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Small molecule binding sites on the Ras:SOS complex can be exploited for inhibition of Ras activation

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ABSTRACT

Constitutively active mutant KRas displays a reduced rate of GTP hydrolysis via both intrinsic and GTPase-activating protein-catalysed mechanisms, resulting in the perpetual activation of Ras pathways. We describe a fragment screening campaign using X-ray crystallography that led to the discovery of three fragment binding sites on the Ras:SOS complex. The identification of tool compounds binding at each of these sites allowed exploration of two new approaches to Ras pathway inhibition by stabilising or covalently modifying the Ras:SOS complex to prevent the reloading of Ras with GTP. Initially, we identified ligands that bound reversibly to the Ras:SOS complex in two distinct sites, but these compounds were not sufficiently potent inhibitors to validate our stabilisation hypothesis. We conclude by demonstrating that covalent modification of Cys118 on Ras leads to a novel mechanism of inhibition of the SOS-mediated interaction between Ras and Raf, and is effective at inhibiting the exchange of labelled GDP in both mutant (G12C and G12V) and wild type Ras.

INTRODUCTION

Members of the Ras family of small GTPases function as simple molecular switches. Ras is transformed into its active, GTP-bound state by interaction with a guanine nucleotide exchange factor (GEF) such as SOS, which catalyses release of GDP and allows binding of the more abundant GTP. In this active conformation, Ras is recognised by a range of direct effectors such as Raf, PI3 kinase and Ral proteins, which in turn can drive proliferation, survival and metastasis. Under normal physiological conditions, the intrinsic GTPase activity of Ras hydrolyses GTP back to GDP, causing inactivation. This process is significantly accelerated by GTPase activating proteins (GAPs) which can increase the intrinsic hydrolysis rate by up to 1000-fold. Mutations in the *KRAS* oncogene are present in a significant proportion of human tumors. Estimates of mutation prevalence vary by tumor type, however, they are known to be particularly associated with carcinomas of the lung, colon and pancreas.¹ Aberrant *KRAS* signaling drives an aggressive proliferative phenotype that is resistant to therapy and results in poor prognosis. These mutations, most commonly G12D and G12V, impair the ability of Ras to hydrolyse the terminal phosphate of GTP, limit sensitivity to GAP stimulation, and result in perpetual activation of Ras pathways.

Ras may be considered the archetypal ‘intractable target’ in oncology. Despite its characterisation over 30 years ago, reports on agents that directly target Ras, or indeed any GTPase, are limited.² Most drug discovery efforts have been directed against its more tractable downstream effectors, for example inhibitors of the kinases Raf and MEK.³ Guanine nucleotides exhibit picomolar affinity for Ras; therefore competitive binding at the nucleoside site is unlikely to be feasible, in contrast to conventional ATP mimetics in the field of kinase inhibition. It is possible that binding to Ras in an allosteric manner may promote loss of nucleotide, and indeed such a mechanism has been speculated for a reported inhibitor of the related GTPase Rac1, although no structural evidence for this is available⁴ and no obvious structural opportunities exist for Ras. A small molecule which is able to bind to Ras-GDP and block SOS binding should be able to limit Ras activation. This approach to Ras inhibition via disruption of a key protein-protein interaction has recently achieved validation by two independent groups. Sun *et al.* have reported a diverse array of small molecules that bind in a hydrophobic pocket located between the $\alpha 2$ helix of switch II (residues 60-74) and the central β sheet of KRas (G12D).⁵ These were identified using an NMR-based screen of 11,000 fragments using ¹⁵N-labelled protein, with the binding mode confirmed by co-crystallization.

Simultaneously, Maurer *et al.* reported structurally distinct small molecules that also bind to this pocket.⁶ Again, an NMR screen of 3,300 fragments using full-length KRas4B (G12D) yielded hits whose structures in

complex with KRas were confirmed by X-ray crystallography. Both approaches have demonstrated that binding at this site in the affinity range of 100s of μM is sufficient to inhibit the SOS-mediated activation of KRas. Since oncogenic KRas is locked in its active GTP-bound state, it is unclear whether such a strategy that prevents activation might be efficacious in tumors harbouring mutations in KRas, although a role in those tumors with active signalling through wild type Ras is plausible. Recent publications by Ostrem *et al.* and Lim *et al.* has shown that inhibition of the GTPase activity of the KRas(G12C) mutant can be achieved by irreversible binding of cysteine-reactive small molecules. Compounds are described which react close to the nucleotide binding site at Cys12, and either allosterically control the GTP affinity of the mutant⁷ or directly block the ability of GTP to bind.⁸ The approach relies on this specific KRas mutation to introduce the reactive cysteine thiolate, resulting in compounds which, while selective over wild type Ras, cannot inhibit other Ras mutants and which may be susceptible to resistance.

