Mutations in the α4-α5 allosteric lobe of RAS do not significantly impair RAS signaling or self-association

Michael Whaby1,2,4, Lauren Wallon3,4, Megan Mazzei1,2, Imran Khan1,2,4, Kai Wen Teng5, Shohei Koide1,5,* and John P. O’Bryan1,2,4,*

From the 1Department of Cell and Molecular Pharmacology & Experimental Therapeutics, and 2Holmes Cancer Center, Medical University of South Carolina, Charleston, South Carolina, USA; 3Department of Biochemistry and Molecular Pharmacology, New York University Grossman School of Medicine, New York, New York, USA; 4Ralph H. Johnson VA Medical Center, Charleston, South Carolina, USA; 5Perlmutter Cancer Center, New York University Langone Health, New York, New York, USA

Edited by Donita Brady

Mutations in one of the three RAS genes (HRAS, KRAS, and NRAS) are present in nearly 20% of all human cancers. These mutations shift RAS to the GTP-loaded active state due to impairment in the intrinsic GTPase activity and disruption of GAP-mediated GTP hydrolysis, resulting in constitutive activation of effectors such as RAF. Because activation of RAF involves dimerization, RAS dimerization has been proposed as an important step in RAS-mediated activation of effectors. The α4-α5 allosteric lobe of RAS has been proposed as a RAS dimerization interface. Indeed, the NS1 monobody, which binds the α4-α5 region of the RAS G domain, inhibits RAS-dependent signaling and transformation as well as RAS nanoclustering at the plasma membrane. Although these results are consistent with a model in which the G domain dimerizes through the α4-α5 region, the isolated G domain of RAS lacks intrinsic dimerization capacity. Furthermore, prior studies analyzing α4-α5 point mutations have reported mixed effects on RAS function. Here, we evaluated the activity of a panel of single amino acid substitutions in the α4-α5 region implicated in RAS dimerization. We found that these proposed “dimerization-disrupting” mutations do not significantly impair self-association, signaling, or transformation of oncogenic RAS. These results are consistent with a model in which activated RAS protomers cluster in close proximity to promote the dimerization of their associated effector proteins (e.g., RAF) without physically associating into dimers mediated by specific molecular interactions. Our findings suggest the need for a nonconventional approach to developing therapeutics targeting the α4-α5 region.

RAS GTPases are important mediators of intracellular signaling cascades that regulate cell proliferation and survival (1, 2). The three RAS genes (HRAS, KRAS, and NRAS) encode four different protein isoforms: HRAS, splice variants KRAS4A and KRAS4B, and NRAS. Each of the RAS isoforms cycle through active (RAS-GTP) and inactive (RAS-GDP and apo-RAS) states that are tightly regulated by GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (3). Prenylation and palmitoylation of cysteine exchange factors of the C-terminal hypervariable region (HVR) of RAS localize it to the inner leaflet of the cell membrane where RAS is typically activated in response to growth factor receptor–mediated stimulation (4). However, activating mutations of RAS stabilize the active RAS-GTP state even in the absence of upstream stimulation (1). Notably, nearly 20% of all human cancers harbor such RAS mutations, making RAS the most frequently mutated oncogene as well as a valuable target for cancer therapy (5).

Clinically available inhibitors of RAS have been elusive. However, the recent FDA approval of the KRASG12C inhibitor, sotorasib, illustrates the feasibility of pharmacological inhibition of KRAS (6, 7). Unfortunately, KRASG12C represents only a fraction of oncogenic RAS mutations in human cancers (8, 9), presenting a critical need for alternative RAS inhibitors. Due to the picomolar affinity of RAS for guanine nucleotides, targeting the nucleotide binding pocket has been considered pharmacologically problematic (10), although recent work provides support for the possibility of targeting the nucleotide-free state of RAS (11). In the absence of direct RAS inhibitors, interfering with RAS membrane localization has been explored as an alternative therapeutic approach (12). However, the ability of KRAS to be alternatively prenylated in response to farnesyl transferase inhibitor treatment rendered this approach ineffective for KRAS-mutant cancers (13), although these inhibitors have shown some efficacy in HRAS-mutant malignancies (14).

More recent studies have highlighted the possibility of inhibiting RAS nanoclustering as a potential approach to inhibit RAS-mediated signaling and biological activity both in vitro and in vivo (15–19). Utilizing monobody technology (20), we previously reported that the NS1 monobody specifically binds the α4-α5 allosteric lobe of HRAS and KRAS, but not NRAS, and inhibits RAS dimers/nanoclusters (15). Additionally, the K13 and K19 DARPins inhibit KRAS via binding of the α3-α4 allosteric lobe and disrupting RAS dimer/


a4-a5 mutations do not impair oncogenic RAS signaling

The importance of RAS dimers/nanoclusters was further highlighted by the endogenous RAS antagonist, DIRAS3, which inhibits RAS nanoclusters through binding of a5 region of RAS (21). These results are consistent with the reported presence of dimers of RAS in a number of crystal structures of RAS (15, 22–24), the GTP-dependent dimerization of the KRAS G domain (25), and the observation that artificial dimerization of the isolated G domain of RAS is sufficient to activate RAF in vitro (26) and increase mitogen-activated protein kinase (MAPK) signaling in cells (27). In contrast, disruption of these higher-order RAS assemblies inhibits downstream signaling and biological function in vivo (15–19).

