Direct small-molecule inhibitors of KRAS: from structural insights to mechanism-based design

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Abstract | KRAS is the most frequently mutated oncogene in human cancer. In addition to holding this distinction, unsuccessful attempts to target this protein have led to the characterization of RAS as 'undruggable'. However, recent advances in technology and novel approaches to drug discovery have renewed hope that a direct KRAS inhibitor may be on the horizon. In this Review, we provide an in-depth analysis of the structure, dynamics, mutational activation and inactivation, and signalling mechanisms of RAS. From this perspective, we then consider potential mechanisms of action for effective RAS inhibitors. Finally, we examine each of the many recent reports of direct RAS inhibitors and discuss promising avenues for further development.

RAS proteins are small, membrane-bound guanine nucleotide-binding proteins; they act as molecular switches by cycling between active GTP-bound and inactive GDP-bound conformations (FIG. 1). RAS signalling is regulated through a balance between activation by guanine nucleotide exchange factors (GEFs) — such as son of sevenless homologue (SOS)[1,2] or RAS guanyl nucleotide-releasing protein (RASGRP)[3] — and inactivation by GTPase-activating proteins (GAPs) — such as p120GAP[4] or neurofibromin[5]. RAS proteins have a crucial role in the regulation of cell proliferation, differentiation and survival by signalling through a number of important pathways, including the RAF–MEK–ERK (RAF–MAPK/ERK kinase–extracellular signal-regulated kinase)[6–11], PI3K–AKT–mTOR (phosphoinositide 3-kinase–AKT–mechanistic target of rapamycin)[12–15] and RALGDS–RAL (RAL guanine nucleotide-dissociation stimulator–RAL)[16–19] pathways, among others.

The major RAS isoforms are encoded by three genes (*HRAS*, *NRAS* and *KRAS*), which give rise to a total of four proteins (HRAS, NRAS, KRAS4A and KRAS4B) owing to a KRAS splice variant. The amino-terminal residues 1–165 of these proteins share 92–98% sequence identity. The remaining 23–24 carboxy-terminal residues diverge substantially in sequence and are therefore termed the hypervariable region (HVR). The HVR contains the membrane anchor sequence for each protein, including a terminal CAAX box. All four proteins undergo prenylation at the CAAX cysteine[20] followed by proteolytic cleavage of the terminal –AAX by RAS-converting enzyme 1 (RCE1) and subsequent carboxymethylation by isoprenylcysteine carboxymethyltransferase (ICMT)[21]. The HRAS CAAX cysteine is modified exclusively by farnesyltransferase, whereas the remaining three proteins can undergo alternative prenylation at this site by geranylgeranyltransferase I when farnesyltransferase activity is blocked[22]. Each protein requires a 'second signal' for proper plasma membrane localization. With the exception of KRAS4B, this second signal includes palmitoylation at a second cysteine in the HVR, resulting in a second hydrophobic anchor[23]. In addition to this palmitoyl group, KRAS4A contains a bipartite polybasic sequence that contributes to membrane attachment through electrostatic interactions. The HVR of KRAS4B contains a polylysine region that similarly anchors the protein to the plasma membrane through electrostatics. Unlike the other three RAS proteins, KRAS4B lacks a second cysteine in the HVR and is therefore not palmitoylated.

The first 166–168 residues of RAS proteins form a single structured domain (the G domain). The remaining residues, including the HVR and lipid tail, seem to be poorly structured when RAS is in solution. The G domain consists of a mixed six-stranded β-sheet and five-α-helix fold, as is typical for α,β-nucleotide-binding proteins (FIG. 1a). The RAS proteins also share a core mechanism of activation and downstream signalling, which has been well-characterized structurally. Four main regions border the nucleotide-binding pocket: the phosphate-binding loop (P-loop, residues 10–17), switch I (residues 30–38), switch II (residues 60–76) and the base-binding loops (residues 116–120 and 145–147). The two switch regions differ in conformation between the GDP and GTP states[24], and they govern all known

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doi:10.1038/nrd.2016.139
Published online 29 Jul 2016

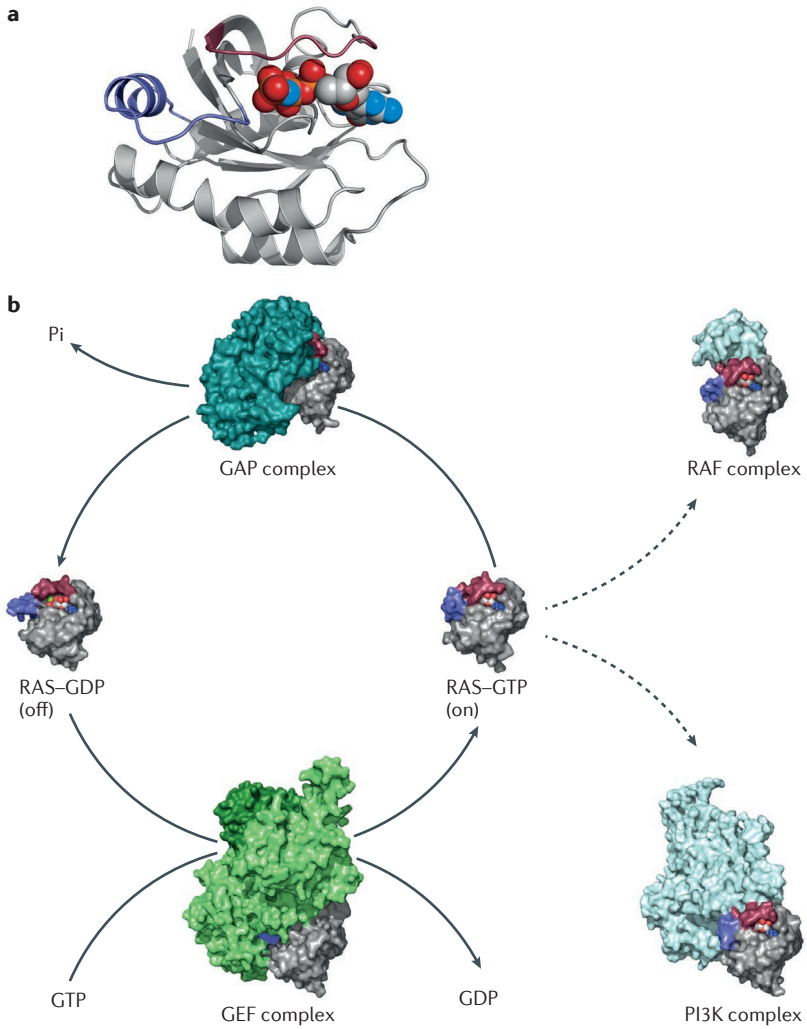


Figure 1 | **RAS secondary structure and the RAS–GTPase cycle. a** | Crystal structure of KRAS (Protein Data Bank identifier (PDB ID): 3GFT) bound to 5′-guanylyl imidodiphosphate (GMPPNP) showing the secondary structure, with switch I coloured red, switch II coloured blue and GMPPNP represented by spheres coloured by element. **b** | RAS cycles between an 'on' state bound to GTP and an 'off' state bound to GDP. The conformations of the switch regions differ substantially between these two states, and as such they control the interactions between RAS and its binding partners. RAS is shown in grey, with switches coloured as in part **a**. PDB IDs: 4Q21 (HRAS–GDP), 6Q21 (HRAS–GTP), 1WQ1 (HRAS–GTPase activating protein (GAP)), 1NVV (HRAS–son of sevenless homologue (SOS)), 1HE8 (HRAS–phosphoinositide 3-kinase (PI3K)) and 4G0N (HRAS–RAF). GEF, guanine nucleotide exchange factor.

nucleotide-dependent interactions with RAS-binding partners. In the GTP state, threonine 35 and glycine 60 make hydrogen bonds with the γ-phosphate, holding the switch I and switch II regions in the active conformation, respectively (FIG. 2). On hydrolysis of GTP and release of phosphate, these two regions are free to relax into the inactive GDP conformation.

Recent evidence suggests that, along with membrane localization and GTP binding, RAS dimerization or multimerization may play an important part in activating certain downstream effectors. It is known that RAF catalytic activity is stimulated through RAF dimerization[25], and several recent studies suggest that RAS facilitates this interaction[26–28]. Although crystal studies have not revealed a RAS–GTP dimer interface, modelling studies suggest two possible interfaces[27].

Hyperactivating mutations in RAS are among the most common lesions found in cancer. Most of these mutations have been shown to decrease GAP-catalysed hydrolysis, intrinsic GTPase activity, or both, leading to an increase in the active GTP-bound population. Mutation of any one of the major RAS isoforms can lead to oncogenic transformation[29–32]; however, KRAS mutations are by far the most common in human cancer[33,34]. Which KRAS splice variant is most important in tumorigenesis remains an area of active debate[35].