An alternative approach to preventing Ras activation may be envisioned in which a small molecule is able to bind at the interface of the Ras:SOS complex. Conceptually, such an agent might be able to stabilise these partners, inhibiting dissociation of SOS, and so prevent reloading of Ras with GTP. In theory this avoids some of the challenges implicit in other approaches, such as nucleotide competition, requiring inhibition of larger protein-protein interfaces, or mutant specificity, and might offer a more tractable binding pocket than the relatively featureless Ras protein alone. Precedent for this approach emerges from the observation that the natural product Brefeldin A (BFA) binds to a related small GTPase, Arf, and its exchange factor, ARNO.⁹ In this complex, GDP is also present, resulting in a quaternary complex where BFA is located in a hydrophobic pocket at the Arf-ARNO interface, one-third of which is contributed by ARNO and two-thirds by Arf. BFA acts as an uncompetitive inhibitor that stabilises an abortive Arf-GDP-ARNO complex resulting in inhibition of the secretory pathway in eukaryotic cells.¹⁰

Compounds have recently been reported by Burns *et al.* which bind to the Ras:SOS complex and increase the rate of SOS-mediated nucleotide exchange.¹¹ This is the reverse of the desired effect for an oncology therapy but it is notable that a small molecule can affect this pathway.

Although there is no reported structure of KRas in complex with an exchange factor, the structure of the closely homologous isoform HRas in 1:1 complex with a catalytic domain construct of SOS is known.¹² Inhibitors of activation of KRas were our primary goal owing to the greater frequency of oncogenic mutations in KRas than for other Ras isoforms, however, molecular modelling predicted high homology to HRas in complex with SOS, so this was utilized as a surrogate. Indeed, in the previously described KRas fragment screen, inhibitors were reported to

cross-react with both wild type K- and HRas.⁵ We therefore sought to use the HRas:SOS binary complex to screen for potential ligands via X-ray crystallography, through a process of soaking ‘cocktails’ of small fragments into crystals of the complex. Such an approach has been reported in inhibitor design against the bacterial enzyme nucleoside 2-deoxyribosyltransferase.¹³

RESULTS

X-ray screening of a fragment library

To obtain a crystal system suitable for X-ray fragment screening, a novel co-expression construct was generated comprising a C-terminal truncated HRas (1-166) and the catalytic domain of SOS encompassing the Ras exchanger motif and cdc25 domain (564-1049). This heterodimeric protein complex was stable during purification and used to reproduce the structural system described by Boriack-Sjodin *et al.*, wherein SOS has HRas bound to its catalytic site,¹² while the allosteric Ras binding site described by Margarit *et al.* is unoccupied.¹⁴

A library of 1160 fragments was organized into four-compound cocktails using an in-house shape fingerprint tool to maximize the shape diversity of compounds in each cocktail.¹⁵ Protein crystals were soaked in a stabilizing solution containing a final concentration of 5 mM of each fragment for 1 hour prior to being flash frozen and stored for data collection using a synchrotron radiation source. Datasets were processed, structures solved and initial refinement carried out using in-house software pipelines. Electron density maps were visually inspected to identify fragment binding. We observed significant attrition where a large number of compound cocktails were observed to abolish diffraction from the soaked HRas:SOS crystals. Of the crystals that survived, 25% were found to have small molecules present at one or more binding sites on SOS and, more intriguingly, at the interface between HRas and SOS. We were able to determine binding affinities for the fragments identified by crystal soaking using TROSY-HSQC solution NMR titrations with stable isotope-labeled HRas:SOS complex. A full description of the experimental methods used can be found in the Supplementary Results section. Crystallographic data collection and refinement statistics are detailed in **Supplementary Table 1**.

This paper describes three distinct small molecule binding sites that were identified on the complex, summarized in **Figure 1**.