Although these examples implicate RAS dimerization as an on-pathway event in RAS-mediated signaling and provide a rationale for drug discovery efforts aimed at development of RAS dimerization inhibitors, the importance of RAS dimerization remains a point of much debate. While active RAS stimulates the dimerization and activation of RAF kinases in the MAPK pathway (28), the G domain of HRAS lacks the propensity to dimerize in solution (23). Furthermore, the isolated HVR of KRAS, devoid of the G domain, is sufficient to drive dimerization of a fluorescent protein–HRV fusion protein in cells, suggesting that KRAS G domain is dispensable for dimerization (27). In light of these studies, many attempts have been made to identify binding interfaces that facilitate higher-order RAS assemblies. Three major RAS dimerization interfaces have been proposed: the a3–a4 interface, the a4–a5 interface, and the β-sheet interface (29). Experimental evidence supporting the validity of these dimerization interfaces, however, has been conflicting. Although RAS was inhibited by the NS1 monobody, point mutations within the NS1-binding interface of HRAS predicted to disrupt dimerization (e.g., R135A, D154Q, R161D) did not impair downstream MAPK pathway activation (15). Similarly, a recent study reported that the KRASG154Q mutation did not affect RAS association in cells (30). In contrast, Ambrogio et al. provided evidence that mutation of D154Q or R161E decreased KRAS association (as measured by FRET), CRAF–BRAF association, and KRAS-mediated signaling and tumor formation in vivo (24). More recent work has suggested that RAF RBD engagement promotes RAS dimerization through the a4–a5 region although mutations in this region did not affect RAS dimerization (31).

Because of the contradictory results with these studies, we sought to characterize a panel of oncogenic HRAS, KRAS, and NRAS mutants to assess the effects of a4–a5 mutations on oncogenic RAS function. These mutations were selected based on previous reports of their importance in RAS dimer formation (15, 22, 25, 32–34). Here, we show that mutations in the a4–a5 allosteric lobe do not affect RAS association in cells. In addition, these mutants did not impair MAPK activation compared with the parental oncogenic RAS. Although downstream signaling was unaffected by these mutations, we observed isoform-specific differences in the transforming activity of selected mutants. Overall, our results are consistent with a model in which RAS protomers associate in close proximity to promote effector dimerization (e.g., RAF) without formation of molecularly defined RAS dimers. Further, we propose that the ability of selected biologics such as NS1 monobody and K13/K19 DARPins to perturb RAS function is due to steric hinderance of RAS rather than disruption of bona fide dimers. This study provides further insights into RAS oligomerization and should inform efforts in developing therapeutics directed at the a4–a5 allosteric lobe of RAS.

Results

Mutations in the a4-a5 region of KRAS do not impair oncogenic activity

Given the contradictory reports on the importance of dimerization in RAS function, we tested whether mutations in the proposed dimerization interface (Fig. 1, A and B; termed a4–a5 mutants hereafter) impaired MAPK pathway activation relative to the parental oncogenic KRAS mutant. To reduce overexpression artifacts, we determined the appropriate amounts of K/H/NRAS DNA that yielded protein expression and ERK phosphorylation below the point of saturation for the signaling assays (Figs. S1–S4). Surprisingly, there were no significant differences in MAPK activation between the KRASG12V a4–a5 mutants and the parental oncogenic KRASG12V with the exception of KRASG12V/K147D, a mutation that has been shown to decrease RAS-GTP levels (Figs. 2, A and B, and S5) (35, 36).

Next, we analyzed CRAF–BRAF association in cells expressing KRASG12V versus KRASG12V harboring a4–a5 mutants, given the well-established role of RAF dimerization in MAPK signaling (37, 38). In addition, a previous study demonstrated that D154Q and R161D KRAS mutants decreased CRAF–BRAF heterodimers (24). In agreement with the results from the MAPK signaling assays described above, there was no significant impairment in CRAF–BRAF interaction in cells expressing the KRAS a4–a5 mutants compared with parental KRASG12V (Fig. 2, C and D). These data indicate that the a4–a5 mutations do not impair the activation of the canonical RAS/MAPK pathway mediated by KRASG12V.

Lastly, we performed transformation assays in NIH/3T3 cells transfected with KRASG12V or the KRASG12V a4–a5 mutants (Fig. 2, E and F). Consistent with the signaling data, the KRASG12V a4–a5 mutants retained the ability to transform cells as well as parental KRASG12V. Taken together, these results suggest that mutations of amino acid residues proposed to be critical for KRAS–KRAS self-association do not significantly impair the signaling or transforming properties of oncogenic KRAS.