Oncogenic mutations in RAS

The most frequent sites of oncogenic mutation in RAS are residues 12 and 13 in the P-loop, and residue 61 in switch II. The frequency of mutation at each site, as well as which RAS isoform is mutated (HRAS, NRAS or KRAS), varies substantially across different types of cancer[33,34]. KRAS mutations are the most common overall and occur most frequently in solid malignancies, including pancreatic, colorectal and lung cancers. The vast majority of these mutations occur at position 12. NRAS mutations occur with highest frequency in melanoma and myeloid leukaemia, with the most common site of mutation being position 61. Finally, HRAS mutations occur with highest frequency in bladder cancer, and mutations are fairly evenly split between position 12 and position 61.

Early structural studies with HRAS revealed the mechanism by which these mutations cause hyperactivation. The structure of HRAS bound to p120GAP shows that the catalytically essential arginine finger of GAP passes immediately adjacent to glycine 12 (REFS 4,36,37). Based on this structure, it was correctly predicted that mutation of glycine 12 to any residue other than proline would cause RAS activation by interfering with GAP binding and GAP-stimulated GTP hydrolysis. Mutations at residue 13 should also sterically clash with the arginine and decrease GAP binding and hydrolysis. Glutamine 61 has a direct role in catalysis by positioning the attacking water molecule and helping to stabilize the transition state of the hydrolysis reaction[38]. Thus, mutations at this position generally block intrinsic and GAP-stimulated hydrolysis without decreasing GAP binding[37,39]. Certain mutations at positions 13 and 61 may also increase the intrinsic nucleotide exchange rate[40].

In vitro studies with HRAS show that oncogenic RAS mutations vary widely in the degree of activation that they induce. Mutation of HRAS glycine 12 to any amino acid other than proline induces colony formation and anchorage-independent growth in rat fibroblasts[41]. By contrast, mutations at glycine 13 tend to be less activating and are more variable in the degree of transformation they induce. This difference can be partially attributed to the larger distance between glycine 13 and the arginine finger of GAP, such that a larger mutation would be required to block GAP binding. Indeed, although mutation to serine at position 12 is strongly activating, at position 13 this mutation has only a small

Effectors
Proteins that bind to the GTP-bound state of RAS in order to transmit downstream signals for proliferation, survival and differentiation.

Intrinsic GTPase activity
GTPase activity (or nucleotide exchange) that is not catalysed by any other protein or chemical.

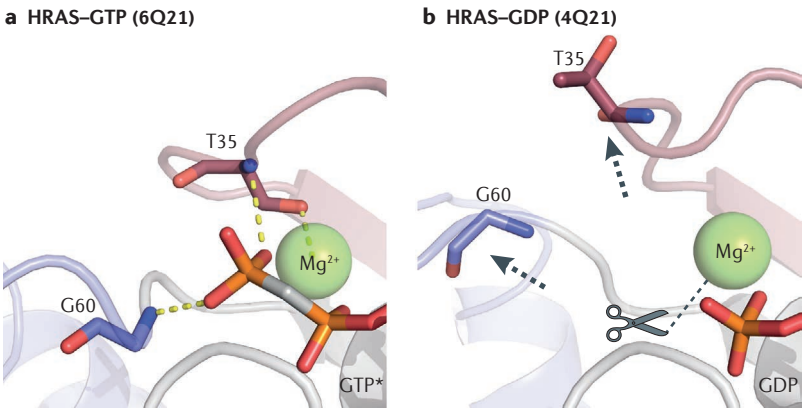


Figure 2 | **Glycine 60 and threonine 35 act as springs anchored at the γ-phosphate of GTP. a** | The GTP state of RAS (Protein Data Bank identifier (PDB ID): 6Q21) shows direct contacts between the γ-phosphate and G60 and T35. **b** | When GTP is hydrolysed the springs are released and the switches recoil into the inactive state (PDB ID: 4Q21). *The nucleotide in the crystal structure 6Q21 is a GTP analog (GMPPCP).

effect on GAP-stimulated hydrolysis and is exceedingly weak in transformation assays[37,41]. Mutation of glycine 13 to a more bulky amino acid, such as valine or aspartic acid, completely blocks GAP activity and induces a transforming phenotype in cells.

Recently, a detailed biochemical analysis by Kenneth Westover and colleagues[42] demonstrated similarly interesting, if less intuitive, differences among oncogenic KRAS mutants. As expected, KRAS proteins with mutations at any of the three common sites (residue 12, 13 or 61) displayed decreased GAP-stimulated GTPase activity, with a similar effect size across all mutants evaluated (partially activating mutations such as G13S were not included). Intrinsic GTPase activity was also decreased, but to widely varying degrees, with KRAS-G12C almost unaffected and the rate for KRAS-G12A decreased by 50-fold. Therefore, the effect on intrinsic GTPase activity may help to explain biological differences between mutants. Curiously, with the exception of KRAS-G12C, mutations at residues 12, 13 and 61 all decreased the affinity for the RAS-binding domain (RBD) of RAF as well, with an effect ranging from two-fold for G12A, G13D and Q61L to sevenfold for G12V. Based on analysis of Cancer Cell Line Encyclopedia data on tumour sensitivity to the MEK inhibitor PD-0325901, the authors hypothesize that KRAS mutants with high RAF affinity and a low intrinsic hydrolysis rate (such as KRAS-G12A and KRAS-Q61L) predominantly depend on RAF pathway signalling. On the contrary, KRAS mutants with high RAF affinity and a high intrinsic hydrolysis rate (such as KRAS-G12C) are less RAF-dependent.

A structural basis for RAS inhibition

Although the transforming potential of RAS was recognized more than three decades ago, drug discovery efforts directed against this oncogene continue. The development of effective RAS inhibitors has been complicated by the fact that RAS activates downstream effectors through the protein–protein binding event itself, and hyperactivation in cancer results from impaired RAS enzymatic activity. Effective RAS inhibitors would therefore need to prevent the productive interaction between RAS and its downstream binding partners. This could be achieved through one or more of the following general mechanisms: decreasing the proportion of RAS in the GTP state; disrupting RAS–GTP–effector interactions; stabilizing non-productive protein complexes; or decreasing the population of RAS at the membrane. If dimerization or multimerization of RAS–GTP truly plays an important part in binding and activation of effectors, then blocking these RAS–RAS interactions might also be an effective strategy for inhibition. Conversely, dimerization or multimerization of RAS–GDP could play a part in autoinhibition, and stabilizing such an interaction might provide a means of blocking RAS signalling. Finally, recent work from Frank McCormick's group[43] suggests that, for KRAS in particular, binding of calmodulin to the HVR may be required for tumorigenesis. Inhibiting this interaction could also be an effective strategy for inhibiting KRAS.

Although inhibitors targeting RAS remain highly sought after for the treatment of cancer, indiscriminate inhibition of both wild-type and mutant KRAS would probably result in substantial toxicity. Methods of inhibiting KRAS that afford specificity for the mutant over the wild-type protein should offer a much higher therapeutic index, and such methods are therefore more desirable. Studies in mice have revealed that *Kras* is an essential gene, with *Kras*-knockout mice dying at 12–14 weeks of gestation with evidence of anaemia, probably from defective liver haematopoesis[44]. A later study demonstrated that modification of the *Kras* gene to express HRAS protein produced viable embryos. However, in adulthood, these mice suffered from dilated cardiomyopathy. Although this suggests the KRAS protein itself is not essential, tissue-specific expression differences for RAS isoforms may make HRAS and NRAS unable to compensate when KRAS is inhibited in normal tissues. In addition, a growing body of literature indicates that wild-type KRAS has a protective role in some KRAS mutant cancers[45–48], although it may be tumour-promoting in others[49]. Efforts to use genetics to predict the therapeutic index of drugging a target are fraught with the many differences between genetics and pharmacology[50]. The most informative measure of therapeutic index for blocking a target of course comes from having highly validated pharmacological agents.

Conformational dynamics of the RAS proteins

Over the past decades, the field of structural biology has shifted from viewing proteins as static structures to recognizing them as flexible and mobile, with parts of proteins moving on different timescales and to different degrees. This understanding of protein structure is important for drug discovery as it suggests that small-molecule binding pockets may not maintain a single shape, or that a pocket may appear on binding to small molecules when no pocket was apparent in a compound-free structure. Some features of crystal structures can hint at these movements. For example, B factors

Transformation
The process by which cells acquire the features of cancer. The degree of transformation refers to the extent to which cells have achieved a cancer-like phenotype.

Tumorigenesis
The initial formation of a cancer, involving the transformation of normal cells into cancer cells.

B factors
Measures of scattering in X-ray crystallography indicating the mobility of the atom. Higher B factors indicate more mobility.

are a measure of variability in the position of each atom within a crystal lattice. A high B factor suggests mobility of the atom, whereas a low B factor suggests that the atom is locked in place. If a region moves substantially within the lattice, the signal is washed out and the protein structure is said to lack electron density for those atoms. Solution structures of proteins determined by nuclear magnetic resonance spectroscopy (NMR spectroscopy) and hydrogen–deuterium exchange measurements also provide useful information on protein dynamics.

