta \text{amid}_{j}$$

**res: mean difference for residue j**

**N_{peps}: number of peptides containing residue j**

**p:** deuterium uptake difference for peptide i that contains residue j amid: number of exchangeable residues within peptide i

**KRAS biochemical coupled assay and KRAS/CRAF RBD assay.** The KRAS biochemical coupled assay was performed as previously described. Briefly, biotinylated KRASG12V was pre-incubated with streptavidin–Europium chelate to form a complex, and in a separate reaction, CRAF RBD-GST (glutathione-S-transferase) was pre-incubated with anti-GST-XL665 to form a second complex. Test samples were incubated with the streptavidin-KRAS complex for 15 min before addition of GTPγS and SOS (to initiate nucleotide exchange) and the CRAF RBD-anti-GST complex. Final concentrations added were 2 mM biotinylated KRAS, 37.5 μg/mL GTPγS and 20 mg/mL SOS. In addition, the buffer for the CRAF RBD: anti-GST complex contained 0.1 mg/mL BSA and 0.1 M potassium fluoride. After 1 h incubation, fluorescent resonance energy transfer (FRET) was measured on an Environ plate reader at emission wavelengths 620 and 665 nm. The ratio of these values was fitted using non-linear regression in the application Prism (GraphPad Software). A assay to measure only the inhibition of KRAS/CRAF RBD was like the coupled assay above, with the exception that test samples were incubated after the completion of the nucleotide exchange step.
medium complemented with 4% FBS. Cells were incubated for an additional 20–24 h at 37 °C before the BRET assay reading. A step-by-step protocol is described elsewhere.  

**BRET2 measurements.** BRET2 signal was determined immediately after addition of coelenterazine 400a substrate (10 μM final) to cells (Cayman Chemicals), using an Envision instrument (2103 Multilabel Reader, PerkinElmer) with the BRET2 Dual Emission optical module (515–30 and 410–80 nm; PerkinElmer). The total GFP fluorescence was detected with excitation and emission peaks set at 405 and 515 nm, respectively. The total mCherry fluorescence was detected with excitation and emission peaks set at 530 and 615 nm, respectively. The total RLuc8 luminescence was measured with the luminescence 400–700 nm-wavelength filter.

The BRET signal or BRET ratio corresponds to the light emitted by the GFP2 acceptor constructs (515–30 nm) upon addition of coelenterazine 400a divided by the light emitted by the RLuc8 donor constructs (410–80 nm). The background signal is subtracted from that BRET ratio using the donor-only negative control, where only the RLuc8 plasmid is transfected into the cells. The normalised BRET ratio is the BRET ratio normalised to a negative control (DARPIn control) during a competition assay. The total GFP2, mCherry and RLuc8 signals were used to control the protein expression level from each plasmid.

**Immunoprecipitation assay.** HEK293T cells were transfected 48 h in duplicate (two wells per condition) with pEF-3xFLAG-RAS and pEF-DARPIn-GFP2 plasmids. Cells were washed once with PBS and lysed in the immunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 10 mM MgCl2, 1% glycerol and 0.5% NP-40) supplemented with protease inhibitors (Sigma) and phosphatase inhibitors (Thermo-Fisher) for 20 min. Lysates were centrifuged for 15 min, and the supernatant incubated with protein G magnetic beads (Life Technologies) and anti-FLAG antibody (Sigma). The complexes were incubated 4 h at 4 °C on a wheel. Beads were washed twice with the IP buffer, the bound proteins were eluted with 1× loading buffer and resolved on 12.5% SDS-PAGE.

**Western blot analysis.** Cells were washed once with PBS and lysed in SDS-Tris buffer (1% SDS, 10 mM Tris-HCl pH 7.4) supplemented with protease inhibitors (Sigma) and phosphatase inhibitors (Thermo-Fisher). Cell lysates were sonicated on Ice and clarified by centrifugation. Equal amounts of protein were loaded for 10 or 15% SDS-PAGE and subsequently transferred onto a PVDF membrane (GE). The membrane was blocked either with 10% non-fat milk (Sigma) or 10% BSA (Sigma) in TBS-0.1% Tween20 and incubated overnight with primary antibody at 4 °C. After washing, the membrane was incubated with HRP-conjugated secondary antibody for 1 h at room temperature (RT, 25 °C). The membrane was washed with TBS-0.1% Tween and developed using Pierce ECL Western Blotting Substrate (Thermo-Fisher) and CL-XPosure films (Thermo-Fisher).

Primary antibodies include anti-phospho-p44/p42 MAPK (ERK) (E10212, CST, Cat#9101 S), anti-p44/p42 MAPK (total ERK) (E10212, CST, Cat#9102 S), anti-phospho-MEK1/2 (E10212, CST, Cat#9154 S), anti-MEK1/2 (E10212, CST, Cat#9101 S), anti-p44/42 MAPK (total ERK) (E10212, CST, Cat#4548 S), anti-phospho-AKT T308 (E10212, CST, Cat#4056 S), anti-AKT (E10212, CST, Cat#4064 S), anti-p44/42 MAPK (total ERK) (E10212, CST, Cat#4068 S), anti-PI3K γ315, RALGDS (E10212, CST, Cat#4064 S), anti-PI3K γ315, RALGDS (E10212, CST, Cat#4064 S), and anti-AKT (E10212, CST, Cat#4064 S). Secondary antibodies include anti-mouse IgG HRP-linked (CST), anti-rabbit IgG HRP-linked (CST) and anti-goat IgG HRP-linked (Santa Cruz Biotechnologies).  

**Confoal microscopy.** HEK293T cells were seeded on coverslips and transfected 24 h with pEF-GFP2–KRASG12V construct. Coverslips were washed in PBS, fixed 10 min in 4% paraformaldehyde and washed twice in PBS. Then the coverslips were mounted with DAPI Fluormount-G overnight. Slides were analysed using a Zeiss 880 Inverted Confoal Microscope with a x63 objective. Confocal images were analysed with ImageJ software.

**Quantification and statistical analysis.** The experiments were performed using Prism 7.0c (GraphPad Software). The data are typically presented as mean ± SD or SEM as specified in the figure legends. Statistical analyses were performed with a one-way ANOVA followed by Dunnett’s post hoc tests, unless otherwise indicated in the figure legends. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** Structure files and coordinates have been deposited to PDB under these accession numbers: 6H46 and 6H47. All relevant data are within the paper and its Supplementary
Information file and the Source data file. The source data underlying Figs. 3c, 4a–d, 5a, b, 7a–c and Supplementary Figs. 7b, 9a, b, 10c and 11a are provided as a Source Data file. Additional data supporting the conclusions are available from the corresponding author on reasonable request.

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Additional information

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Competing interests: S.L., I.D., J.B., K.E., C.S., P.K.-Z., N. Barrett, R. Marwood, J.W., J.T., R.O., C.P. and R. Minter are employees of the AstraZeneca Group and may have stock/options in AstraZeneca. N. Bery, A.M. and T.H.R. have no conflicts of interest to declare.

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