Mutations in the a4-a5 allosteric lobe do not affect KRAS association in cells

Next, we addressed whether these a4–a5 mutations affected RAS–RAS association in cells. We employed Live-Cell NanoLuc Binary Technology (NanoBit), a protein–protein interaction (PPI) detection system where one protein partner is tagged with an 11-amino acid peptide (SmBiT), while the other protein partner is tagged with a 17.6 kDa NanoLuc fragment.
When expressed in cells, PPIs between the two protein partners allows for complementation of the SmBiT and LgBiT tags to generate a luminescent signal upon substrate addition.

To avoid potential interference of endogenous RAS proteins with the SmBiT/LgBiT-tagged KRAS protein partners, we performed these assays in RAS-less MEFs transformed with BRAFV600E, a cell line that lacks all 3 RAS isoforms (39). To account for variations between transfections, cells were lysed after measuring the live-cell luminescence (Fig. S8A), and LgBiT-tagged proteins were quantified using HiBiT, an 11 amino acid peptide with high affinity (K_D = 0.7 nM) for the LgBiT peptide. The HiBiT peptide out-competes the SmBiT peptide for LgBiT binding while still generating luminescent signal, allowing for a fast and sensitive method to quantify total LgBiT peptide levels in cells (Figs. 3A and S8B).

When coexpressed in cells, SmBiT-KRAS G12V and LgBiT-KRASG12V reconstituted luciferase activity (Fig. 3, A and B). In contrast, coexpression of SmBiT-KRASG12V with EGFRLgBiT, also a membrane-localized protein which served as a negative control, generated a weak luminescent signal (Fig. 3B). The introduction of the α4-α5 mutations to LgBiT-

**Figure 1. A model of an α4-α5 dimer of KRAS and the locations of residues tested in this study.** A, The model was built by fitting the structure of KRAS4B•GMPPNP (residues 1–169; PDB ID 6VC8; (49)) onto the crystallographic dimer of HRAS•GMPPNP (residues 1–166; PDB ID 5P21; (50)). The Switch I and II regions are colored in tan. The α4 and α5 helices in protomer 1 are labeled, and those in protomer 2, that is, the symmetry-related copy, are labeled as α4' and α5'. The residues subjected to mutational studies are shown in cyan and labeled for protomer 1 and shown in blue for protomer 2. The side chains of E49, K128, and R135 are disordered in the 6VC8 model and thus not depicted. B, RAS α4-α5 mutants included in this study.

**Figure 2. Mutations in the α4-α5 allosteric lobe of KRAS do not impair oncogenic RAS signaling.** A, ERK/MAPK activity assay in HEK 293 cells cotransfected with HA-tagged KRASG12V mutants and MYC-tagged ERK. B, Normalized pERK signal from panel (A). C, Immunoprecipitation (IP) of endogenous CRAF in HEK 293 cells transfected with KRASG12V mutants. D, Normalized BRAF signal from the CRAF IP in panel (C). E, NIH/3T3 transformation assay in cells transfected with KRASG12V mutants. F, Normalized foci number from the NIH/3T3 transformation assays in panel (E). All experiments were repeated at least three times (n = 3) and results quantified using Welch's t test; error bars representing SEM (***p < 0.0005, **p < 0.005, and *p < 0.05). MAPK, mitogen-activated protein kinase.
KRAS<sup>G12V</sup> resulted in no significant decreases in luciferase activity (Fig. 3B). These results are consistent with the results from signaling assays (Fig. 2), indicating that not only do the α4-α5 mutations have little to no effect on KRAS signaling and biology, they also do not impair RAS–RAS interactions in cells.

**RAS α4-α5 mutations are still susceptible to inhibition by the NS1 monobody**

NS1 inhibits oncogenic RAS-mediated signaling, biological transformation, and tumor formation (15, 18, 19) and disrupts higher-order RAS associations at the plasma membrane (15). Through binding of the α4-α5 region of RAS, NS1 allosterically inhibits RAS function irrespective of its nucleotide state (15, 16). We hypothesized that if the α4-α5 mutations decreased RAS–RAS interaction in cells, then NS1 would have less of an inhibitory effect on these mutants compared with the parental RAS oncogenic mutants. As illustrated in Figures S6 and S7, α4-α5 mutations in KRAS<sup>G12D</sup> did not impact NS1 inhibition. Furthermore, coexpression of NS1 effectively inhibited downstream ERK phosphorylation in cells expressing KRAS<sup>G12D</sup> and KRAS<sup>G12D</sup> α4-α5 mutants (Figs. 4A and S9). The inhibitory effect of NS1 was highly specific as KRAS<sup>G12D/R135E</sup>, which does not bind NS1 (15), was refractory to inhibition by NS1 (Figs. 4A and S9). These results further support the notion that these α4-α5 mutations are not sufficient to impair RAS association or oncogenic RAS-mediated signaling in cells.