Structural studies of RAS in the GDP state indicate increased flexibility in the switch regions, especially switch II, relative to the GTP state. Analysis of B factors from crystal structures of HRAS–GDP indicates that loop L4 (residues 60–68) in switch II is particularly dynamic[51,52]. Comparison of multiple GDP-bound structures reveals wide conformational variability in this region, and two solution structures of HRAS confirm extensive mobility of loop L4 (REFS 53,54). Interestingly, multiple KRAS structures, including the GDP state structures of KRAS-G12C[55] and KRAS-Q61L[42] as well as the GTP state structure of wild-type KRAS (Protein Data Bank identifier (PDB ID): 3GFT), lack electron density for much of switch II, suggesting even greater mobility in KRAS. Molecular dynamics simulations comparing HRAS and KRAS provide further support for this conclusion[56]. In comparison with the GDP state, the GTP state is much more rigid in the switch II region. Although B factors remain highest for the residues of loop L4 in the structure of HRAS–GTP[24], glycine 60 is anchored at the γ-phosphate, and NMR studies indicate that motions in switch II are substantially slowed in the GTP state relative to the GDP state[54]. Westover and colleagues[42] recently reported the GDP state structures of wild-type KRAS and four mutants, in addition to the KRAS-Q61L structure discussed above. However, with the exception of KRAS-Q61L, all of these structures show extensive stabilization of switch II by crystal symmetry mates (neighbours in the crystal lattice), confounding analysis of switch II mobility.

When bound to GTP, RAS is believed to exist in at least two rapidly interconverting conformations, state 1 and state 2 (REF. 57), of which only state 2 is thought to be capable of binding to effectors. Studies of wild-type HRAS using ^{31}P NMR at 0 °C suggest that state 1 and state 2 are present in a 1:2 ratio. This equilibrium shifts toward state 2 in the presence of the RAF RBD, implicating this state as the active conformation. Conversely, certain mutations in switch I (specifically T35S and T35A) and switch II (G60A) that impair effector binding push the equilibrium towards state 1. Structural analysis of RAS-G60A revealed loss of contact formation between the backbone of residue 60 and the γ-phosphate, which is likely to be due to the decreased rotational freedom of alanine compared to glycine[58]. The contact between threonine 35 and the γ-phosphate is also lost in this structure.

Three distinct crystal forms of RAS-T35S have also been solved[57,59]. The first of these structures lacks electron density for both switch regions, indicating a high degree of mobility for these residues[57]. Kataoka and colleagues[59] later solved two crystal forms with switch I ordered,

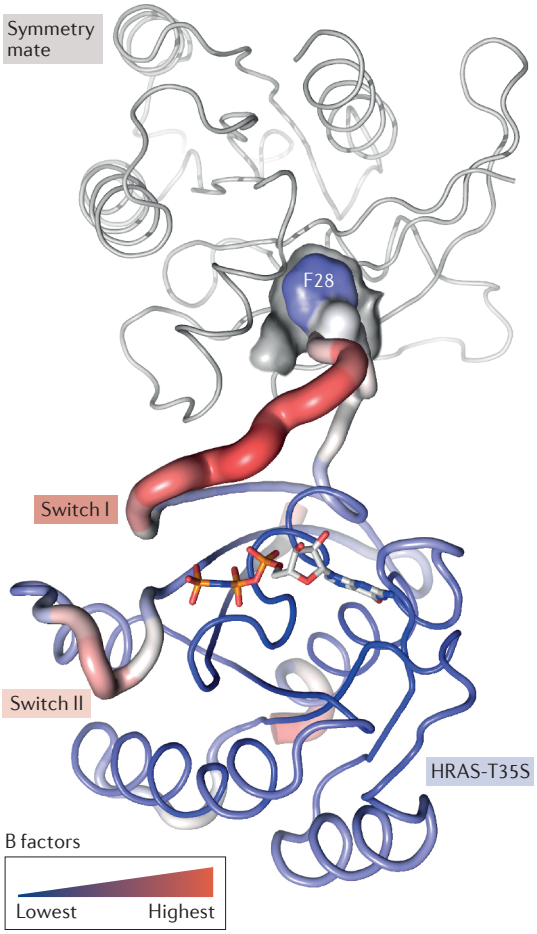


Figure 3 | **Crystal form 1 of HRAS-T35S shown with symmetry mate.** The unit cell of the form 1 structure of HRAS-T35S (Protein Data Bank identifier: 3KKN) contains a single RAS molecule and shows substantial displacement of the amino-terminal portion of switch I. Although most of switch I is highly mobile (B factors represented by heat map and diameter), the side chain of F28 (coloured surface contour) is tightly bound in a pocket (grey surface contour) on a crystal symmetry mate (grey).

one of which (form 1) overlays extremely well with the structure of RAS-G60A[58]. The structures of both RAS-T35S form 1 and RAS-G60A show a massive displacement of the N-terminal portion of switch I (particularly phenylalanine 28) away from the guanine base. Analysis of B factors in these structures suggests that although the C-terminal portion of switch I is highly mobile, phenylalanine 28 is rigidly held in place. The generation of crystallographic symmetry mates reveals that, in both of these structures, phenylalanine 28 binds tightly in a pocket on a neighbouring RAS molecule. Therefore, although these structures demonstrate flexibility in switch I, this open conformation with large displacement of phenylalanine 28 is probably a result of crystal packing forces (FIG. 3).

The remaining crystal form of RAS-T35S (form 2) shows high B factors for the segment of switch I surrounding serine 35. This finding parallels the solution structure of HRAS-T35S, which shows a large degree

Electron density
A measure of the probability of electrons being present in a region. Because electrons are concentrated around atoms and bonds, in X-ray crystallography a model of the protein structure is built by fitting atoms within an electron density map calculated from the X-ray diffraction pattern.

Nuclear magnetic resonance spectroscopy. (NMR spectroscopy).
A technique based on the absorption and re-emission of electromagnetic radiation by certain isotopes in a magnetic field. This technique may be carried out in solution and may be used to study protein structure, dynamics and interactions.

Hydrogen–deuterium exchange
A technique in which solvent-accessible, labile hydrogen atoms (such as amide hydrogen atoms or side-chain hydrogen atoms) are exchanged for deuterium by incubating a protein in deuterated water (D_2O). The exchange can then be evaluated using mass spectrometry or nuclear magnetic resonance spectroscopy. This technique can be used to determine whether regions of the protein are solvent accessible, and by proxy, whether they are mobile.

Dynamics
Proteins are no longer thought to exist as a static shape, but rather as sets of shapes that interconvert on a range of timescales. Dynamics refers to the study of these movements within a protein structure, including the transitions themselves as well as the range of conformations a protein adopts.

of conformational variability around residue 35, while phenylalanine 28 remains minimally displaced[60]. Similar to the initial structure of HRAS-T35S, in crystal form 2, no electron density is visible for switch II.

Both T35S and G60A mutations abolish the transforming activity of viral HRAS (vHRas), which differs from HRAS by G12V and A59T mutations[61,62]. Therefore, these studies illustrate a crucial vulnerability in the conformational stability of the GTP state of RAS. Even small perturbations are capable of destabilizing contacts between the γ-phosphate and residues 35 and 60, and these effects can have drastic consequences for RAS signalling.

Experimental mutations that impair RAS

One potential mechanism of inhibition is interference with effector binding. Mutagenesis studies provide some indication of which regions of RAS are important for the formation and stabilization of effector interactions. In addition to T35S and G60A, several experimentally introduced mutations have been shown to impair effector binding (FIG. 4; Supplementary information S1 (table)). The majority of information on the RAS–RAF interaction comes from work using HRAS and CRAF, and most mutations shown to affect CRAF association are located within switch I[61,63]. Indeed, mutation of almost any residue in RAS switch I can decrease binding to CRAF. Many of these mutations also decrease the transforming ability of RAS in the context of a G12V mutation[61]. Switch II mutations seem to have a greater effect on other RAS interactions, such as PI3K, neurofibromin and exchange factors[63], although a few exceptions are worth noting. Both Y64W and G60A mutations decrease binding of RAS to the cysteine-rich domain in CRAF[64], and similar to G60A, the Y64W mutation, when introduced into vHRas, impairs the capacity of vHRas to induce oncogenic transformation in NIH 3T3 cells[62,65].

Mutation of aspartate 57 to alanine decreases binding to CRAF and blocks transformation by RAS[61]. Interestingly, this mutant shows an increased rate of nucleotide exchange as well as a new preference for GDP over GTP. Serine 17 coordinates the Mg^{2+} ion in the active site of RAS, and, similar to the D57A mutant, mutation of this residue to asparagine results in a reduced affinity for GTP without having a substantial effect on GDP affinity[66]. This protein has a dominant-negative effect, which probably results from increased affinity for the GEF and reduced affinity for effectors[67]. RAS-D119N is similar to RAS-S17N and RAS-D57A, in that it decreases nucleotide affinity and increases affinity for the GEF. However, unlike these other mutants, RAS-D119N still productively binds to effectors[68]. Therefore, when this mutant is expressed at concentrations lower than the GEF it has a dominant-negative effect, but at concentrations higher than the GEF it is activating.