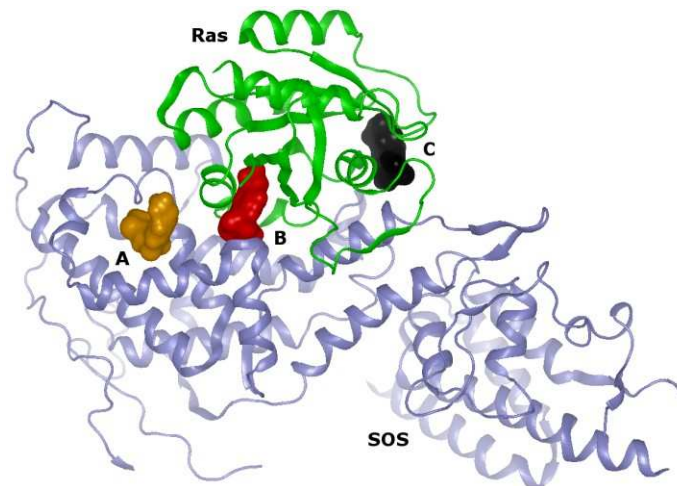


Figure 1 | Three fragment binding sites on the HRas:SOS complex. HRas in green, SOS in lilac. Fragment binding site A (gold) is located on SOS. Site B (red) is at the HRas:SOS binding interface. On the opposite face of the complex, covalent binding site C (black) is found on HRas.

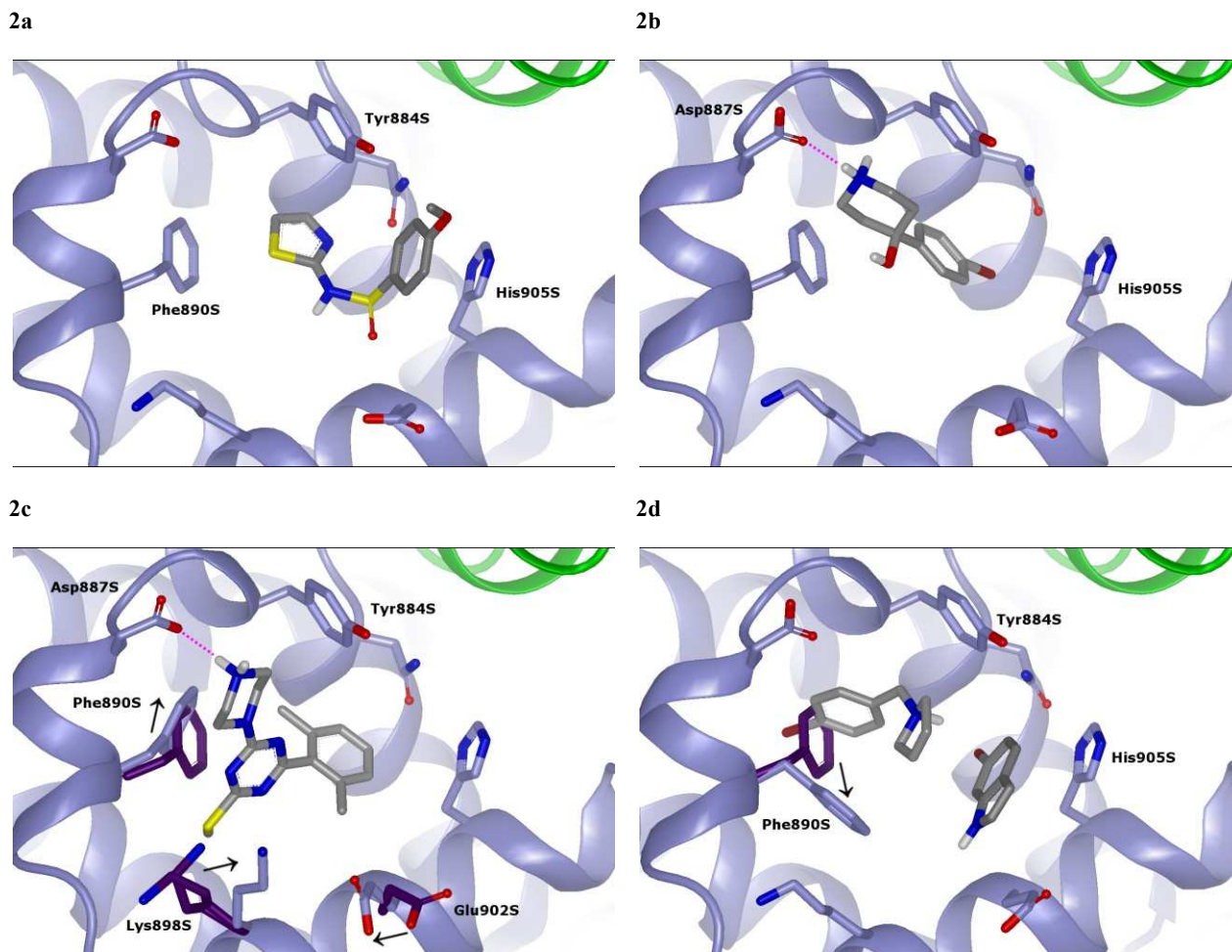


Figure 2 | Fragments binding at HRas:SOS site A. HRas colored green, SOS in lilac. Where side chain movements occur on ligand binding, their original positions in the HRas:SOS unbound structure *IBKD* are shown in dark purple for comparison, with arrows indicating direction of movement. **(a)** Binding of **1** results in no significant side chain movements. **(b)** The binding of **2** also causes no significant side chain perturbation. **(c)** Upon binding of **3**, side chain reorganization occurs at the front of the pocket opening a channel to accommodate the methylsulfanyl. **(d)** Simultaneous binding of **4a** and **4b** is accompanied by movement of Phe890S, opening up the back of the pocket.

