The Q61H mutation decouples KRAS from upstream regulation and renders cancer cells resistant to SHP2 inhibitors

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Cancer cells bearing distinct KRAS mutations exhibit variable sensitivity to SHP2 inhibitors (SHP2i). Here we show that cells harboring KRAS Q61H are uniquely resistant to SHP2i, and investigate the underlying mechanisms using biophysics, molecular dynamics, and cell-based approaches. Q61H mutation impairs intrinsic and GAP-mediated GTP hydrolysis, and impedes activation by SOS1, but does not alter tyrosyl phosphorylation. Wild-type and Q61H-mutant KRAS are both phosphorylated by Src on Tyr32 and Tyr64 and dephosphorylated by SHP2, however, SHP2i does not reduce ERK phosphorylation in KRAS Q61H cells. Phosphorylation of wild-type and Gly12-mutant KRAS, which are associated with sensitivity to SHP2i, confers resistance to regulation by GAP and GEF activities and impairs binding to RAF, whereas the near-complete GAP/GEF-resistance of KRAS Q61H remains unaltered, and high-affinity RAF interaction is retained. SHP2 can stimulate KRAS signaling by modulating GEF/GAP activities and dephosphorylating KRAS, processes that fail to regulate signaling of the Q61H mutant.
RAS proteins are small GTPases that are regulated in a switch-like manner. They are turned "on" by binding a molecule of guanosine triphosphate (GTP) and turned "off" upon GTP hydrolysis. As the intrinsic rates of nucleotide exchange and GTP hydrolysis are slow, cells regulate these processes via proteins called guanine nucleotide-exchange factors (GEFs) and GTPase-activating proteins (GAPs). Activated RAS interacts with and activates effector proteins (e.g., RAF and phosphatidylinositol 3-kinase (PI3K) kinases), which drive cellular processes such as growth, proliferation, and metabolism. The RAS genes were among the first discovered oncogenes and RAS mutation or amplification is associated with over 30% of all human cancers1,2.

Of the three RAS isoforms (H-, K-, and N-RAS), 85% of oncogenic RAS mutations occur in KRAS3. More than 97% of these mutations occur at three hotspots (codons 12, 13, and 61) and multiple specific substitutions have been observed at each hotspot2. Codons 12 and 13 are the most frequently mutated sites in KRAS, whereas mutations in codon 61 are less common. By contrast, codon 61 is the most frequently mutated site in NRAS and HRAS, with Q61R/K being predominant3. Although all oncogenic mutations cause accumulation of the GTP-loaded form, each specific KRAS mutation exhibits some unique biochemical and structural properties3-6. Certain specific KRAS mutants have been associated with differential prognosis and therapeutic response of patients9-13. Thus, in-depth mutation-specific biochemical characterization of mutant RAS proteins is of paramount importance to understand the underlying mechanisms of pathogenesis, identify specific mutation-dependent therapeutic approaches, and, importantly, to identify patients who are likely to benefit from personalized or precision medicines14,15.

SHP2 is a SH2 domain-containing protein tyrosine phosphatase encoded by the gene PTPN11, which has been known for over two decades to promote RAS-driven mitogen-activated protein kinase (MAPK) signaling, and activating PTPN11 mutations are one of the common causes of RASopathies, developmental syndromes defined by hyperactive RAS-MAPK signaling16. In recent years, there has been much excitement surrounding SHP2 inhibition as a potential therapeutic for KRAS-driven cancers. Our group and others have presented encouraging preclinical results that demonstrate the potential use of SHP2 inhibitors to impair growth and induce death of KRAS-driven cell lines, patient-derived organoids, and xenografts, alone or in combination with MEK, ERK, or ALK inhibitors17-25. We recently presented a model that demonstrates a direct catalytic role of SHP2 in reversing Src phosphorylation of KRAS. This model adds an additional layer to previously proposed roles of SHP2 in RAS-MAPK signaling and provides a molecular mechanism by which SHP2 inhibition prevents cell growth. Although SHP2 modulation of the KRAS GTPase cycle favors KRAS activation by promoting GEF26,27 and restraining GAP activities28,29, the phosphatase also acts directly on KRAS (Fig. 1a). Src kinase phosphorylates two tyrosine residues in the switch regions of KRAS, which impacts the GTPase cycle by disrupting regulation by GAPs and GEFs, as well as impairing binding to effector proteins17,20,30. Dephosphorylation by SHP2 releases KRAS from this "dark state," unleashing unleash (Fig. 1b). Interestingly, the nucleotide cycling properties of KRAS mutants have been proposed as predictors of sensitivity to SHP2 inhibition18,20. It has been reported, based on a limited number of cases, that cancer cells that harbor KRAS G13D or K/N-RASQ61X mutations are resistant to SHP2 inhibition21,23,25,31. However, the underlying mechanistic explanation for the differential sensitivity of distinct KRAS mutants towards SHP2 inhibition remains to be elucidated.

Here we show that pancreatic cancer cells that harbor KRAS Q61H mutation exhibit resistance to both catalytic and allosteric SHP2 inhibitors. KRAS Q61H, the most prevalent mutation occurring at codon 61 of KRAS3, has been found in about 5% of pancreatic ductal adenocarcinoma (PDAC) patients32,33 and has also been reported as a mechanism of acquired anti-epidermal growth factor receptor (EGFR) drug resistance in both lung and colorectal cancers34-36. Here we propose a detailed mechanistic explanation for the resistance of KRAS Q61H mutant cells to SHP2 inhibitors based on data from in vitro and cell-based biochemical analyses, as well as molecular dynamics (MD) simulations.