Although much work has focused on signalling downstream of RAF in cancer, it remains unclear which effectors of RAS are most important under various cellular contexts[69–71]. Several RAS mutants studied by Shirouzu *et al.*[61] show normal RAF binding but do not induce a transformed phenotype (specifically RAS-Y32F, RAS-K42A, RAS-L53A and RAS-Y64G). Still other mutants bind poorly to RAF but retain a transforming capacity (RAS-S39A, RAS-Y40W, RAS-R41A, RAS-L56A, RAS-T58A and RAS-A59T). Along with residues in switch I, tyrosine 64 seems to be particularly important in signalling downstream. The structure of HRAS in a complex with PI3Kγ shows a direct contact between tyrosine 64 and the PI3K RBD[72], and mutation of this residue to glycine, aspartate, glutamate, glutamine or tryptophan blocks transformation by vHRas[65].

Targeting a GTPase

As discussed above, potential mechanisms for directly inhibiting RAS can be organized into four general categories: decreasing the proportion of RAS in the GTP state, disrupting RAS–GTP–effector interactions, stabilizing non-productive protein complexes and decreasing the population of RAS at the membrane.

Decreasing levels of GTP-bound RAS. The high affinity of RAS for its substrate GTP (dissociation constant (K_d) ~10^{-11} M)[73,74], combined with the high concentration of GTP in the cell (~0.5 mM)[75], makes successful competition with the nucleotide exceedingly unlikely. Comparison to kinase inhibitors suggests that reversible-binding competitive RAS inhibitors would require an affinity in the low femtomolar range, surpassing even the affinity of streptavidin for biotin (K_d ~10^{-13} M) — one of the tightest known reversible interactions. A more feasible approach to decreasing RAS–GTP levels may involve GEF inhibition or allosterically interfering with nucleotide binding or release.

Biochemical analyses of HRAS and KRAS[42,76] suggest that maintaining high levels of RAS–GTP would require substantial flux through the RAS cycle, even for oncogenic mutants. This conclusion is based on comparing the rates of intrinsic GTP hydrolysis and intrinsic nucleotide exchange for various RAS mutants. For HRAS-G12V and HRAS-G12R, the rate of hydrolysis approximately equals the rate of exchange[76]. Although the rates of uncatalysed exchange have not been measured for the respective KRAS mutants, the rates of hydrolysis correspond well to HRAS-G12V and HRAS-G12R[42]. KRAS mutants vary widely with respect to their rates of intrinsic GTP hydrolysis, and KRAS-G12V and KRAS-G12R lie at the slow end of the spectrum, implying that other mutants should favour hydrolysis even more. However, even for KRAS-Q61L, which has the slowest intrinsic hydrolysis reaction, the rates of exchange and hydrolysis should be fairly balanced. Furthermore, many oncogenic KRAS mutants retain some degree of sensitivity to stimulation by p120GAP[42]. In the absence of activating signals through the GEF, this predicts a cycle equilibrium that favours the GDP state with only a small proportion of oncogenic KRAS in the GTP state. Stimulation by the GEF should then induce prolonged, high-level activation of KRAS.

Cellular studies are consistent with this analysis, indicating an essential role for upstream signals in maintaining oncogenic KRAS in a highly active state[77]. Similarly, mouse models of KRAS mutant pancreatic

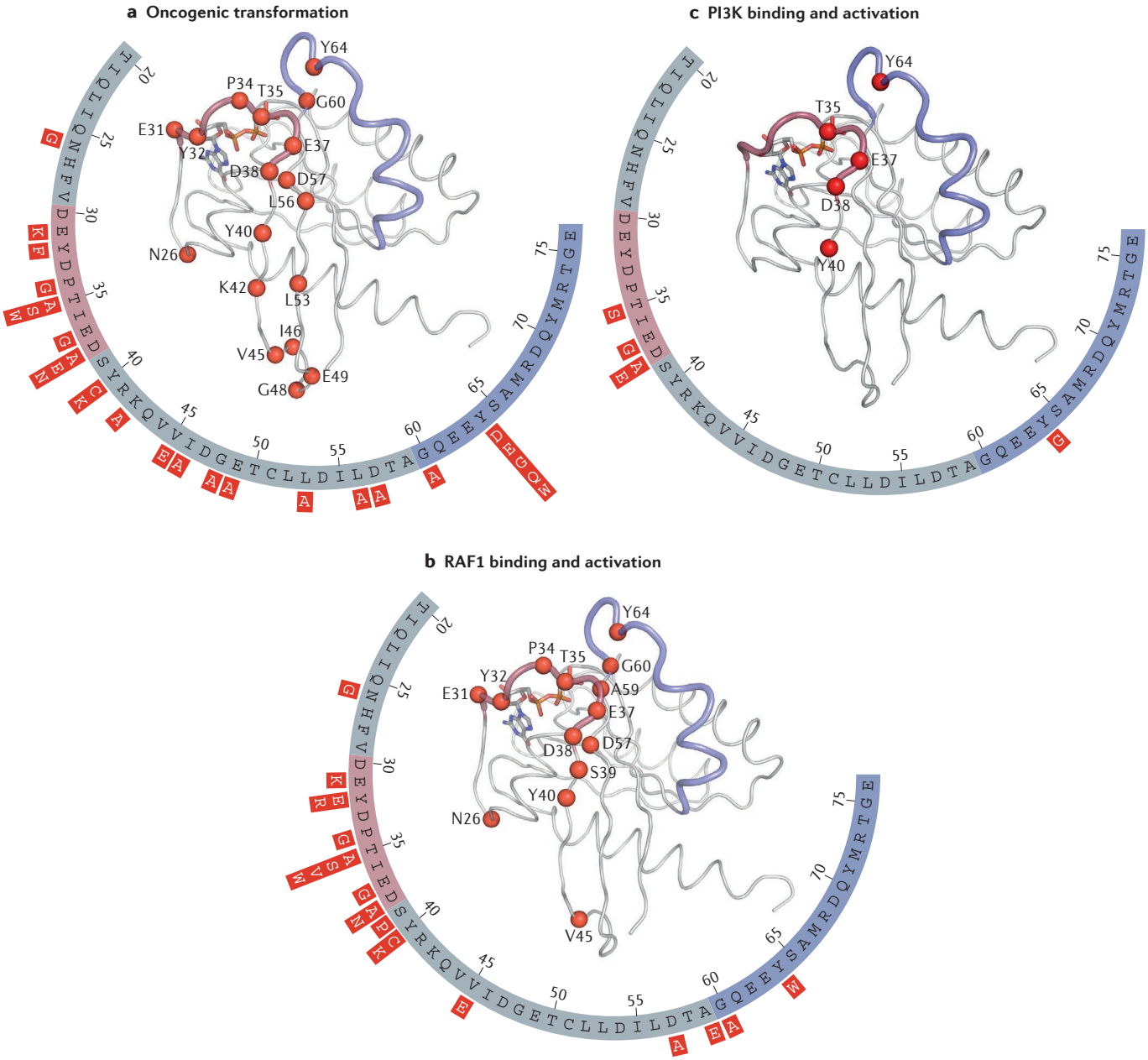


Figure 4 | **Mutations that block oncogenic transformation, CRAF binding, or phosphoinositide 3-kinase binding.** Crystal structures and partial sequences of HRAS GTP (Protein Data Bank identifier: 6Q21) showing mutations that block oncogenic transformation (part **a**), CRAF binding and activation (part **b**), or phosphoinositide 3-kinase (PI3K) binding and activation (part **c**). Residues with identified inhibitory mutations are marked with red spheres at the α-carbon. A partial protein sequence is shown in the arc with switches coloured as in structure. Inhibitory mutations at each site are listed in red, outside the arc.

cancer demonstrate a crucial role for epidermal growth factor receptor (EGFR)-mediated activation in tumorigenesis[78,79]. Unfortunately, this finding has not translated into clinical efficacy of EGFR inhibitors in KRAS-driven pancreatic cancer, suggesting a role for EGFR in tumorigenesis but not tumour maintenance in this disease[80]. RAS is known to induce production of a number of EGF-like peptides that function in an autocrine fashion to upregulate RAS activation through multiple different receptors[81]. The resulting redundancy in upstream activators may help to explain why KRAS mutations confer resistance to EGFR inhibitors in other cancers as well, including non-small cell lung cancer (NSCLC) and colorectal cancer, despite the apparent requirement for upstream activation[82–84].