Fragment binding site A on SOS

The fragment binding site we identified on the SOS protein (site A in **Fig. 1**) is a flexible bowl-shaped pocket lined with several residues showing mobile side chains. Upon binding of **1** to the SOS site A (**Fig. 2a**), no significant side-chain movements are observed relative to the unbound HRas:SOS structure (PDB *IBKD*). Binding appears to be

driven by aromatic ring interactions such as the edge-face interactions of the ligand thiazole with the phenyl ring of Phe890 of SOS ('Phe890S')¹⁶ (~3.6 Å), and a π -stacking conformation between His905S (~3.8 Å) and the ligand phenyl ring.

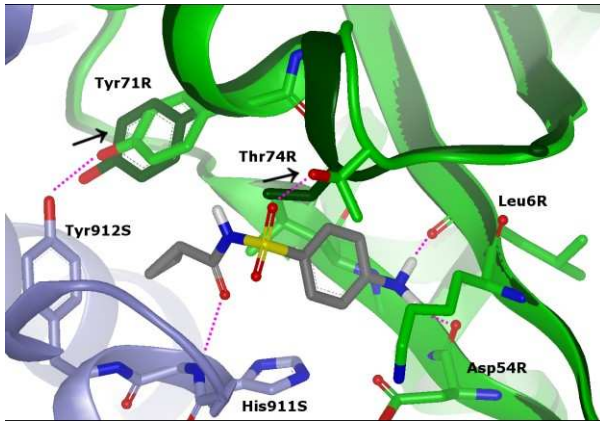
Binding of **2** in site A occurs without significant perturbation of protein side chains (**Fig. 2b**). The key interaction is a hydrogen bond between the piperidine nitrogen and Asp887S (2.8 Å). The bromine atom of **2** is situated in a lipophilic pocket near His905S.

Binding of **3** at site A appears to be 'shape-driven' (**Fig. 2c**). An edge-face interaction is observed between Tyr884S and the ligand phenyl (~4 Å), and a π -stacking interaction between Phe890S and the triazine (~3.7 Å). Phe890S is displaced by ~1.8 Å and rotated compared with the unbound structure *IBKD*. The basic nitrogen of the ligand piperidine forms a hydrogen bond with the carboxylic acid of Asp887S (3.0 Å). Lys898S changes from a dual to a single occupancy state, moving 4.3 Å to avoid a steric clash with the methylsulfanyl group of **3**, with concomitant movement of Glu902S which forms a salt bridge with Lys898S (4.0 Å).

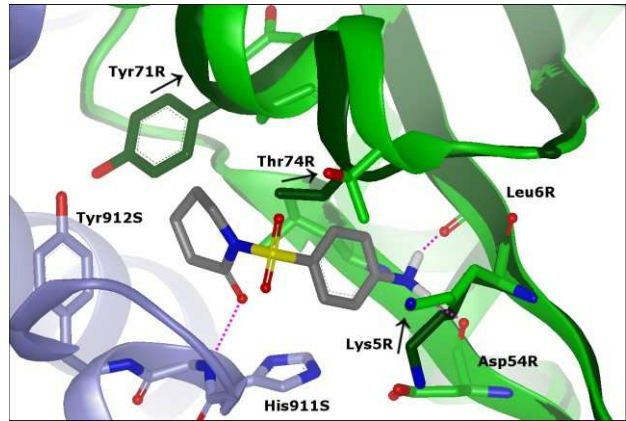
The crystal structure solved for **4a** and **4b** is notable because it comprises two fragments from the same cocktail which each bind to site A only in the presence of their partner (**Fig. 2d**). An NMR-derived pK_D was measurable for each fragment independently, however, given the small number of shifts observed, especially for **4b**, the binding sites monitored in the solution NMR experiments may not exactly coincide with that of the partnered binding observed in the crystal structure. In this structure Phe890S rotates 6.3 Å out towards solvent opening up a lipophilic pocket at the back of site A which is filled by the **4a** bromine. **4b** is stacked between His905S and the pyrrolidine of **4a**, whilst **4a** is sandwiched between Phe890S and Tyr884S, and lies perpendicular to the plane of **4b**.