Results

PDAC cells harboring KRAS Q61H are resistant to SHP2 inhibitors. We and others have shown that dampening KRAS signaling by targeting SHP2 may provide a tractable strategy for the treatment of cancers driven by the major (codon 12) oncogenic KRAS mutants, including non-small-cell lung cancer (NSCLC), gastroesophageal cancer, and PDAC17-22. However, recent data suggest that some KRAS mutants may not respond to SHP2 inhibition, raising important questions about the mechanisms of resistance. We observed that PDAC and PDAC patient-derived xenograft (PDX) cell lines harboring the KRAS Q61H mutation are less sensitive to the SHP2 catalytic inhibitor 11a-135, as well as the allosteric inhibitor SHP0999, in comparison to cells harboring mutations at the hotspot codon 12 (G12D/V/C/R) (Fig. 1b and Supplementary Fig. 1). Spheroids prepared in ultra-low attachment, surface-coated round well-bottom microplates exhibited consistent results. Although the viability of CFPAC1 spheroids, which harbor KRAS G12V, was significantly decreased in the presence of SHP2i 11a-1, Hs766T spheroids, which harbor the KRAS Q61H mutant, were insensitive to the SHP2 inhibitor (Fig. 1c). Furthermore, this trend was also observed in PDAC patient-derived organoids grown in three-dimensional (3D) culture conditions. An organoid with KRAS Q61H mutation was less sensitive to SHP099 (GI50 = 46.1 μM) compared to an organoid bearing a G12D KRAS mutation (GI50 = 35.7 μM), whereas MiaPaCa-2, which is highly dependent on KRAS G12C was most sensitive (GI50 = 22.1 μM) (Supplementary Fig. 2). Concordantly, phosphorylation of ERK (pERK) in SHP2i-sensitive codon 12-mutant PDAC and NSCLC, but not in Q61H-harboring resistant cells, was attenuated upon treatment with SHP2 inhibitors (Fig. 1d and Supplementary Fig. 1d). Furthermore, molecular ablation of SHP2 via CRISPR/Cas9-mediated knockout of PTPN11 in PDAC cell lines harboring KRAS G12V or G12D markedly reduced the level of pERK in response to epidermal growth factor (EGF) stimulation, whereas cells harboring KRAS Q61H exhibited no appreciable difference in pERK (Fig. 1e). SHP2 inhibitor treatment also attenuated the level of pERK in human embryonic kidney epithelial HEK293 cells transiently overexpressing wild-type (WT) KRAS or empty plasmid (i.e., mock-transfected cells expressing endogenous KRAS) in a dose-dependent manner, but failed to reduce pERK in cells overexpressing KRAS Q61H (Fig. 1f). Consistent with these results, HEK293 cells stably overexpressing GFP-KRAS Q61H exhibited resistance to SHP2 inhibitor treatment, whereas iso- genetic cells ectopically expressing GFP-KRAS WT or GFP alone were sensitive (Fig. 1g and Supplementary Fig. 3). These results suggest that some unique feature(s) of the KRAS Q61H mutant can render cancer cells independent of SHP2 function(s) and therefore resistant to pharmacologic (or molecular) inhibition of SHP2, unlike cells with WT-KRAS or the more common codon 12 mutations.
Fig. 1 KRAS Q61H mutant cells exhibit insensitivity to pharmacologic inhibition of SHP2 (SHP2i). a Schematic cartoon illustrating proposed catalytic and scaffolding roles of SHP2 in modulating KRAS signaling. SHP2 scaffolds the GRB2:SOS complex to activated receptor tyrosine kinases to promote RAS activation, whereas its phosphatase activity inhibits Sprouty negative regulation of SOS (left) and dephosphorylates a pTyr docking site for p120RASGAP on GAB1, thereby reducing inactivation of KRAS (right). Top: SHP2 also dephosphorylates KRAS, release it from the "dark state" and restoring KRAS signaling. b PDAC cell lines (left) or PDX cells (right) were plated in 96-well plates in triplicate, treated with increasing concentrations of the SHP2 inhibitor 11a-1 for 48 h, then cell viability was assessed at 48 h using alamarBlue. Data represent mean ± SEM of three independent experiments. 

PDAC cell lines (left) or PDX cells (right) were plated in 96-well plates in triplicate, treated with increasing concentrations of the SHP2 inhibitor 11a-1 for 48 h, then cell viability was assessed at 48 h using alamarBlue. GI50 values were determined using GraphPad Prism 6.0. Data represent mean ± SEM of three independent experiments.

c CFPAC1 or Hs766T cells were seeded at 1000 cells/well in 96-well spheroid microplates. Spheroids were cultured for 48 h and then exposed to vehicle (DMSO) or 50 µM 11a-1, a 3D cell viability assay was performed. Left: representative images obtained using a VWR Vista Vision inverted microscope. Scale bar, 100µm. Right: data represent mean ± SEM. RLU (relative light units) of three independent experiments. Pancreatic cancer

Hs766T (Q61H) cells were seeded at 1000 cells/well in 96-well plates in triplicate, treated with increasing concentrations of the SHP2 inhibitor 11a-1 for 48 h, then cell viability was determined using alamarBlue. GI50 values were determined using GraphPad Prism 6.0. Data represent mean ± SEM of three independent experiments.

d CFPAC1 or Hs766T cells were transfected with mock, HA-KRAS WT, or HA-KRAS Q61H plasmids, as indicated. Cells were cultured for 48 h and then exposed to increasing concentrations of SHP099 (0, 2, 5, 10, 20 µM) for 2 h. Equal amounts of lysates were resolved on SDS-PAGE and immunoblotted with the indicated antibodies.

e CFPAC1 (G12V) or HPAFII (G12D) or Hs766T (Q61H) cells were treated with increasing concentrations of 11a-1 and cell viability was assessed at 48 h using alamarBlue. Equal amounts of lysates were resolved on SDS-PAGE and immunoblotted with the indicated antibodies. Numbers indicate pERK levels normalized to vinculin based on densitometry.

f HEK293 cells were transfected with mock, HA-KRAS WT, or HA-KRAS Q61H plasmids, as indicated. Cells were cultured for 48 h and then exposed to increasing concentrations of SHP099 (0, 2, 5, 10, 20 µM) for 2 h. Equal amounts of lysates were resolved on SDS-PAGE and immunoblotted with the indicated antibodies. Numbers indicate pERK levels normalized to vinculin based on densitometry. HEK293 cells stably overexpressing GFP-KRAS WT, GFP-KRAS Q61H, or GFP with 11a-1 and cell viability was assessed at 48 h using alamarBlue. Data represent mean ± SEM of three independent experiments. The GFP and GFP-KRAS WT groups were independently compared with GFP-KRAS Q61H group using two-tailed Student’s t-test. The respective p-values are indicated on the figure. 