Blocking stimulation through individual receptors, such as EGFR, may be effective in only a minority of KRAS-mutant cancers. However, interrupting all GEF-catalysed nucleotide exchange by directly targeting RAS could be sufficient to halt aberrant RAS signalling.

The success of this approach will depend on whether the remaining low levels of RAS–GTP are adequate for tumorigenesis. Interfering with GDP release or GTP binding may prove to be a more viable mechanism, as this approach has the potential to completely deplete RAS–GTP.

An instructive example of impaired GDP release comes from the heterotrimeric G protein $G_q\alpha\beta\gamma$. The α-subunit of this protein, $G_q\alpha$, contains a GTPase domain that is structurally related to RAS, with switch I and switch II equivalents (FIG. 5). Similar to RAS, this GTPase cycles between an active GTP-bound state and an inactive GDP-bound state. In the GDP state, $G_q\alpha$ forms a ternary complex with β- and γ-subunits. Switching to the GTP-bound state induces dissociation of $G_q\alpha$ from $G_q\beta\gamma$ and allows it to bind to effector proteins. The natural product YM-254890 binds to and stabilizes the GDP state of $G_q\alpha$ in complex with $G_q\beta\gamma$[85]. The co-crystal structure of $G_q\alpha\beta\gamma$ and YM-254890 shows the inhibitor bound adjacent to switch I, occupying the equivalent of the RAF-binding site on RAS[86]. In binding to switch I of $G_q\alpha$, YM-254890 stabilizes the region and blocks release of GDP. Although mutations in RAS show that destabilizing the switch regions in the 'on' state can impair function, the case of YM-254890 suggests that stabilizing the switches in the 'off' state can similarly impair the function of a GTPase.

The finding that RAS exists in flux between GDP and GTP states makes targeting the GDP state quite attractive. Binding to the GDP state could allow the stabilization of the inactive RAS–GDP complex or the allosteric blocking of GTP binding. The GTP state of the protein is likely to be sequestered in active complexes with RAF and other effectors in the cell, potentially making it less susceptible to inhibition by small molecules. Also, as discussed above, the more pronounced conformational dynamics exhibited by the GDP state might afford greater opportunity for small molecules to bind.

Blocking effector binding. Interfering with protein–protein interactions is inherently difficult. In particular, binding of small molecules to protein–protein contact surfaces remains a substantial challenge[87]. In the case of RAS and other GTPases, allosteric inhibition of protein–protein interactions may be a more tenable approach. Multiple lines of evidence point to this being the case for RAS. First, switch I acts as the main protein–protein interface, and it is known to be highly dynamic, undergoing large conformational rearrangements between nucleotide states. Second, mutations at sites outside the interface have been shown to affect the conformation in ways that impair effector binding. Finally, structural and NMR studies have enabled the identification of multiple inactive conformations. Compounds that bind to and stabilize these inactive states have the potential to potently and effectively inhibit RAS. An example of this type of blockade comes from small molecules that bind to the pseudokinase KSR[88]. The kinase inhibitor ASC24 binds to an inactive conformation of KSR and blocks the allosteric transition that is induced on heterodimerization of KSR with BRAF.

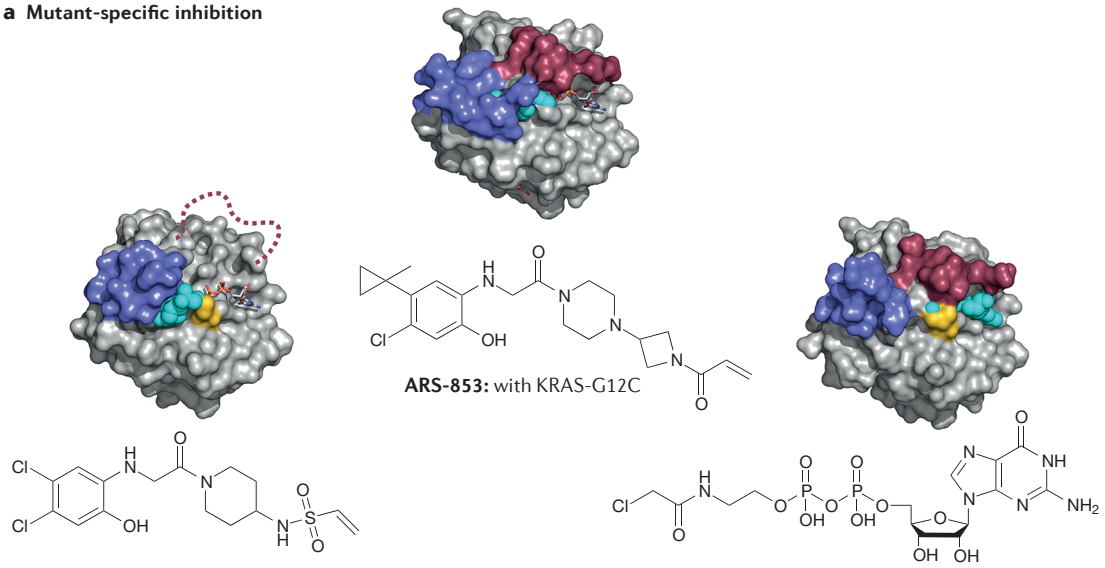
A recent report suggests that a compound currently in clinical trials, rigosertib, works as an indirect inhibitor of RAS by binding to the RBDs of RAS effectors and preventing interaction with RAS[89]. NMR evidence for binding of rigosertib to the RBD of RAF is compelling. However, the *in vitro* binding of rigosertib to the RAF RBD is orders of magnitude more potent (0.18 nM) than the cellular effects of blocking the RAF RBD (>1 μM). The real test of this mechanism will probably be whether rigosertib has efficacy in patients with KRAS mutant tumours. As it is currently in phase III clinical trials, this question will be answered relatively soon.

Stabilizing non-productive interactions. Several natural product inhibitors stabilize non-productive protein–protein interactions. Three of the best known of such inhibitors are cyclosporin A, FK506 and rapamycin. All three act by inducing the association of a receptor protein with a target. Cyclosporin A and FK506 bind to their respective immunophilin targets, cyclophilin and FK506-binding protein (FKBP), and the resulting complexes inhibit the phosphatase calcineurin[90]. Similar to FK506, rapamycin binds to FKBP[91]. However, instead of binding to calcineurin, the FKBP–rapamycin complex associates with and inhibits the protein kinase mTOR[92]. It may be possible to modify these or related natural products to force the association between RAS and a receptor protein.

Another approach could be to develop small molecules that stabilize a native complex between RAS and a non-effector protein, such as the nucleotide exchange factor. The natural product brefeldin A (BFA), an inhibitor of the GTPase ADP-ribosylation factor 1 (ARF1), provides an instructive example of this mechanism of action. This inhibitor blocks the function of ARF1 in a manner dependent on the SEC7 family exchange factor ARF nucleotide-binding site opener (ARNO; also known as cytohesin 2). Structural analysis revealed that BFA makes direct contacts with both ARF1 and ARNO, stabilizing the ARF1–GDP–ARNO ternary complex[93] (FIG. 5).

Altering localization. Finally, it may be possible to prevent downstream signalling by decreasing the population of RAS at the membrane. Localization of RAS to the inner leaflet of the plasma membrane is known to be crucial for activation of RAF and other downstream effector pathways. Inhibitors that prevent post-translational modification, impair proper trafficking, or displace RAS from the membrane may effectively block aberrant signalling by this oncogene. Early attempts to block signalling by oncogenic KRAS focused on this approach through the development of farnesyltransferase inhibitors (FTIs) following the discovery that prenylation of the CAAX cysteine is required for oncogenic transformation[20,94–97]. However, in cells treated with FTIs, KRAS and NRAS undergo alternative prenylation by geranylgeranyltransferase I[22]. Phase II and phase III clinical trials of FTIs were disappointing, failing to demonstrate antitumor activity in KRAS-driven cancers, probably owing to this alternative prenylation. Simultaneous genetic inactivation of