**HRAS and NRAS α4-α5 mutants exhibit isoform-specific biological properties**

We then examined whether α4-α5 mutations in the other RAS isoforms had similar effects on their signaling and biological properties. In contrast to previously reported results with KRAS<sup>D154Q</sup> (24), we reported that mutations at D154 or R161 of HRAS<sup>G12V</sup> had no effect on ERK-MAPK activation (15), which might suggest isoform-specific effects of these mutations. Consistent with prior findings (15, 30) and the results with KRAS<sup>G12V</sup> (Fig. 2), mutations at D154 or R161 had no effect on either HRAS<sup>G12V</sup> or NRAS<sup>G12V</sup> signaling (Figs. 5, A–D and S10).

Next, we assessed the impact of these mutations on the biological activity of H/NRAS<sup>G12V</sup>. Whereas the KRAS<sup>G12V</sup> mutants showed no significant differences in foci formation (Fig. 2, E and F), all of the HRAS<sup>G12V</sup> α4-α5 mutants (Fig. 5, E and F) and one of the NRAS<sup>G12V</sup> α4-α5 mutants (R161D) (Fig. 5, G and H) resulted in significantly fewer foci than those of the parental control. Together, these data show that mutations in the α4-α5 allosteric lobe of all three RAS isoforms have no effect on ERK-MAPK signaling but impair the transforming activity of HRAS<sup>G12V</sup> and to a lesser extent NRAS<sup>G12V</sup>.
activated the MAPK pathway (27), while inhibition of RAS clusters at the plasma membrane is associated with inhibition of the MAPK pathway (15, 21). Although there is still debate surrounding the exact mechanism of RAS nanoclustering at the plasma membrane, the α4-α5 region of RAS has been proposed in several studies to be an important interface contributing several stabilizing interactions to facilitate formation of RAS dimers (15, 22, 25, 32–34, 41). Based on these

**Figure 4. RAS α4-α5 mutants are still susceptible to inhibition by the NS1 monobody.** A, ERK/MAPK signaling assay in HEK 293 cells cotransfected with indicated HA-tagged KRASG12D mutants and FLAG-tagged NS1 or a negative control monobody (Mb (neg)). Results are representative of one of three biological replicates. Graph represents the relative pERK levels. The mean and sd (n = 3) for the normalized pERK/ERK in the NS1 compared to Mb (neg) sample is shown. Dotted line at 1 represent pERK levels in Mb (neg) samples. All p values were generated using an unpaired Student’s t test (**p < 0.005, ***p < 0.0005). MAPK, mitogen-activated protein kinase.

**Figure 5. HRAS and NRAS α4-α5 mutants exhibit isoform-specific biological properties.** A, ERK/MAPK activity assay in HEK 293 cells transfected with indicated HA-tagged HRASG12V mutants. B, Normalized pERK signal from panel (A). C, ERK/MAPK activity assay in HEK 293 cells transfected with HA-tagged NRASG12V mutants. D, Normalized pERK signal from panel (C). E, NIH/3T3 transformation assay in cells transfected with HRASG12V mutants. F, Normalized foci number from the NIH/3T3 transformation assays in panel (E). G, NIH/3T3 transformation assay in cells transfected with NRASG12V mutants. H, normalized foci number from the NIH/3T3 transformation assays in panel (E). All experiments were repeated at least three times (n = 3) and results quantified using Welch’s t test; error bars representing SEM (***p < 0.0005, **p < 0.005, and *p < 0.05). MAPK, mitogen-activated protein kinase.
α4-α5 mutations do not impair oncogenic RAS signaling

Our results are consistent with a model in which RAS promaters rely on proximity, but not direct association with one another to form a signaling-competent complex (Fig. 6). The required proximity may be in the form of loosely associated nanoclusters where RAS promoters are close enough to promote RAF dimerization but do not require well-defined interactions between amino acid side chains of residues within the α4-α5 allosteric lobe. Disruption of these nanoclusters may require larger molecules, such as NS1 or DIRAS3, which may reduce the density of RAS on the membrane surface and/or distort the RAS–RAF complex into an inactive conformation (46). In contrast, point mutations of specific amino acid residues on the α4-α5 lobe of RAS do not appear sufficient to disrupt the interactions necessary for downstream pathway activation. The implications of these results for drug design are that smaller molecules targeting the α4-α5 allosteric lobe may be insufficient to impair downstream pathway activation. Instead, an approach to bring a large molecule to the α4-α5 region utilizing “glue” compounds (47) may be required to exploit this vulnerability of RAS.

Experimental procedures

Cell culture and cloning

Freshly thawed HEK 293 (MUSC Tissue Culture Facility) and NIH/3T3 (National Institutes of Health) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Corning) supplemented with 10% fetal bovine serum (FBS) or 10% calf serum, respectively. RAS-less MEFs (BRAFV600E) were obtained from the National Cancer Institute and maintained in DMEM supplemented with 10% FBS and 4 μg/ml blasticidin. RAS α4-α5 mutants were generated via site-directed mutagenesis using pCGN-HA-RASG12V or pCGN-HA-RASG12D as templates for each isoform. Primers used to generate RAS α4-α5 mutants are listed in Table 1. All monobodies were subcloned into CMV-driven expression vectors containing an mCherry-tag followed by a FLAG-tag on the N-terminus. KRASG12V was subcloned into a high-expression, CMV-driven vector downstream of the SmBiT cDNA sequence. Similarly, KRASG12V and the α4-α5 mutants were cloned downstream of LgBiT-containing vectors; however, these vectors were low-expression, HSV-driven vectors. Lastly, EGFR-LgBiT clone was in a high-expression, CMV-driven vector. All NanoBiT vectors were provided by Matt Robers and Dr Jim Vasta (Promega).