g PDAC cell lines and lung cancer cell lines were treated with the increasing concentrations of the SHP2 inhibitor 11a-1 or without (−) EGF (10 ng/ml). Equal amounts of lysates were resolved on SDS-PAGE and immunoblotted with the indicated antibodies. Numbers indicate pERK levels normalized to vinculin based on densitometry. Data represent mean ± SEM of three independent experiments. The GFP and GFP-KRAS WT groups were independently compared with GFP-KRAS Q61H group using two-tailed Student’s t-test. The respective p-values are indicated on the figure.
KRAS Q61H is insensitive to SOS1-mediated nucleotide exchange. To investigate potential mechanisms for the SHP2 inhibitor resistance associated with Q61H, we first compared the nucleotide-exchange properties of six oncogenic KRAS mutants, as one of the established functions of SHP2 is to promote the GEF activity of Son of Sevenless (SOS)19. To monitor the exchange reaction, a non-hydrolysable analog of GTP guanosine (guanosine 5′-O-[γ-thio] triphosphate, GTPγS) was added at tenfold molar excess to fully guanosine diphosphate (GDP)-loaded 15N-KRAS samples (250 µM) and nucleotide exchange was observed using our real-time nuclear magnetic resonance (NMR) GTPase assay30. Initially, we determined the intrinsic nucleotide-exchange rate for WT and each of six KRAS mutants (Fig. 2a, b and Supplementary Table 1a). KRAS G13D exhibited the fastest intrinsic exchange, with a rate ~13-fold higher than WT, whereas KRAS Q61L was the next fastest (~4-fold faster than WT). The remaining mutants (G12V, G12D, G12C, and Q61H) exhibited intrinsic nucleotide-exchange rates similar to that of WT (Fig. 2a, b and Supplementary Table 1a). These trends are largely consistent with previously reported measurements that used fluorescently labeled nucleotide analogs5, except the faster exchange we observed for Q61L was less pronounced in that study. The mutation at residue 61 (Q61H) did not appreciably accelerate the nucleotide exchange as measured by either method. In cells, the activation of RAS is catalyzed by GEFs such as SOS1, which is recruited to receptor complexes upon growth factor stimulation. Although the intrinsic nucleotide exchange of common KRAS mutations was reported previously5, the effects of specific KRAS mutations on GEF-mediated exchange have not been studied systematically. To address this gap, we investigated the impact of KRAS mutations on SOS1-assisted nucleotide-exchange kinetics mediated by recombinant SOS1 catalytic domain (residues 564–1049, hereafter referred to as SOScat). Interestingly, all oncogenic mutations tested reduced the sensitivity of KRAS to GEF activity. With the addition of SOScat to KRAS at a molar ratio of 1:600, the WT protein exhibited >20-fold increase in exchange rate relative to the intrinsic rate, whereas G12V and G12D showed >5-fold increases. The G12C, G13D, and Q61L mutants exhibited strong impairment in their ability to be activated by SOScat (two- to threefold enhancements). Notably, the Q61H mutant was completely non-responsive to the catalytic activity of SOScat, as its exchange rate was not increased above the intrinsic level (Fig. 2c, d, Supplementary Fig. 4a, and Supplementary Table 1a).

The crystal structure of the RAS:SOS complex shows that Gln61 is involved in a direct interaction with Thr935 of SOS (Fig. 2e, f), suggesting how its mutation to His may affect this interaction. To further investigate potential mechanism(s) underlying our experimental data, we performed MD simulations of WT-KRAS and the Q61H mutant in complex with the catalytic domain of SOS (residues 753–1046), based on crystal structures listed in Supplementary Table 3. Previous structural studies showed that RAS Switch II forms an extensive network of interactions with SOS to stabilize the KRAS:SOS complex41, and that mutation of several Switch II residues hinders interaction with SOS and/or catalysis of nucleotide exchange42,43. Our MD simulations reveal that when complexed with SOScat, the P-loop, switch II, and alpha3 regions are more dynamic in WT-KRAS vs. the Q61H mutant, on the basis of root mean square deviation and root mean square fluctuation measurements of C-α atoms (Supplementary Fig. 5a–d). Fewer interactions were observed between KRAS residues, including Ala59 and Tyr71, with SOS in the Q61H mutant vs. WT (Supplementary Fig. 6a, b). The substitution of the His sidechain for Gln61 severely disrupted the Gln61:Thr935 interaction (Fig. 2e, f). Hydrophobic interactions between RAS and SOS also make important contributions to stabilizing the RAS:SOS complex. For example, RAS Tyr64 becomes buried in a hydrophobic patch of SOS formed by Ile825 and Phe92944, and mutation of Tyr64 to Ala was shown to reduce the binding affinity of HRAS to SOS by at least 50%42. Consistently, the sidechain of Tyr64 of WT-KRAS remained clamped between SOS Ile825 and Phe929 during our entire MD simulation, whereas Tyr64 of the Q61H mutant dissociated from this hydrophobic groove after 690 ns (Supplementary Fig. 6c). These simulations strongly suggest that the Q61H mutation reduces the stability of the SOS:RAS complex, consistent with our observation that the mutation disrupts SOS-mediated nucleotide exchange (Fig. 2a–d and Supplementary Fig. 4a).

KRAS Q61H is insensitive to the GAP activity of RASA1. To characterize the other side of the KRAS Q61H GTPase cycle, we measured its intrinsic GTP hydrolysis and sensitivity to the GAP activity of RASA1. Consistent with the known role of Gln61 in hydrolysis, our results demonstrate that the Q61H mutation decreases the intrinsic GTP hydrolysis rate by about threefold compared to the WT and, importantly, the mutant was completely resistant to stimulation of GTP hydrolysis by the GAP domain of RASA1 (1 : 3000 molar ratio), whereas the hydrolysis rate of WT-KRAS was enhanced by over 300% in the same condition (Fig. 2g, Supplementary Fig. 4b, and Supplementary Table 1b).

To investigate potential mechanisms underlying our experimental observation of the exceptional GAP resistance of KRAS Q61H at the atomic level, we conducted all-atom MD simulations of KRAS Q61H in complex with RasGAP using crystal structures listed in Supplementary Table 3. The simulation revealed residues that may stabilize the protein–protein interaction through a network of hydrogen bonds, salt bridges, and hydrophobic interactions between RAS and the GAP domain. Unlike the mutant-KRAS:SOS complex, the mutant-KRAS:RasGAP complex remained intact, stabilized by several long-lived salt bridges, hydrogen bonds, and hydrophobic interactions (details in Supplementary Fig. 7a–h). These data suggest that the Q61H mutation does not disrupt the formation of the KRAS:RasGAP complex. Rather, KRAS Q61H was predicted to form a mutant-specific interaction with the extra domain of RasGAP (Supplementary Fig. 8a–c), which could potentially enhance stability of the complex. Thus, we focused on the local structure of the catalytic regions in the simulations. Previously, it has been proposed that a hydrogen bond between the “arginine finger” (Arg789) and Gln61 promotes activation of the catalytic water molecule44,45. In our simulation of the Q61H mutant, the backbone and sidechain of His61 were pointed away from the catalytic site during most of the simulation and did not interact with the arginine finger (Fig. 2h and Supplementary Fig. 9). Taken together, our simulation strongly suggests that the Q61H mutation alters the local structure of the catalytic site and directly impairs the previously proposed roles of Gln61 in stabilizing the catalytic residues and/or acting as a general base to extract a proton from the catalytic water molecule44,46,48.

In summary, these findings demonstrate that the KRAS Q61H GTPase cycle is severely decoupled from regulation by the GEF and GAP activities of SOS1 and RASA1, whereas the properties of its intrinsic GTPase cycle would lead to the accumulation of the GTP-loaded form.