a Mutant-specific inhibition

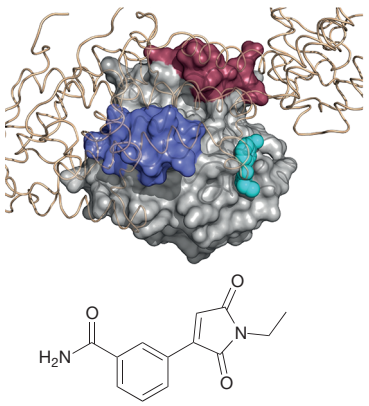


ARS-853: with KRAS-G12C

Compound 8: with KRAS-G12C

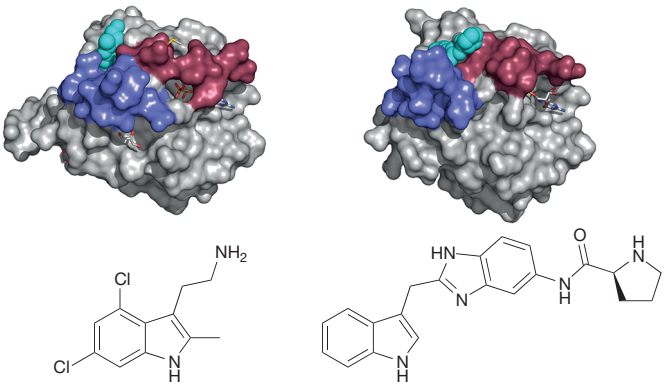
SML-8-73-1: with KRAS-G12C

b Inhibit nucleotide association



Compound 12: with KRAS and SOS

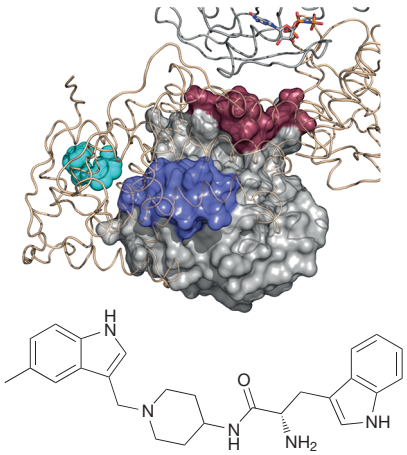
c Inhibit exchange factor



DCAI: with HRAS

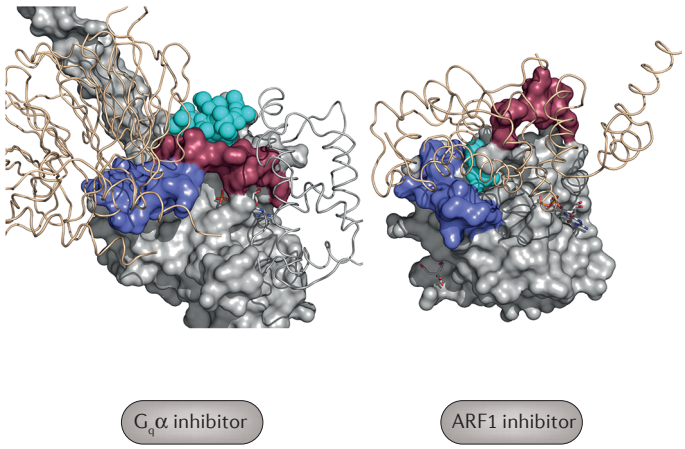
Compound 13: with HRAS

d Active exchange factor



Compound 3: with HRAS and SOS

e Natural product inhibitors of other GTPases



YM-254890: with $G_q\alpha\beta\gamma$

Brefeldin A: with ARF1 and ARNO

Figure 5 | **Crystal structures and mechanisms of action for reported RAS inhibitors.** **a** | Molecules that specifically block oncogenic mutant RAS. **b** | Molecules that impair nucleotide association. **c** | Molecules that slow SOS-catalysed nucleotide exchange. **d** | Molecules that increase RAS-GTP while blocking downstream signalling. **e** | Examples of effective inhibitors of other GTPases. Inhibitors are shown in light blue, switch I is shown in red and switch II is shown in dark blue. In structures of the RAS–son of sevenless homologue (SOS) complexes, SOS is represented by a ribbon diagram (tan). The structures of G_q with YM-254890 and ARF1–ARNO (ADP-ribosylation factor 1–ARF nucleotide-binding site opener) with Brefeldin A are included for comparison. The core G protein domain is represented similarly as surface contour, with $G_q\beta\gamma$ and ARNO represented as ribbon diagrams (tan). Relative to the structure of RAS, $G_q\alpha$ residues 67–177 constitute a large insertion amino-terminal to switch I. To facilitate comparison to RAS, these residues have been represented as a ribbon diagram (grey) as well.

farnesyltransferase and geranylgeranyltransferase I was shown to reduce KRAS-driven lung tumorigenesis in mice[98]. However, combined inhibition of these enzymes by small molecules has yet to show efficacy in KRAS-driven cancers. This topic has been reviewed in depth elsewhere[99,100].

Small molecules that directly target RAS

Several groups have reported the development of small molecules that directly bind to RAS[55,101–109]. The most common mechanism of inhibition shared by these small molecules involves blocking GEF-catalysed nucleotide exchange. As discussed above, it is not known whether exclusively inhibiting the GEF will be sufficient to stop RAS-driven malignancies. However, owing to an apparent high degree of sensitivity of this function, nucleotide exchange inhibition seems to be an accompanying feature of molecules with other, potentially more efficacious mechanisms of action. As expected, targeting the interaction between RAS and its effectors has proven more challenging, although some molecules have been reported that can achieve this. Efforts directed at blocking post-translational processing or localization of RAS continue to hold promise, although few of these approaches involve targeting RAS directly. The crucial role of KRAS in normal physiology makes mutant-specific inhibition particularly desirable, but achieving this specificity will probably not be possible for all mutants.

Targeting RAS nucleotide exchange and binding. The first series of molecules reported to bind to and inhibit RAS directly was designed by Schering-Plough in collaboration with Agouron Pharmaceuticals[101,110]. These compounds were designed to compete with nucleotides for RAS binding, but crosslinking studies and preliminary NMR analysis suggested a new binding site located beneath switch II. Despite continued efforts, it has not been possible to obtain a crystal structure with inhibitors based on this scaffold. However, more-detailed NMR analysis did confirm binding in the region of switch II[111]. Compounds from this original series were shown to block intrinsic nucleotide exchange, similar to the neutralizing anti-RAS monoclonal antibody Y13-259 (REFS 112,113), which binds to residues of switch II. Optimized derivatives of these compounds were also shown to inhibit GEF-catalysed exchange *in vitro*[114] and decrease the viability of the KRAS-G13D mutant cancer cell line HCT116 at high concentrations (half-maximal inhibitory concentration (IC_{50}) = 68 μM)[115]. Although these cellular studies suggested higher sensitivity of KRAS-G13D mutant cells compared to KRAS wild-type cells, biochemical analysis revealed much more potent inhibition of wild-type KRAS than of the G13D mutant in GEF-catalysed nucleotide exchange assays. The fact that no X-ray co-crystal structures were determined for the hits from Schering-Plough probably made subsequent optimization challenging.

Using a directed peptidomimetic approach, Patgiri and colleagues[102] developed a synthetic peptide inhibitor based on an α-helix from the exchange factor son of sevenless homologue 1 (SOS1) that binds between switch I and switch II. To stabilize the helix, the authors used the hydrogen bond surrogate approach, which involves replacement of the first turn of the helix with a macrocycle. Similar to the compounds from Schering-Plough, this synthetic peptide slows GEF-catalysed nucleotide exchange *in vitro*. At high concentrations, this peptide was able to attenuate the increase in RAS–GTP and phosphorylated ERK (pERK) in response to EGF stimulation in cells expressing wild-type RAS.

Walensky and colleagues[108] took a similar approach, developing a hydrocarbon-stapled helix, named SAH-SOS1A, which corresponds to the same α-helix from SOS. This molecule blocks nucleotide association with KRAS *in vitro*. Cellular studies demonstrated attenuation of EGF-stimulated phosphosignalling downstream of RAS and decreased viability of KRAS-G12D Panc 10.05 cells, both with an IC_{50} of ~10 μM. Further optimization will be necessary to improve potency, and it is not yet clear whether such molecules will have sufficient tumour penetration and cellular uptake to be effective treatments. Efforts by other investigators to make stapled peptides more drug-like have led to questions about inherent barriers to cell permeability[116].

Gorfe and colleagues[106] used molecular dynamics simulations that sampled the state 1 conformation of KRAS to evaluate binding of derivatives of the natural product andrographolide. These simulations suggested multiple potential binding sites for these compounds in the switch I and switch II regions. Cellular studies showed decreased accumulation of KRAS–GTP following EGF stimulation, with concomitant attenuation of ERK activation. These compounds showed growth-inhibitory effects in both KRAS mutant and wild-type cells at low micromolar concentrations, with twofold greater potency in KRAS mutant cells. Based on their results, the authors propose a mechanism of action involving inhibition of GEF-catalysed nucleotide exchange. However, this mechanism was not evaluated biochemically, and direct binding to RAS was never verified experimentally.