Transfections and cell signaling assays

Transfections and cell signaling assays were performed as previously described (11, 48). Briefly, HEK 293 cells were
transfected with HA-tagged RAS using polyethylenimine (PEI). Typically, we transfected cells using 3 μl of PEI for every 1 μg of DNA. When indicated, HA-tagged RAS was cotransfected with MYC-tagged ERK for signaling assays. Transfected cells were incubated for 30 h in complete media (DMEM with 10% FBS), then serum-starved overnight. MYC-tagged ERK was immunoprecipitated from the cell lysates using α-MYC antibody (Millipore-Sigma), then ERK and pERK levels were analyzed via Western Blot using α-ERK (Cell Signaling Technology) and α-pERK (Cell Signaling Technology) antibodies. ERK and pERK protein levels were quantified using Image Studio Lite (Ver 5.2) software. pERK/ERK ratio was determined for each mutant and normalized to the parental oncogenic RAS mutant. Each experiment was performed three times (n = 3).

To analyze CRAF–BRAF association, HEK 293 cells were transfected with the indicated RAS mutants using the same conditions as described above. After cell lysates were collected, a coimmunoprecipitation was done by pulling down endogenous CRAF using α-CRAF (BD Biosciences) antibody and probing for CRAF and BRAF via Western Blot with α-CRAF (BD Biosciences) and α-BRAF (Santa Cruz) antibodies. BRAF/CRAF ratio was determined for each mutant and all values were normalized to the parental oncogenic RAS mutant. Each experiment was performed three times (n = 3).

For signaling assays performed with KRASG12D and the NS1 monobody, 1 × 10⁶ HEK293T cells were cultured 24 h before transfection on a 6-well plate using DMEM supplemented with 10% FBS. Cells that were between 70 and 90% confluent were then serum starved and transfected with either KRAS variants

### Table 1

| α4–α5 mutation | Forward Primer (5’ to 3’) | Reverse Primer (5’ to 3’) |
|----------------|---------------------------|---------------------------|
| KRASG12V       |                           |                           |
| K128E          | CCAGAACAGTAGAACAGAGAGCTCACGAGACTTAGCA | TGCTAAGTCCTGAGGCTGTTCGCTTCTACGTGTTCTG |
| Q131E          | CAGTAGACAAAAACACGGCTGAGGACCGTACGAGTA | ACTCTTGCTGAGAAGCTACGCTGAGGCTG |
| R135E          | CAGGCTCGAGGAGAAGAAGCTGAGGACTTAGCA | AAAAGAATTCCAACTTTCTGCTACTGAGGCTG |
| K147D          | TTATTTAAGAATGACAGAAGAGGCTACGAGTA | TCAAGACCCCTTGCTGGTCTGCTGAGGCTG |
| D154Q          | ACCAGACAGGGCTGAGGACTTAGCA | AACTAAATGTGAGGGCTCAGTACAGMCCCTGCTGGT |
| R154R          | ACAAGACAGGGCTGAGGACTTAGCA | AACTAAATGTGAGGGCTCAGTACAGMCCCTGCTGGT |
| R161R          | GCCCTCTACCTATATTAGTGGAAATTCGAACACTAAAA | TTTAATGTGAGGGCTCAGTACAGMCCCTGCTGGT |
| HRASG12V       |                           |                           |
| D154Q          | ACCCGGCAGGGAGGCTGAGGACTTAGCA | CACCAACGTTGCTGGTCTGCTGAGGCTG |
| D154R          | ACCCGGCAGGGAGGCTGAGGACTTAGCA | CACCAACGTTGCTGGTCTGCTGAGGCTG |
| R161R          | GATGCCCTTCTACCTATATTAGTGGAAATTCGAACACTAAAA | TTTAATGTGAGGGCTCAGTACAGMCCCTGCTGGT |
| NRASG12V       |                           |                           |
| D154Q          | ACCAGAACAGGGCTGAGGACTTAGCA | TACACGTTGCTGGTCTGCTGAGGCTG |
| D154R          | ACCAGAACAGGGCTGAGGACTTAGCA | TACACGTTGCTGGTCTGCTGAGGCTG |
| R161R          | GCTTTTACCTATATTAGTGGAAATTCGAACACTAAAA | TTTAATGTGAGGGCTCAGTACAGMCCCTGCTGGT |
**a4-a5 mutations do not impair oncogenic RAS signaling**

or in a 1:1 ratio with monobodies using lipofectamine according to the manufacturer’s protocol. Raw band intensity was evaluated using Image Studio Lite Version 5.2. For calculating the normalized (pERK/ERK)/HA, each band was first normalized to vinculin. For calculating the NS1/Mb (neg) ratio, pERK/ERK ratio was first calculated then used to generate the NS1/Mb (neg) ratio. Statistical analysis was performed using GraphPad Prism 9.