KRAS Q61H is phosphorylated by Src and dephosphorylated by SHP2. Previously, we described a molecular mechanism by which SHP2 inhibition can dampen KRAS signaling47. In our model, Src phosphorylation of the switch regions of KRAS stalls its GTPase cycle and impairs its binding to effector proteins.
whereas SHP2 dephosphorylates these sites to restore KRAS signaling to MAPK17. Thus, SHP2 inhibitors prevent the reactivation of KRAS by SHP2. To dissect the molecular mechanisms of resistance to SHP2 inhibitors in cells that harbor KRAS Q61H mutation, we initially examined whether the mutation directly altered the phosphorylation profile of KRAS. HEK293 cells transfected with plasmids encoding Src together with HA-KRAS WT, G12D, G12V, or Q61H were immunoprecipitated using anti-hemagglutinin (HA) antibody and probed with an antibody recognizing phosphorylated tyrosine (pan pTyr). Less phosphorylated KRAS (pKRAS) was detected for all mutants compared to WT; however, the amount of phosphorylated Q61H was not less than the other mutants (Fig. 3a), suggesting that SHP2 resistance is not due to a lack of phosphorylation of this mutant.

Fig. 2 KRAS Q61H is insensitive to GEF and GAP activities. a Intrinsic nucleotide-exchange curves of wild-type (WT) and mutant KRAS obtained from real-time NMR experiments. A tenfold molar excess of GTPγS was added to GDP-loaded KRAS at t = 0 and the GDP-bound fraction remaining was monitored over time by NMR. The data were fit to one-phase exponential decay functions. The nucleotide-exchange curves do not go to zero, because the nucleotide exchange equilibrates at a certain ratio, which is a function of the relative affinity of GDP:GTPγS. b Histogram of intrinsic exchange rates of each mutant determined in a, presented as fold changes for the mutants relative to wild-type KRAS. The error bars indicate the SD of the fold changes. c GEF-assisted nucleotide-exchange curves in the presence of SOSca(1:600 molar ratio SOS:KRAS), measured using real-time NMR. Data were fit as in a. d Histogram representing the mean intrinsic and SOSca (1:600) assisted exchange rates for wild-type KRAS and mutants obtained from three independent fitting curves for each mutation. Three pair of peak intensities for each group were used to generate the fitting curves. The error bars represent SEM of the three exchange rates. e A snapshot ribbon diagram of SOS1 (red) in complex with WT-KRAS (blue) (top panel). Time evolution of hydrogen bonds formed between the Thr935 of SOS and the sidechain of KRAS Q61 (bottom panel). f A snapshot ribbon diagram of SOS1 (red) in complex with KRAS Q61H (orange) (top panel). Time evolution of hydrogen bonds formed between the Thr935 of SOS and the sidechain of KRAS His61. g Intrinsic and GAP-assisted GTP hydrolysis curves for the wild type and Q61H mutant. The GAP domain of RASA1 was added at a ratio of 1:3000 to KRAS-GTP (a, c, g). h A ribbon diagram of RasGAP (blue) and KRAS Q61H (orange) catalytic site. Dots represent the fraction of GDP- and GTP-specific peaks (I_{GDP}/I_{GDP}+I_{GTP}). a, b, g Error bars represent SEM of the three independent pairs of peak intensities used for the calculation.
The differential phosphorylation of WT-KRAS vs. mutants may be related to their states of activation in the cell. To investigate the effect of activation on phosphorylation, we performed in vitro phosphorylation by incubating recombinant KRAS Q61H preloaded with GDP or the GTP-analog Gpp(NH)p (Guanosine 5'-[β,y-imido]triphosphate trisodium salt hydrate) with c-Src (1 mol of Src to 125 mol of KRAS), 2 mM of ATP, and phosphatase inhibitors. The reactions were analyzed by mass spectrometry (MS) to investigate the extent of phosphorylation. The GDP- and GTP-analog-loaded KRAS Q61H samples were both phosphorylated at one to two sites with similar distributions of mono- and diphosphorylated forms (Fig. 3b), which is similar to our previous observations for WT-KRAS17.

This in vitro observation was confirmed using a newly developed phospho-(p)Y64-specific anti-RAS antibody. The specificity of this antibody was verified using purified KRAS in the presence of active Src and pY64 RAS signal was strongly competed away by excess pY64 peptide, but not the non-phosphorylated form of the same peptide (Supplementary Fig. 10a). We further validated the antibody by phosphorylating purified WT-KRAS and mutants with phenylalanine substitutions at Tyr32, 40 or 64, as well as a double mutant (32/64) with Src. The antibody generated robust signals in WT pKRAS, Y32F, and Y40F, but not in Y64F or the Y32/64F double mutant, thereby demonstrating the specificity of the antibody for pY64 (Supplementary Fig. 10b). This pY64 antibody detected higher levels of Y64 phosphorylation in the antibody for pY64 (Supplementary Fig. 10b).
Src phosphorylation promotes the intrinsic exchange rate of KRAS Q61H. As the resistance to SHP2i associated with the KRAS Q61H mutation does not appear to be related to differential phosphorylation, we examined the impacts of phosphorylation on the biochemical properties of the mutant. To understand the role of phosphorylation in regulating the biochemical properties of the mutant, we performed all-atom MD simulations of four KRAS-RAF1 complexes (KRAS-RAF1, pKRAS-RAF1, KRAS Q61H-RAF1, and pKRAS Q61H-RAF1) (Supplementary Table 3 and Supplementary Fig. 15). The unmodified sample exhibited ~4-fold weaker binding than the unmodified sample, whereas the same modification reduced WT-KRAS binding to the RBD by more than 15-fold (Fig. 5a and Supplementary Fig. 14c, d). Further, RBD pulldown experiments of KRAS expressed in HEK293 cells produced consistent results. In cells co-transfected with KRAS G12V and increasing amounts of Src, the amount of KRAS pulled down by immobilized RAF1-RBD decreased with increasing Src expression; however, the KRAS Q61H mutant did not exhibit such a marked impairment (Fig. 5b).

To better understand the impact of phosphorylation of KRAS on its interaction with RAF-RBD at the atomic level, we performed all-atom MD simulations of four KRAS-RAF1 complexes (KRAS-RAF1, pKRAS-RAF1, KRAS Q61H-RAF1, and pKRAS Q61H-RAF1) (Supplementary Table 3 and Supplementary Fig. 15). The unmodified KRAS-RAF1 and the KRAS Q61H-RAF1 systems share common KRAS-RAF contact points that persist throughout our simulation. Several hydrogen bonds
between KRAS switch I and RAF were observed in our MD simulation (Supplementary Table 4), whereas Tyr32 and Tyr64 were not interacting with the RBD. In the unmodified WT-KRAS-RAF1 simulation, Tyr32 was directed away from the RBD interaction site and formed an interaction with the γ-phosphate of GTP stabilized by a long-resident Na\(^+\) ion (Supplementary Fig. 16a, b). Tyr64 is positioned far from the KRAS-RAF1 contact region. Upon phosphorylation of WT-KRAS, the model predicted that pTyr32 and pTyr64 forms new interactions with RAF1 (with Arg73 and Arg59/Thr54, respectively), whereas the modification disrupted the interaction between Glu37 of WT-KRAS and Arg59 of RAF1 (Supplementary Fig. 17 and Supplementary Table 4). In our simulation, the distinctive characteristics of the pKRAS-RAF1 complex can be attributed to the dynamics of the pKRAS switch regions. The simulations revealed that phosphorylation increases the mobility of switch I and II of WT-KRAS, whereas the KRAS Q61H protein becomes stiffer upon phosphorylation (Fig. 5c).