Recent advances in fragment-based drug discovery have helped to reinvigorate efforts to directly target RAS. Two groups almost simultaneously reported results from structure–activity relationship by NMR (SAR by NMR) screens. One group at Vanderbilt[104], under the guidance of Stephen Fesik (the developer of SAR by NMR), and another group at Genentech[103] independently discovered small molecules that bind in a new shared site at the base

of the switches (FIG. 5). The screening hits from these two groups are structurally related, sharing an indole moiety with a substitution at position 3. Their co-crystal structures revealed that they bind to the same pocket, although the indole moieties adopt different orientations within the pocket. Similar to earlier inhibitors, binding to this new site impairs GEF-catalysed nucleotide exchange *in vitro*. The Genentech group also reported decreased activation of wild-type RAS following EGF stimulation of HEK 293T cells in the presence of their most effective compound, DCAI[103]. However, whereas NMR titration experiments gave a K_d of 1.1 mM for this compound, the cellular IC_{50} was 15 μM, which is suggestive of off-target activity in their cellular assays. The pocket identified by these two groups has no known biological function. Interestingly, though, it is the same pocket occupied by phenylalanine 28 in the HRAS-T35S crystal symmetry mates discussed above (FIG. 3). It is possible that this pocket plays a part in the dominant-negative effect of certain state 1-stabilizing mutants, such as RAS-G60A.

Stabilizing non-productive RAS complexes. A second series of compounds described by Fesik and colleagues[117] bind in a pocket on SOS that is adjacent to switch II, but they do not directly contact RAS. These compounds increase the acceleration of nucleotide exchange by SOS. Evaluation in cellular assays confirmed a sustained increase in RAS–GTP levels on treatment with compound 4. Surprisingly, rather than enhancing proliferation as shown for activating SOS mutations[118,119], these compounds decrease viability of both RAS mutant and RAS wild-type cells. Treatment decreased AKT phosphorylation with an IC_{50} 0.4–1.6 μM, whereas phosphorylation of ERK showed a biphasic response, with induction at low concentrations and inhibition at higher concentrations. The peak of pERK induction correlated well with the *in vitro* IC_{50} for nucleotide exchange as well as the IC_{50} for cell viability. The authors note a similarity between this biphasic response and RAF inhibitor-induced paradoxical activation.

Interestingly, comparison of this pocket in crystal structures of SOS with and without compound 4 shows no change in any of the residues lining the pocket[117,120]. The compounds may accelerate nucleotide exchange by stabilizing the RAS-bound conformation of SOS; however, a comparison of crystal structures of the SOS catalytic domain in the absence and presence of RAS demonstrates no changes in any of these residues either, with the single exception of rotation of the side chain of histidine 905 away from the pocket[117,120–122]. It is also possible that binding of compound 4 to this pocket alters the conformational dynamics at the RAS-binding interface. An example of such an effect comes from the thrombin–thrombomodulin interaction, wherein binding of thrombomodulin distal to the active site results in allosteric changes in conformational dynamics that contribute to the shift in thrombin substrate specificity[123].

Researchers at AstraZeneca also identified a set of compounds that bind in this pocket on SOS by using a crystallography-based fragment screen against RAS–SOS[109]. They also identified molecules that bind at the interface between RAS and SOS at a site near, although not equivalent to, the BFA binding site at the ARF1–ARNO interface. Although these two sets of molecules provide important starting points for further development, they are not potent enough to detect biological activity. However, using a series of maleimide-containing fragments, the authors were able to identify a third set of compounds, which irreversibly bind to cysteine 118 and block RAS signalling (FIG. 5). The authors only observed efficient inhibition of biological activity when RAS pre-incubation and labelling with compound were carried out in the presence of SOS. Relative to the structure of RAS alone, in the RAS–SOS complex, loop L8 of RAS (which contains cysteine 118) is rotated 180° such that cysteine 118 is directed toward the nucleotide pocket and is much more solvent-exposed. This probably allows for bulky substitution by the AstraZeneca compounds, which may in turn interfere with nucleotide binding. It is unclear how specifically these compounds bind to RAS. Cysteine 118 is the most exposed cysteine in the RAS–SOS complex, and the maleimide functional group is highly reactive, as evidenced by complete abrogation of binding in the presence of 1 mM dithiothreitol. By contrast, the reaction of acrylamide compounds developed by our group with KRAS-G12C is not impaired by 1 mM dithiothreitol[124], and we do not detect significant dithiothreitol Michael addition product by mass spectrometry under these conditions (unpublished data). Importantly, this work from AstraZeneca suggests that modification of cysteine 118 could be a viable approach to inhibiting RAS, and that this might be best achieved by targeting the RAS–SOS complex.

Decreasing RAS effector binding. The non-steroidal anti-inflammatory drug (NSAID) sulindac sulfide has been shown to impair RAS-driven transformation and, at much higher concentrations, to impair the association between RAS and its binding partners *in vitro*[125]. Waldmann and colleagues[126,127] published back-to-back articles describing the development of sulindac-derived inhibitors of the RAS–RAF interaction. In the first paper, they describe a phenotype-based screen monitoring the reversion of HRAS transformation in Madine–Darby canine kidney (MDCK) cells[126]. The second paper described the evaluation of the same compound library on the RAS–RAF interaction *in vitro*[127]. Analysed independently, the results of each paper look promising. However, the cellular and biochemical potencies for individual compounds in their library correlate poorly, with the most potent compounds from their cellular assay having the least effect on the RAS–RAF interaction *in vitro*. In addition, there is limited evidence that these compounds bind directly to the RAS protein. Recent evidence suggests that their effects on the RAS pathway may arise through increased phosphorylation of an inhibitory site on CRAF and activation of ERK phosphatases, which may be a general effect of NSAIDs[128].

Another set of putative RAS–RAF interaction inhibitors was identified in a yeast two-hybrid screen[129]. These compounds block pull-down of RAS by the RAF RBD

in G12V-mutant NIH-3T3 cells[130]. The compounds also attenuate ERK phosphorylation and revert the RAS-transformed phenotype of multiple RAS-mutant cell lines. Similar to the sulindac-derived compounds, it is still unclear whether or not these molecules directly bind to RAS or RAF, and, in the absence of structural data, further chemical optimization has been challenging.

Researchers from Kobe University reported the discovery of two RAS-binding compounds, Kobe0065 and Kobe2602, through *in silico* screening[105]. Both Kobe0065 and Kobe2602 seem to interfere with binding of RAS–GTP to SOS and CRAF *in vitro*. Interestingly, molecular modelling and NMR data suggest that the binding site of these compounds overlaps with the pocket reported by Maurer *et al.*[103] and Sun *et al.*[104]. Unfortunately, the authors were only able to obtain a solution structure of Kobe2601, an inactive control compound, bound to RAS-T35S, an inactive mutant of RAS. Unlike the structures from these other two reports, no pocket is apparent in the structure with Kobe2601. Rather, the molecule sits on the surface of the protein. Evaluation of Kobe0065 and Kobe2602 in cellular assays demonstrated impaired phosphosignalling downstream of RAS and inhibition of NIH 3T3 cell transformation by HRAS-G12V. These compounds also decreased growth in a tumour xenograft model.

Although these compounds have promising effects on RAS-driven cancer cells, there is substantial discord between their *in vitro* and *in vivo* potencies. Both compounds are vastly more potent in colony formation assays (IC_{50} = 0.5 μM for Kobe0065) than in blocking SOS binding or RAF binding *in vitro* (IC_{50} ~25 μM and 46 μM for Kobe0065, respectively). Furthermore, the relative potency of the two compounds reverses across different assays, wherein Kobe0065 is significantly more potent and effective *in vitro*, and Kobe2602 is superior or equivalent in cellular assays. Combined, these discrepancies implicate off-target activities for their compounds *in vivo*.

Targeting RAS localization. Another approach to targeting RAS signalling is to prevent localization to the membrane. In an attempt to overcome alternative prenylation by geranylgeranyltransferase I, Spielmann and colleagues designed analogues of farnesyl pyrophosphate that could be transferred to the CAAX cysteine of HRAS by protein farnesyltransferase[131]. By increasing the hydrophilicity of these analogues they were able to block RAS-induced ERK phosphorylation and germinal vesicle breakdown in *Xenopus* sp. oocytes, using endogenous farnesyltransferase to catalyse transfer of their molecules to RAS. While these results are promising, their compounds are unlikely to be chemically stable enough to function effectively as drugs, and they would probably have poor bioavailability owing to the highly charged phosphate groups.

In addition to the FTIs discussed above, several attempts have been made to target RAS localization indirectly by inhibiting RAS processing and transport machinery. Among these, perhaps the most promising is a series of inhibitors developed by Waldmann and colleagues[132] to target phosphodiesterase δ (PDEδ), a protein chaperone required for transport of farnesylated KRAS4B to the membrane. Recently, the same group described a second series of inhibitors with similar biochemical potency[133]. These compounds display less nonspecific cytotoxicity than the original series, but they are not pharmacologically stable enough for *in vivo* evaluation. Therefore, further chemical optimization will be required to fully evaluate this strategy.