**Immunoblotting and antibodies**

Following experimental endpoints as described above, cell lysates were made by washing cells with PBS followed by addition of PLC lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 1.5 mM magnesium chloride, 100 mM sodium fluoride supplemented with 1 mM vanadate, 10 mg/ml leupeptin, and 10mg/ml aprotinin). Lysates were nated at 4 °C for 1 h, then centrifuged at 14K RPM for 10 min. Supernatant-containing protein was transferred to new tubes and stored at −80 °C. The Pierce BCA Protein Assay Kit (ThermoFisher) was used to quantify protein in cell lysates before Western Blot analyses. The following antibodies were used: monoclonal HA (clone 16B12, Biolegend #90154), monoclonal FLAG (Clone M2, Sigma #F1804), phospho-ERK (Thr202/Tyr204, CST #9101), total ERK (CST #9102), Vinculin (SC #73614), Anti-MYC (Clone A46, Millipore-Sigma #05–724), CRAF (BD Biosciences #610151), BRAF (Santa Cruz #sc-9002).

**NIH/3T3 transformation assays**

For NIH/3T3 transformation assays, 2.5 × 10^5 cells were split into 60 mm tissue culture plates and seeded overnight. The following day, cells were transfected with the indicated RAS mutants using PEI transfection method (48). Media on cells was changed every 2 days following transfections. Oncogenic RAS induced foci formation approximately 2 to 3 weeks following transfections, and cells were fixed and stained with 0.1% crystal violet before quantification of foci. Assays were performed three times each (n = 3).

**NanoBiT protein–protein interaction assays**

For NanoBiT PPI assays, 3.0 × 10^4 cells per well (RAS-less MEFs) were plated in a white-wall, clear-bottom 96-well plate (Thermo Scientific 165306) and incubated at 37 °C overnight. The next day, all wells were transfected with SmBiT-KRASG12V, and selected wells were transfected (technical replicates per experiment = 6) with either EGFR-LgBiT, LgBiT-KRASG12V, or LgBiT-KRASG12V α4-α5 mutants using PEI transfection method. Twenty four hours after transfection, media was aspirated from wells, and luminescence was measured using NanoGlo Live-Cell Substrate (Promega; Cat # N2012) suspended in Opti-MEM reduced serum media (Gibco; cat # 31985070). After the live-cell luminescence measurement, cells were lysed with 1.0% Triton X-100 and incubated with HiBiT peptide (0.1 μM) for 10 min on orbital shaker. Then, luminescence was measured to quantify LgBiT peptide levels. Live-cell luminescence was normalized to luminescence after HiBiT peptide addition, and all samples were normalized to SmBiT-KRASG12V/LgBiT-KRASG12V. Assays were performed three times each (n = 3).

**Statistical analysis**

All statistical analyses were performed using GraphPad Prism 9 software.

**Data availability**

All reagents in this article are available upon request and completion of an MTA with the Medical University of South Carolina and/or New York University.

**Supporting information**—This article contains supporting information.

**Acknowledgments**—We wish to thank members of the O’Bryan and Koide laboratories for comments on the article. We also thank Matt Robers and Dr Jim Vasta (Promega Corporation) for providing the NanoBiT reagents along with helpful advice on establishing the NanoBiT assay in the laboratory.

**Author contributions**—M. W., L. W., I. K., S. K., and J. P. O. conceptualization; M. W., L. W., I. K., K. W. T., S. K., and J. P. O. methodology; M. W., L. W., M. M., and I. K. investigation; M. W., L. W., I. K., S. K., and J. P. O. data interpretation; M. W., L. W., M. M., I. K., S. K., and J. P. O. writing—original draft; M. W., L. W., M. M., S. K., and J. P. O. writing—review and editing; S. K. and J. P. O. project administration; S. K. and J. P. O. resources; S. K. and J. P. O. supervision.

**Funding and additional information**—M. W. was supported by an NIH T32 (GM132055). L. W. was supported in part by T32 GM 88118-10. K. W. T. was supported in part by NIH fellowship (F32 CA225131) and ACS fellowship (PF-18-180-01-TBE). J. P. O. was supported by grants from the Department of Veterans Affairs Biomedical Laboratory Research and Development Service MERIT Award (1101BX002095), the NCI (P30 CA138313), and the NIGMS (P20 GM103542). J. P. O. and S. K. were supported by a grant from the NCI (R01CA12608). S. K. was supported by a grant from the NCI (R01 CA154356). The contents of this article do not represent the views of the U.S. Department of Veterans Affairs or the United States government. The contents of this article are solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

**Conflict of interest**—J. P. O., A. K., and S. K. are listed as inventors on a patent application on Monobodies targeting the nucleotide-free state of RAS files by the Medical University of South Carolina and New York University (No. 62/862924). K. W. T., A. K., and S. K. are listed as inventors on a patent application on mutant RAS targeting Monobodies filed by New York University (Application No. 63/121903). A. K. and S. K. are listed as inventors on issued and pending patents on Monobody technology filed by The University of Chicago (US Patent 9512199 B2 and related pending applications). S. K. was an SAB member and holds equity in and received consulting fees from Black Diamond Therapeutics and receives research funding from Black Diamond Therapeutics,
a4-a5 mutations do not impair oncogenic RAS signaling

Puretech Health, and Argex BVBA. The other authors declare no competing interests.