Our result is consistent with a previous finding that tyrosyl...
phosphorylation alters the conformational dynamics of the KRAS protein. Furthermore, the phosphorylation of WT-KRAS caused a substantial allosteric effects on the dynamics of the bound RAF1-RBD.

These experimental and MD simulation results indicate that Src phosphorylation of KRAS at Y32 and Y64 positions has less impact on RAF-RBD binding of the Q61H mutant compared to WT protein and the G12V mutant, which is consistent with the failure of SHP2 inhibition to reduce ERK phosphorylation in cells with endogenous or exogenous KRAS Q61H.

Discussion

Our results show that cells with KRAS Q61H mutation are resistant to SHP2 inhibition as measured by ERK
phosphorylation and cell viability (Fig. 1). The finding is consistent with recent reports20,21 showing the sensitivity of cells with specific KRAS mutations to SHP2 inhibitors is associated with the properties of the KRAS GTPase cycle of each mutant. Cells with codon 12 mutations exhibit higher sensitivity than cells with codon 13 and 61 mutations25,52, but the underlying mechanistic explanation of this phenomenon has remained unclear21,53.

SHP2 is an SH2 domain-containing phosphatase that activates the MAPK pathway. Earlier investigations show that both catalytic and scaffolding roles of SHP2 regulate the RAS GTPase cycle (Fig. 1a). SHP2 acts as a scaffold to recruit the GRB2:SOS complex to the plasma membrane to promote RAS activation26,27, whereas its phosphatase activity (i) inhibits Sprotty’s negative regulation of SOS to maintain GEF function54, (ii) eliminates a docking site for p120RASGAP on EGFR and GAB128,29 to prevent rapid inactivation of KRAS-GTP, and (iii) directly dephosphorylates Src-phosphorylated RAS to restore the canonical GTPase cycle and RAF-binding affinity17. Distinct biochemical properties of specific KRAS mutants likely determine whether they confer resistance or exhibit sensitivity to SHP2 inhibitors, based on whether their activation and signaling depend on these SHP2 functions.

To characterize and compare the properties of the GTPase cycle of KRAS Q61H with the other common mutants, we first profiled the intrinsic and SOScat catalyzed nucleotide exchange of six KRAS mutants. Of the samples we tested, KRAS Q61H was the only mutant that was completely insensitive to the GEF activity of SOScat, whereas nucleotide exchange of the other mutants was accelerated by SOScat, albeit less efficiently than WT-KRAS (Fig. 2 and Supplementary Fig. 4a). In addition, the Q61H mutation reduced the intrinsic GTPase activity by about threefold compared to the WT and rendered KRAS Q61H resistant to RASA1 GAP stimulation. These properties suggest that the KRAS Q61H mutant is not dependent on SOS or on the upstream Receptor Tyrosine Kinases (RTKs) signaling that stimulates SOS and is thus decoupled from the aforementioned mechanisms by which SHP2 function promotes KRAS signaling through SOScat or by restricting RASA1 GAP activity26–29. As the catalytic domains of all RAS GAPs and GEFS are structurally and mechanistically similar30, we anticipate the Q61H mutation would similarly confer resistance to the activities of other RAS GApS and GEFS. In parallel to these findings, previous studies have reported frequent emergence of KRAS Q61H mutation as a molecular mechanism of acquired resistance to EGFR inhibition34,35. Interestingly, cell-free DNA sequencing detected the Q61H mutation in 52% of colorectal cancer patients who developed resistance to anti-EGFR monoclonal antibody (mAb) treatment (Cetuximab and Panitumumab)30. These results suggest that targeting upstream signaling molecules such as RTKs, SOS, and SHP2 may be ineffective against tumors harboring the KRAS Q61H mutation.

At the atomic level, residue 61, located in switch II, is involved in a direct interaction with SOS. A crystal structure of the RAS:SOS complex (1BKD) shows that Thr935 forms a hydrogen bond with the sidechain amide of Gln6141 (Fig. 2e), which was captured in the MD simulation. The MD simulations showed that the substitution of Glu to His disrupts this interaction by altering the electrostatic and surface complementarity at the key molecular interface between KRAS and SOS (Fig. 2f).

A previous study also reported that mutation of Thr935 to Glu decreased the exchange activity of the yeast homolog of SOScat (SCDC25)56. Furthermore, a hydrophobic interaction between KRAS Q61H and SOScat was distinctively compromised by the mutation (Supplementary Fig. 6c). Likewise, the Q61H mutation has been reported to disrupt the sensitivity of HRAS to the GEF activity of SCDC25, whereas Q61L had little effect57. In addition to the specific sidechain interactions described above, SOS makes extensive interactions with switch I, which exhibits evidence of structural perturbations in the HSQC spectrum of the KRAS Q61H mutant. Clearly, the substitution to His at the 61 position has dramatic effects on GEF-mediated exchange in both KRAS and HRAS. We did not observe any substantial differences in the pattern of Src-mediated phosphorylation or SHP2-mediated dephosphorylation of the KRAS Q61H mutant vs. WT. Similar to WT, KRAS Q61H can be phosphorylated at Tyr32 and Tyr64 by Src, and dephosphorylated by SHP2 (Fig. 3). However, we found that Src phosphorylation has less impact on signaling downstream of KRAS Q61H relative to WT or other KRAS mutants, largely because its binding to RAF-RBD is uncompromised by phosphorylation (Fig. 5a, b). In agreement with our findings, KRAS Q61H was shown to bind more tightly to RAF-RBD than p110y-RBD58. As all three RAF RBDs bind RAS in the same structural mode and the contacting residues are highly conserved59, we expect the modest effect of KRAS Q61H phosphorylation on affinity for RAF-RBD to extend to other RAF isoforms. However, binding to other effector proteins, in particular those that interact with Switch II regions such as PI3K56, Nore161, and AGO252, may be more strongly impacted.

Phosphorylation favors accumulation of the GTP-loaded state due to enhanced intrinsic nucleotide-exchange rate and impaired GTP hydrolysis. We propose here that resistance to SHP2 inhibitors of KRAS Q61H cells stems from these unique features, which maintain MAPK signaling even when KRAS is phosphorylated, together with SOS GEF and RASA1 GAP insensitivity (Fig. 5d). Various clinical trials of SHP2 inhibitor therapies for solid tumors have taken different approaches to using RAS
mutation status in their eligibility criteria, for no exclusions (e.g., JAB-3068) to excluding specific mutation types (RASQ61X for BRB-398; RASQ61X and KRASG13X for RMC-4630) or all known activating RAS mutations (except KRAS G12X for TNO155). A better understanding of the mechanistic details underlying resistance or sensitivity of each specific Q61X mutation should help guide selection of therapeutic opportunities.