Mutant-specific RAS inhibitors. Indiscriminate inhibition of both wild-type and mutant KRAS is likely to result in substantial toxicity. Inhibitors that target only the mutant protein could circumvent this potential obstacle and are therefore particularly desirable. One method of achieving mutant specificity is through covalent attachment of an inhibitor to the mutant residue itself. Cysteine is the most reactive amino acid, acting as a nucleophile to form covalent bonds with a variety of chemical warheads. A number of inhibitors of other proteins form covalent attachments through native active-site cysteines, including the FDA-approved Bruton tyrosine kinase (BTK) inhibitor ibrutinib[134,135]. KRAS-G12C is one of the three most common RAS mutants in cancer, and it harbours a non-native cysteine residue that can be targeted to achieve specificity over the wild-type protein. Wild-type RAS lacks any cysteines in the active site, but the mutant cysteine 12 is conveniently positioned for inhibitor design, sitting at the edge of the nucleotide pocket and adjacent to the highly dynamic switch II region. The G12C mutation accounts for 29,700 new cancer diagnoses annually (lung, colon and pancreatic), occurring most frequently in NSCLC and colorectal cancer. For comparison, the two most common KRAS mutations, G12D and G12V, annually account for 53,700 and 39,100 new cancer diagnoses, respectively[136].

We have recently developed a series of inhibitors that irreversibly target KRAS-G12C by forming a covalent attachment to the mutant cysteine[55] (FIG. 5). By depending on the mutant residue for binding, our compounds specifically target KRAS-G12C while sparing the wild-type protein. These compounds bind in an allosteric pocket beneath switch II, which we have named the switch II pocket (S-IIP). In doing so, these compounds displace glycine 60 toward switch I, causing disordering of residues 30–38 and loss of Mg^{2+} in several co-crystal structures. The GTP state of RAS is exquisitely sensitive to conformational disturbances in the region of glycine 60 and threonine 35, as demonstrated by the dominant-negative effects of the conservative mutants RAS-G60A and RAS-T35S. Although we have been unable to obtain a co-crystal structure in the GTP state, it is clear that the presence of our inhibitor in the S-IIP would have pronounced effects on the position of both these residues.

Biochemical analysis showed that our compounds preferentially bind to the GDP state of RAS, impair SOS-catalysed nucleotide exchange and decrease the affinity of RAS for GTP relative to GDP. These compounds block RAS–RAF association in G12C mutant cells, a result that is probably due to a combination of effects on nucleotide exchange, relative nucleotide affinities and, perhaps most critically, conformation effects on switch I

and switch II. Importantly, the SAR that was determined *in vitro* tracks well with cellular viability assays, with IC_{50} values corresponding very well with biochemical potencies. Finally, viability is selectively decreased in cancer cell lines expressing KRAS-G12C, indicating on-target activity. This unprecedented tractable pocket on KRAS, and the mutant-specific nature of the approach, led to the out-licensing of the compounds to a new start-up company, Wellspring Biosciences.

Further optimization of this scaffold by researchers at Wellspring Biosciences yielded the much more broadly KRAS-G12C cell-specific active inhibitor, ARS-853. The KRAS-G12C inhibitor we originally reported (compound 12 in REF. 55) is very close in chemical structure to ARS-853, further supporting the druggability of the S-IIP. Similar to our initial series of inhibitors, ARS-853 only targets the GDP state of KRAS-G12C[124]. However, this optimized inhibitor rapidly modifies KRAS-G12C at lower concentrations than our initial series of inhibitors, and after covalently attaching to the GDP state of RAS, it blocks exchange of GDP for GTP with much higher efficacy. Using mass spectrometry, the authors were able to demonstrate specific modification of KRAS-G12C in cells, with resulting blockade of downstream signalling through RAF–MEK–ERK and PI3K–AKT[124,137]. As expected for irreversible covalent interactions, target engagement by ARS-853 is both time- and concentration-dependent[124]. Inhibition of downstream signalling probably occurs owing to failure of GTP loading on RAS.

ARS-853 potently inhibits growth of G12C mutant cells in ultra-low adherent 3D culture, but it does not inhibit growth of any non-KRAS-G12C cells tested. As predicted, activation of upstream signalling and second-site mutations in RAS that slow flux through the RAS cycle impair ARS-853 activity[137]. These results provide further evidence against the classical view of oncogenic KRAS residing in a constitutively active state and confirm the analysis above, at least for KRAS-G12C, that the RAS cycle is highly dynamic. Although RAS mutants almost certainly vary in the degree of cycle flux they undergo, it is highly likely that this finding of a dynamic cycle will extend to many non-RAS-G12C mutants as well. This work also further validates the approach of targeting KRAS-G12C through binding to the S-IIP and demonstrates that continued optimization of this scaffold is possible.

Gray and colleagues[107] reported the development of an electrophilic GDP analogue that similarly reacts with the mutant cysteine of KRAS-G12C (FIG. 5). Using their molecule, SML-10-70-1, they were able to achieve a small decrease in pERK and pAKT at 100 μM. However, they do not see any therapeutic window between G12C mutant and G12S mutant (control) cell lines with regard to viability. Owing to the exceptionally high affinity of RAS for guanine nucleotides, improving the potency of this scaffold may be quite challenging.

Because both of these approaches to targeting KRAS-G12C rely on cysteine-reactive chemical groups for binding and inhibition, reaction with free cysteine residues on other cellular proteins is a reasonable concern. Importantly, treatment of cells with ARS-853 at relatively high concentration (30 μM) resulted in modification of only two other targets, FAM213A and reticulon 4 (REF. 124). Continued optimization of this scaffold should improve potency, enabling the use of lower drug concentrations and thus making off-target reactions even less of a concern.

Conclusions

Mutations in the RAS genes are among the most common lesions in cancer, and the resulting hyperactivated RAS proteins act as potent drivers of tumorigenesis and tumour growth. Efforts to target these key proteins have spanned more than three decades, but have so far yielded no approved RAS-directed therapies. Given the limited success of downstream inhibitors in RAS-driven cancers, the recent development of new tools in drug discovery has led to renewed efforts to directly inhibit RAS. Although the bulk of RAS structural, mutational and biochemical data come from studies using HRAS, the major driver in most RAS-mutant cancers is KRAS. These proteins are highly similar in sequence and structure, particularly in the catalytic domain where direct inhibitors are most likely to bind. However, small but important differences exist. For example, KRAS shows increased flexibility relative to HRAS[56], a feature that may prove to be advantageous in the development of a direct inhibitor. Whereas oncogenic RAS was traditionally thought to exist almost exclusively in the GTP-bound state, re-analysis of rate constants for the HRAS and KRAS cycles predicts a more balanced cycle equilibrium. Recent cellular studies have confirmed this prediction[77,124].

Of the various steps of RAS activation and signalling, GEF-catalysed exchange seems to be the most sensitive to inhibition by RAS-binding small molecules. However, it remains unknown whether blockade of GEF-catalysed exchange will be sufficient to stop cancers driven by oncogenic RAS. To date, inhibitors exclusively targeting GEF-catalysed exchange have not been sufficiently potent to answer this question. Although oncogenic RAS mutants do depend on the GEF to a degree, wild-type RAS is entirely dependent on the GEF for activation. Molecules that indiscriminately block GEF-catalysed exchange for both mutant and wild-type RAS may lead to substantial toxicity.

Although it may be possible to develop inhibitors of KRAS that depend solely on oncogene addiction for selective toxicity against cancer cells, mutant-specific inhibition holds the promise of a much larger therapeutic window. The RAS-G12C mutant is particularly amenable to this approach, owing to the ability of cysteine to act as a nucleophile. In principle, it should be possible to achieve specificity for other mutants, such as G12D or G13D, through this or similar strategies. However, for some mutants, such as G12V, this is unlikely to be possible. It is unclear yet whether it will be possible to develop S-IIP-binding molecules that do not depend on covalent attachment to the mutant, but our experience optimizing S-IIP inhibitors has yielded promising results. The deep switch II pocket targeted by our G12C-specific molecules is only fully formed on compound binding.

Electrophilic
An electron-poor chemical group that interacts with an electron-rich nucleophile to form a covalent bond. In this reaction, the nucleophile donates a pair of electrons to form a bond with the electrophile.

Through our medicinal chemistry efforts, we have shown that the pocket can expand to accommodate even larger molecules, and by exploiting this feature we were able to improve binding by multiple orders of magnitude. The increased flexibility of KRAS relative to HRAS might provide sufficient difference to establish isoform specificity.

Directly blocking RAS–effector interactions has also been quite challenging. Although several small molecules have been reported to block this interaction by binding to RAS, all of them suffer from low potency and lack important structural and mechanistic information that will be crucial for further optimization. Allosterically blocking RAS–effector interactions will probably be more feasible than directly competing for the effector interface. Targeting the GDP state may be the most viable approach given the increased flexibility of RAS–GDP and the likelihood that RAS–GTP is tightly bound in effector complexes and therefore less accessible.

Targeting RAS in human cancer remains a substantial challenge. Since the discovery of this oncogene, a wealth of knowledge has accrued on its structure, dynamics and signalling biochemistry, and this information will play a crucial part in facilitating inhibitor development. Although RAS has long been considered an undruggable target, recent advances suggest this may yet be an attainable goal.

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The authors declare competing interests: see Web version for details.

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