Abbreviations—The abbreviations used are: FBS, fetal bovine serum; GAP, GTPase activating protein; HVR, hypervariable region; MAPK, mitogen-activated protein kinase; PEI, polyethylenimine; PPI, protein–protein interaction.

References
1. Moore, A. R., Rosenberg, S. C., McCormick, F., and Malek, S. (2020) RAS-Targeted therapies: is the undruggable drugged? Nat. Rev. Drug Discov. 19, 533–552
2. Karnoub, A. E., and Weinberg, R. A. (2008) Ras oncogenes: split personality. Nat. Rev. Mol. Cell Biol. 9, 517–531
3. Khan, I., Bhrett, J. M., and O’Byran, J. P. (2020) Therapeutic targeting of RAS: new hope for drugging the "undruggable". Biochim. Biophys. Acta Mol. Cell Res. 1867, 118570
4. Simanshu, D. K., Nissley, D. V., and McCormick, F. (2017) RAS proteins and their regulators in human disease. Cell 170, 17–33
5. Bryan, J. P. (2019a) Pharmacological targeting of RAS: recent success with direct inhibitors. Pharmacol. Res. 139, 503–511
6. Rowell, C. A., Kowalczyk, J. J., Lewis, M. D., and Garcia, A. M. (1997) MAPK, mitogen-activated protein kinase; PEI, polyethylenimine; PPI, protein–protein interaction.