The decoupling of the Q61H mutation from upstream regulation is consistent with the lack of observed efficacy of inhibitors of upstream processes. The present findings suggest that the targeted inhibition of downstream effectors (e.g., RAF, MEK, ERK, PI3K, AKT, and mTOR) or combinations thereof may be a more rational and effective approach for the treatment of cancers driven by KRAS Q61H. As a proof of principle, we treated PDAC cells harboring KRAS G12V or -Q61H mutations with the MEK inhibitor trametinib, the ERK inhibitor ulixertinib, or a combination of both drugs (Supplementary Fig. 18a). Treatment with either single agent resulted in slight inhibition of growth, whereas the combination treatment significantly reduced the viability of PDAC cells with either KRAS mutation compared to vehicle control. Similar results were observed in HEK293T cells engineered to overexpress KRAS WT, -G12V, or -Q61H (Supplementary Fig. 18b). Concordantly, it was recently found that in lung cancers, KRAS Q61H signals more strongly through MAPK than PI3K/AKT, and that these cells responded to RAF and MEK inhibitors.

We treated patient-derived organoids with KRAS-G12D or -Q61H mutations with trametinib and the AKT inhibitor MK-2206, and found organoids with either KRAS mutation responded to both inhibitors (Supplementary Fig. 18c). Together, our findings suggest that a rational approach for treating cancers with KRAS Q61H mutation should consider the inhibition of targets downstream of KRAS.

The GTPase cycle of RAS proteins is often described as a two-state ON/OFF “switch” by virtue of definitive dependency on the nucleotide bound (GTP/GDP) and the remarkable acceleration of nucleotide cycling mediated by GEF and GAP activities. In our model of the modulation of KRAS signaling, the regulator-dependent rapid ON/OFF switching function is largely compromised by tyrosyl phosphorylation and the system becomes highly dependent on intrinsic nucleotide exchange and hydrolysis activities, as well as SHP2-mediated tyrosyl dephosphorylation, which are slower processes. Due to the impairment of effector activities, as well as SHP2-mediated tyrosyl dephosphorylation, the system becomes highly "tuned by bound GTP, tyrosyl phosphorylation by Src can "dim" its signaling output, whereas dephosphorylation by SHP2 restores signaling. The Q61H mutation effectively short circuits this system by locking the switch "ON" and bypassing the dimmer, as this mutant does not evade phosphorylation by Src, but the posttranslational modification has little impact on its ability to signal. In contrast, KRAS G12 mutations cause the switch to stick in the ON position, but signaling from these mutants can still be downregulated by the dimmer (i.e., phosphorylation); thus, they are sensitive to SHP2 inhibitors. Thus, RAS signaling output is finely tuned by both a nucleotide-dependent ON/OFF binary switch and a tyrosyl phosphorylation-dependent "dimmer" switch, and both switch functions appear to be impacted by specific mutations.

Methods

Cells. HEK293, Capan1, HPAF-II, SW1990, HUPT3, MiaPaCa-2, and Hs766T cells were obtained from the American Type Culture Collection. P411TI were generated from PDAC PDX©, OCI-P236, OCI-P347, and PPTO.93 were generated by the Princess Margaret Living Biobank Organoid core facility (https://www.livingbiobank.ca, Toronto, Canada). HEK293, MiaPaCa-2, and Hs766T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Wisent) at 37 °C in a humidified 5% CO2 atmosphere. CFCAP1, Capan-1, HPAF-II, SW1990, HPAC, and HUPT3 cells were maintained similarly in RPMI-1640 (Wisent) medium supplemented with 10% (v/v) FBS. P411TI cells were maintained in DMEM/F12 medium (Thermo Fisher Scientific, 11330-032) supplemented with 5 μg/ml EGF (R&D Systems, 236-EG-01M) and 10 μg/ml insulin (Thermo Fisher Scientific, 12585-014), and 1% (v/v) FBS.

Plasmids for mammalian cell expression. A plasmid cDNA encoding human KRASG134B WT was subcloned from a pBabe plasmid (generously provided by Dr. Channing Der, University of North Carolina, Chapel Hill) into pcDNA3. KnpI and NotI restriction enzymes were used to integrate an N-terminal HA tag to the amplified KRAS cDNA from RKO conducted site-directed (Dulbecco’s Roche) to generate single and double KRAS mutants (G12V, G12D, Q61H, Y32F, and Q61H/Y32F). pCMV5-Src (WT or K295RY527F), pCMV5-SHP2 (WT and C459S), pCGN-HA-HRAS WT, pCGN-HA-KRAS WT, and pCGN-HA-NRAS WT were obtained from Addgene. Gateway Cloning technology (Invitrogen) was used to subclone Flag-SHP2 constructs into pcDNA3. All the plasmids were verified by direct DNA sequencing before they were used in this study.

Antibodies. A 12-mer phospho-peptide comprising the KRAS sequence flanking pTyr64 (TAGQEEYPSAMDR) was used to generate anti-pRAS Y64 rabbit polyclonal antibody (1:1000; Bethyl Laboratory, Inc.). Pan-Ras (OP400, 1:500), mAbs against HA (12CA5, 1:500), KRAS (OP24, 1:200, 1:500), and pTyr (4G10) (50-321, 1:1000) were obtained from Boehringer Ingelheim and Millipore, respectively. Monoclonal FLAG-M2 (F1804, 1:2000), β-actin (A5316, 1:2000) and Vinculin (V9264, 1:2000) antibodies were obtained from Sigma. Rabbit polyclonal antibodies against Src (#2109, 1:5000), pERK (#9101, 1:1000), ERK (#9102, 1:1000), pTyr (P-Tyr-1000) (#9954, 1:2000), Ras (36965, 1:1000), and HA (37321, 1:5000) were obtained from Cell Signalling Technologies. HA#11612, 1:1000) was obtained from Covance. Polyclonal IgG (sc-2027), HRAS (sc-520, 1:1000), NRAS (sc-519, 1:1000), and SHP2 (sc-280, 1:1000) were obtained from Santa Cruz Biotechnology.

Chemicals. SHP099, Trametinib, and Ulixertinib were obtained from Selleck Chemicals. MK-2206 was obtained from Cayman Chemicals. EGF was obtained from Promega. Compound 11a-1, 6-Hydroxy-3-iodo-1-methyl-2-(3-(2-oxo-2-(((4-thiophen-3-yl)phenyl)amino)acetaldo)phenyl)-1H-indole-5-carboxylic acid was synthesized as described.

CRISPR/Cas9-mediated gene editing. Gene editing was conducted using pLentiCRISPR (49535) from Addgene, with a guide sequence derived from exon 1 of SHP2 (human) and the non-target control sequence (Supplementary Table 5).