21. Sullivan, M. N., Lu, Z., Li, Y. C., Zhou, Y., Huang, T., Rege, A. S., et al. (2019) DIRAS3 (ARHI) blocks RAS/MAPK signaling by binding directly to RAS and disrupting RAS clusters. Cell Rep. 29, 3448–3459.e6
22. Guillemant, J., Rudack, T., Bachler, P., Mann, D., Triola, G., Waldmann, H., et al. (2012) N-Ras forms dimers at POPC membranes. Biophys. J. 103, 1585–1593
23. Kovrigina, E. A., Galakhmetov, A. R., and Kovrigin, E. L. (2015) The ras G domain lacks the intrinsic propensity to form dimers. Biophys. J. 109, 1000–1008
24. Ambrogio, C., Köhler, J., Zhou, Z. W., Wang, H., Paranal, R., Li, J., et al. (2018) KRAS dimerization impacts MEK inhibitor sensitivity and oncogenic activity of mutant KRAS. Cell 172, 857–866.e15
25. Muratcioglu, S., Chavan, T. S., Freed, B. C., Jang, H., Khavrutskii, L., Freed, R. N., et al. (2015) GTP-dependent K-Ras dimerization. Structure 23, 1325–1335
26. Inouye, K., Mizutani, S., Koide, H., and Kaziyo, Y. (2000) Formation of the Ras dimer is essential for Raf-1 activation. J. Biol. Chem. 275, 3737–3740
27. Nan, X., Tamgumne, T. M., Collinson, E. A., Lin, L. J., Pitt, C., Galeas, J., et al. (2015) Ras-GTP dimers activate the mitogen-activated protein kinase (MAPK) pathway. Proc. Natl. Acad. Sci. U. S. A. 112, 7996–8001
28. Weber, C. K., Slupszy, J. R., Kalmes, H. A., and Rapp, U. R. (2001) Active Ras induces heterodimerization of crAf and BrAf. Cancer Res. 61, 3595–3598
29. Rudack, T., Teuber, C., Scherlo, M., Guldenhaupt, J., Schartner, I., Lübben, M., et al. (2021) The Ras dimer structure. Chem. Sci. 12, 8178–8189
30. Grozavu, L., Stuart, S., Lyakisheva, A., Yao, Z., Pathmanathan, S., Ohh, M., et al. (2022) D154Q mutation does not alter KRAS dimerization. J. Mol. Biol. 434, 167392
31. Pack, M. R., Parker, J. A., Chung, J. K., Li, Z., Lee, Y. K., Cookis, T., et al. (2021) Raf promotes dimerization of the Ras G-domain with increased allosteric connections. Proc. Natl. Acad. Sci. U. S. A. 118, e2015648118
32. Mysore, V. P., Zhou, Z. W., Ambrogio, C., Li, L., Kapp, J. N., Lu, C., et al. (2021) A structural model of a Ras-Raf signallingosome. Nat. Struct. Mol. Biol. 28, 847–857
33. Lee, K. Y., Fang, Z., Enomoto, M., Gasmri-Seabrook, G., Zheng, L., Koide, S., et al. (2020) Two distinct structures of membrane-associated homodimers of GTP- and GDP-bound KRAS4B revealed by paramagnetic relaxation enhancement. Angew. Chem. Int. Ed. Engl. 59, 11037–11045
34. Lee, K. Y., Enomoto, M., Gebregiorgis, T., Gasmri-Seabrook, G. M. C., Ikura, M., and Marshall, C. B. (2021) Oncogenic KRAS G12D mutation promotes dimerization through a second, phosphatidylyserine-dependent interface: a therapeutic vulnerability for KRAS oligomerization. Chem. Sci. 12, 12827–12837
35. Baker, R., Wilkerson, E. M., Sumita, K., Isom, D. G., Sasaki, A. T., Dohlmans, A. G. H., et al. (2013) Differences in the regulation of K-Ras and H-Ras isoforms by monoubiquitination. J. Biol. Chem. 288, 36856–36862
36. Sasaki, A. T., Carracedo, A., Locasale, J. W., Anastassiou, D., Takeuchi, K., Kahoud, E. R., et al. (2011) Ubiquitination of K-Ras enhances activation and facilitates binding to select downstream effectors. Sci. Signal. 4, ra13
37. Rajakulendran, T., Sahmi, M., Lefrancois, M., Söcheri, F., and Therrien, M. (2009) A dimerization-dependent mechanism drives RAF catalytic activity. Nature 461, 542–545
38. Freeman, A. K., Ritt, D. A., and Morrison, D. K. (2013) Effects of Raf dimerization and its inhibition on normal and disease-associated Raf signaling. Mol. Cell. Biol. 49, 751–758
39. Drosten, M., Dhawahir, A., Sum, E. Y., Urosevic, J., Lechuga, C. G., Esteban, L. M., et al. (2010) Genetic analysis of Ras signalling pathways in cell proliferation, migration and survival. EMBO J. 29, 1091–1104
40. Santos, E., Nebreda, A. R., Bryan, T., and Kempner, E. S. (1988) Oligomeric structure of p21ras proteins as determined by radiation inactivation. J. Biol. Chem. 263, 9853–9858
41. Herrero, A., and Crespo, P. (2021) Ras dimers: the novice couple at the Ras-ERK pathway ball. Genes (Basel) 12, 1556
42. Hobbs, G. A., Gunawardana, H. P., Baker, R., and Campbell, S. L. (2013) Site-specific monoubiquitination activates Ras by impeding GTPase-activating protein function. Small GTPases 4, 186–192
43. Baker, R., Lewis, S. M., Sasaki, A. T., Wilkerson, E. M., Locasale, J. W., Cantley, L. C., et al. (2013) Site-specific monoubiquitination activates Ras
by impeding GTPase-activating protein function. Nat. Struct. Mol. Biol. 20, 46–52

44. Song, H. Y., Biancucci, M., Kang, H. J., O’Callaghan, C., Park, S. H., Principe, D. R., et al. (2016) SIRT2 deletion enhances KRAS-induced tumorigenesis in vivo by regulating K147 acetylation status. Oncotarget 7, 80336–80349

45. Yan, J., Roy, S., Apolloni, A., Lane, A., and Hancock, J. F. (1998) Ras isoforms vary in their ability to activate Raf-1 and phosphoinositide 3-kinase. J. Biol. Chem. 273, 24052–24056

46. Fang, Z., Marshall, C. B., Nishikawa, T., Gossert, A. D., Jansen, J. M., Jahnke, W., et al. (2018) Inhibition of K-RAS4B by a unique mechanism of action: stabilizing membrane-dependent occlusion of the effector-binding site. Cell Chem. Biol. 25, 1327–1336.e4

47. Shigdel, U. K., Lee, S. J., Sowa, M. E., Bowman, B. R., Robison, K., Zhou, M., et al. (2020) Genomic discovery of an evolutionarily programmed modality for small-molecule targeting of an intractable protein surface. Proc. Natl. Acad. Sci. U. S. A. 117, 17195–17203

48. Khan, I., and O’Bryan, J. P. (2021) Probing RAS function with monoclonal antibodies. Methods Mol. Biol. 2262, 281–302

49. Ingólfsson, H. I., Neale, C., Carpenter, T. S., Shrestha, R., López, C. A., Tran, T. H., et al. (2022) Machine learning-driven multiscale modeling reveals lipid-dependent dynamics of RAS signaling proteins. Proc. Natl. Acad. Sci. U. S. A. 119, e2113297119

50. Pai, E. F., Krenfel, U., Petsko, G. A., Goody, R. S., Kabsch, W., and Wittinghofer, A. (1990) Refined crystal structure of the triphosphate conformation of H-ras p21 at 1.35 Å resolution: Implications for the mechanism of GTP hydrolysis. EMBO J. 9, 2351–2359

α4-α5 mutations do not impair oncogenic RAS signaling