Lentivirus production and infection of cell lines. Lentivirus was produced by transient transfection of HEK293FT cells (Thermo Fisher Scientific) with pPACK2, pMDG1.vsg, and pLentiCRISPR transfer vector. The supernatant, which contains lentivirus, was collected at 72 h post transfection and filtered before being used. Efficient retroviral infection required addition of 5 μg/ml Polybrene (Millipore) for HEK293, PFCAP1, HPAF-II, and Hs766T. Selective growth was performed using puromycin (5 μg/ml) (Wisent) or hygromycin (100 μg/ml) (Invitrogen) 24 h after infection and polyclonal populations were generated.

Immunoprecipitation and immunoblotting. Immunoprecipitation and western blotting were performed as follows. Cells were collected in EBC lysis buffer (50 mM Tris pH 8, 120 mM NaCl, 0.5% NP-40) supplemented with protease and phosphatase inhibitors (Roche). Lysates were immunoprecipitated using the indicated antibodies along with protein A Sepharose (Repligen). Bound proteins were washed five times in NETN buffer (20 mM Tris pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40), eluted by boiling in sample buffer and resolved by SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were electrotransferred onto polyvinylidene difluoride membrane (Bio-Rad), blocked, and probed with the indicated antibodies.

Cellular RAS/RAF-RBD-binding assay. RAS/RAF binding was assessed using the RAS activation assay kit from Millipore (17-218). Briefly, KRAS-GTP protein was pulled down from lysates of cells treated under various conditions using an agarose-bound Glutathione S-transferase (GST) fusion protein corresponding to the RBD of human RAF1. KRAS bound to RAF-RBD was detected by western blotting using an anti-RAS antibody (Millipore #05-516, 1:2000).

Cell proliferation assay. Equal numbers of cells were plated in quadruplicate in 96-well plates in the presence of a range of concentrations of the indicated inhibitors for 48 h and cellular proliferation was assessed using Alamar Blue proliferation assay as per the manufacturer’s instructions (Invitrogen #DAL1100).
3D spheroid assay. In vitro 3D spheroid culture was performed using an Ultra-Low Attachment surface-coating 96-well spheroid microplate (Corning). Briefly, equal numbers of cells were plated in 96-well spheroid microplates and cultured in the presence or absence of indicated inhibitors. Spheroid viability was determined using the CellTiter-Glo 3D Cell Viability assay following the protocol provided by the manufacturer.

Organoid culture and drug screening. Organoids were propagated in hPDC media modified from Bo et al. Additionally, for drug screening, organoids were dissociated to single-cell suspensions on a thin layer of Matrigel (Corning, NY, USA) in 384-well plate (3000/well). Next day, organoids were treated with a range of MK-2266 (0.05–30 μM) and Trametinib (0.001 mM to 10 μM) or SHP099 (0.05–400 μM) concentrations for 96 h and cell viability was determined by CellTiter-Glo 3D viability assay (Promega, Madison, USA). Drug-response curves were generated and the minimal inhibitory concentration values were calculated using GraphPad Prism 8.0 (San Diego, CA, USA).

Recombinant protein expression. A synthetic gene encoding KRAS4B residues 1–173 (with C118S mutation) optimized for Escherichia coli expression (GenScript) was cloned into a pET-28 vector and expressed in E. coli (BL21) (residue 1–173, with C118S mutation). QuickChange site-directed mutagenesis was used to produce specific KRAS mutants (G12V, G12C, G12D, G13D, Q61H, and Q61L, Y32F, Y40F) (Supplementary Table 5). The 15N-labeled proteins were expressed by cultivating the bacteria in M9 minimal media supplemented with 1 g/L of 15 ammonium chloride and at 37 °C until the OD of 0.6 reached 0.6. Protein expression was induced with 0.2 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) at 16 °C overnight. The culture was centrifuged and the cell pellets were re-suspended in 400 µL buffer to collect (50 mM Tris, 150 mM NaCl, 0.1% NP-40, 10% Glycerol, 10 mM Imidazole, 5 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol, and lysozyme at pH 8.0) and lysed by sonication. Cell debris was removed by centrifugation (23,708 × g for 45 min) and the resulting lysate was clarified by the manufacturer. Using the CellTiter-Glo 3D Cell viability assay according to the protocol provided by the manufacturer.

The kinase domain of Src was produced as a recombinant His-tagged protein in E. coli. Briefly, cDNA encoding GAP domain of human RASA1 (residues 715–173, with C118S mutation) optimized for E. coli expression was subcloned into pRSF Ek/LIC. In both constructs, a thrombin cleavage site was added downstream of the coding region for the protease, as well as phosphatase inhibitors (1 mM activated sodium molybdate, and 1.15 mM sodium molybdate at pH 7.4). The GAP constructs were transformed into Escherichia coli BL21 (DE3) cells. Cell cultures were grown to an OD600 of 0.8 at 37 °C and recombinant protein expression was induced with a 1 : 300 molar ratio to KRAS. The tyrosyl-phosphorylated 15N-KRAS Q61H sample was prepared by incubating the GDP-loaded mutant with a catalytic amount of recombinant Src (i.e., 1 : 250 molar ratio) in the presence of 2 mM ATP, 1 mM activated sodium vanadate, 2 mM imidazole, 1 mM sodium fluoride, and 1.15 mM sodium molybdate. Phosphorylation was confirmed using 1H-15N HSQC spectrum and before the excess ATP was removed from the KRAS sample by gel filtration chromatography (Superdex T75 10/300, GE Healthcare) to remove excess nucleotide and sequential HSQC spectra were collected as hydrolysis proceeds. The GAP assays were performed by adding recombinant GAP domain of RASA1 (GAP-334) at a 1 : 3000 molar ratio to KRAS. The tyrosyl-phosphorylated 15N-KRAS Q61H sample was prepared by incubating the GDP-loaded mutant with a catalytic amount of recombinant Src (i.e., 1 : 250 molar ratio) in the presence of 2 mM ATP, 1 mM activated sodium vanadate, 2 mM imidazole, 1 mM sodium fluoride, and 1.15 mM sodium molybdate. Phosphorylation was confirmed using 1H-15N HSQC spectrum and before the excess ATP was removed from the KRAS sample by gel filtration chromatography (Superdex T75 10/300, GE Healthcare).

NMR data were processed using NMRPipe and analyzed using NMRFAM–SPARKY software60. The nucleotide-exchange and hydrolysis rates were calculated based on the fractional GDP-bound GTP KRAS peaks intensity obtained from at least three cross-peaks, which were plotted as a function of time and fit to a one-phase exponential association curve40 using GraphPad Prism 7.

Mass analysis of intact pKRAS. To obtain accurate masses of KRAS Q61H and pKRAS Q61H samples in 20 mM HEPS, 100 mM NaCl, 5 mM MgCl2, 2 mM TCEP, 1 mM activated sodium vanadate, 2 mM imidazole, 1 mM sodium fluoride, and 1.15 mM sodium molybdate at pH 7.4 were diluted 1 : 5 with 20 mM Tris-Base, 5 mM MgCl2, 2 mM TCEP pH 5.5, to obtain a final protein concentration of 500 µM. 1H-15N HSQC spectra were obtained on Agilent 600 MHz NMR Micro involving a Quadrupole time-of-flight mass spectrometer. The experiment was run in positive mode with electrospray ionization.

Ion-exchange chromatography. The mono-, di-, and triphosphorylated forms of KRAS WT and Q61H were separated by anion exchange chromatography using a Mono Q 5/50 GL column run with 20 mM HEPS pH 7.0, 5 mM MgCl2, and 1 mM TCEP (Buffer A), and 20 mM HEPS pH 7.0, 5 mM MgCl2, 1 mM TCEP, and 1 M NaCl (Buffer B). The separation of the three forms was achieved with an 80-column-volume gradient of 0–40% buffer B. To maintain the phosphorylation of the samples, phosphatase inhibitors were added to the wash and elution buffer (1 mM activated sodium vanadate, 2 mM imidazole, 1 mM sodium fluoride, and 1.15 mM sodium molybdate).

Biolayer interferometry. An Octet RED-384 biolayer interferometry instrument equipped with the Octet Data Acquisition 9.0.0.37 and FortéBio Data software (Pall) was used to measure the affinity of monomeric, dimeric, and triphosphorylated forms of KRAS WT and Q61H. GST-tagged BRAF-RBD (residues 150–233) was immobilized on anti-GST-conjugated biosensors (Pall FortéBio) by incubating the sensor with 5 µg/mL of BRAF-RBD for 10 min. The sensor was then dipped into wells containing increasing concentration of the KRAS for 30 s. The sensor was then dipped in 20 mM HEPS, 100 mM NaCl, 5 mM MgCl2, and 2 mM TCEP supplemented with 0.5% bovine serum albumin (BSA) and 0.05% buffer to monitor dissociation of KRAS from BRAF-RBD.

The assay was performed using 96-well plates at 25 °C with 1000 r.p.m. agitation in a buffer comprising 20 mM HEPS, 100 mM NaCl, 5 mM MgCl2, and 2 mM TCEP supplemented with 0.5% BSA and 0.05% Tween-20 to minimize nonspecific binding, as well as phosphatase inhibitors (1 mM activated sodium vanadate, 2 mM imidazole, 1 mM sodium fluoride, and 1.15 mM sodium molybdate) and BFA-RBD immobilized on the sensor to a one-to-one binding stoichiometry model using steady-state analysis.
Liquid chromatography and mass spectrometry. Following phosphorylation by Src kinase domain, KRAS Q61H samples were reduced and alkylated with DTT (5 mM, β-mercaptoethanol (10 mM), and digested with trypsin (10 μg/mL in 50 mM NH₄HCO₃ pH 8.3) at 37 °C for 16 h. The resultant tryptic peptides were de-salted using reverse-phase C18 columns and lyophilized in a vacuum centrifuge. The dried samples were reconstituted in 0.1% HCOOH and analyzed by LC-MS. An EASY-nLC 1200 pump was used to load and resolve samples on an Acclaim PepMapTM 100 nanoViper pre-column (75 μm × 2 cm, 3 μm) and in-line Acclaim PepMapTM RSLC nanoViper analytical column (75 μm × 50 μm, 3 μm) with a gradient of 5–30% acetonitrile over 120 min in a 0.1% HCOOH mobile phase. Positive-mode electrospray ionization was applied and ions were analyzed using an Orbitrap Exactive HRMS instrument set to perform MS/MS HCD fragmentation scans on ≤20 most intense ions (ion count ≥ 500 for activation) from an MS parent ion scan (390–1800 m/z range; 60,000 full-width half-maximum resolution at 200 m/z). Fragmented ions were placed on a dynamic exclusion list for 5 s. Acquired raw files were converted to mzML format using Proteowizard (v3.8.10800), then searched using X/Tandem (v2013.06.15) against Human RefSeq Version 45 (36,113 entries). The search parameters specified tolerances of 15 p.p.m. for the parent ion and 0.4 Da for fragment ions. One missed trypsin cleavage was allowed and carbamidomethylation [C] was set as a fixed modification, whereas oxidation [M], deamidation (N, Q), acetylation (protein N-term), and phosphorylation (STY) were allowed as variable modifications. All data are publicly available through the Massive archive (https://massive.ucsd.edu).

MD simulations. MD simulations were conducted using NAMD 2.137. The revised CHARMM36 force field71-73 was used for the proteins and CHARMM-GUI server74 was used to parameterize the nucleotide. The starting coordinates for the preparation and production simulations, we used a non-bonded pair list cutoff of 11 Å and the short-range non-bonded interactions were switched off between 10 and 12 Å. Periodic boundary conditions were applied in all simulations. Each system was padded size of 20 Å in each dimension. NaCl (150 mM) was then added to each system to maintain physiological ion concentrations and neutralize the system. The starting coordinates for the preparation and production simulations, we used a non-bonded pair list cutoff of 11 Å and the short-range non-bonded interactions were switched off between 10 and 12 Å. Periodic boundary conditions were applied in all simulations. Each system was padded size of 20 Å in each dimension. NaCl (150 mM) was then added to each system to maintain physiological ion concentrations and neutralize the system. The preparation and production simulations, we used a non-bonded pair list cutoff of 11 Å and the short-range non-bonded interactions were switched off between 10 and 12 Å. Periodic boundary conditions were applied in all simulations. Each system was padded size of 20 Å in each dimension. NaCl (150 mM) was then added to each system to maintain physiological ion concentrations and neutralize the system.

Statistical analyses. All experiments were performed in triplicate with mean and SE reported. Unpaired two-tailed Student’s t-test was used to compare between treatment groups and cell types. All statistical analysis was performed using GraphPad PRISM 4.0 or 7.0 software. p-value < 0.05 was considered statistically significant.

Data availability

Mass spectrometry data are available in the MassIVE repository under accession number MSV00084657. Other data not supporting this study are included in the article and the supplementary files. Data are provided with this paper.

Code availability

The study utilized all-atom molecular dynamics simulation codes as described in CHARMM-GUI74 https://www.charmm-gui.org/. The details of the simulation parameters are described in the “Methods” section.

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Author contributions
T.G., Y.K., C.B.M., M.O., and M.I. conceptualized the project and wrote the manuscript. T.G., Y.K., J.S.-G., N.R., M.L.U., R.H., B.P.K.P., W.H., J.V.-S., C.M.R., and M.H. conducted experiments. A.M. provided technical expertise for MD simulations. J.M., Z.-Y.Z., and J.J.Y. provided reagents or cell lines. C.B.M., M.O., M.S.I., J.E.L., M.-S.T., and B.R. supervised. All authors reviewed and edited the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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