Binding site A corresponds to the site recently reported by Burns, *et al.*¹¹ The three X-ray crystal structures they describe (*4NYI*, *4NYJ*, *4NYM*) show minimal side chain movements in site A compared with the unbound structure of SOS, and correspond most closely with our structures of **1** and **2** in complex with HRas:SOS (**Figs. 2a & 2b**).

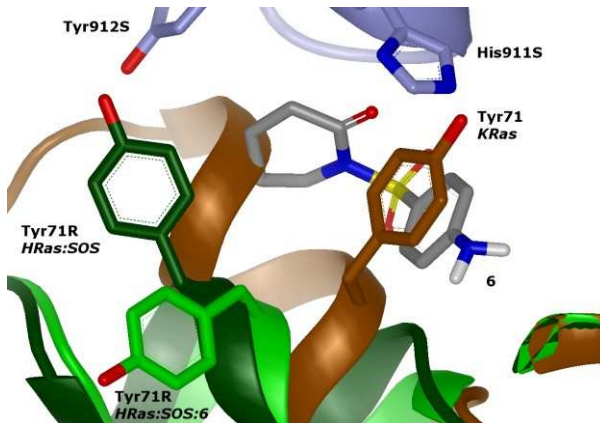
3a



3b



3c



3d

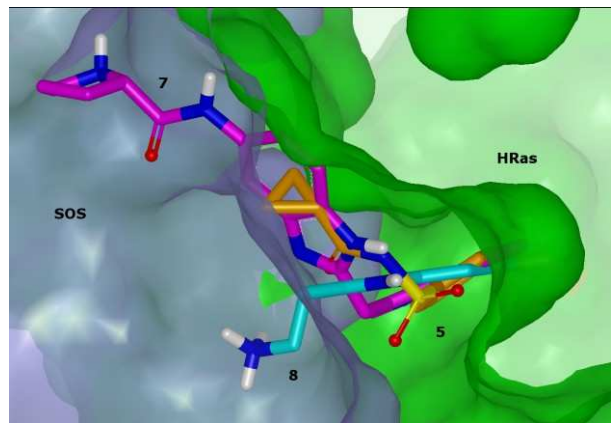


Figure 3 | Fragments binding at HRas:SOS site B. HRas colored green, SOS in lilac. Where side chain movements occur on ligand binding, their original positions in the HRas:SOS unliganded structure are shown in dark green for comparison. (a) **5** binds in site B at the interface between HRas and SOS, with resultant movements in HRas protein side chains shown. (b) **6** binding at the same location causes a larger shift in Tyr71R opening up a new pocket. (c) The Tyr71R side chain shows a wide range of conformations, as illustrated by an overlay of the KRas unbound structure *4EPR* (brown), HRas:SOS unliganded structure (dark green) and HRas:SOS:**6** complex structure (light green). (d) **5** (orange carbon atoms) binds in site B between the HRas (green) and SOS (lilac) surfaces in the HRas:SOS complex. In comparison, the KRas ligands **7**⁵ (magenta) and **8**⁶ (cyan) bind to KRas site 2 and place side chains into the space SOS would occupy in the HRas:SOS complex.

Fragment binding site B at the HRas:SOS interface

The binding site identified at the interface of HRas and SOS ('site B') corresponds to a site previously described in studies of uncomplexed Ras protein^{5,6} ('site 2'). However, while Ras ligands **7** and **8**, which were identified in these studies, inhibit SOS-mediated nucleotide exchange by preventing the binding of SOS to Ras, the fragments **5** and **6** we identified as binding to site B interact with both Ras and SOS, and we hoped to show that this additional binding interaction may serve to stabilise the protein-protein interaction.

Fig. 3a shows **5** in complex with HRas:SOS occupying the largely solvent-inaccessible site B at the HRas:SOS interface. Hydrogen bonding interactions are observed between the aniline of **5** and the backbone carbonyls of Leu6R (3.2 Å) and Asp54R (3.0 Å). The carbonyl of the acylsulfonamide group forms an H-bond with the backbone NH of His911S (2.8 Å). An edge-face interaction between the phenyl ring of **5** and His911S is also observed (~3.5 Å). The hydroxyl group of Thr74R is positioned within H-bonding distance (2.8 Å) of one of the S=O bonds of the acylsulfonamide. The cyclopropyl ring is positioned between Leu56R (~3.4 Å) and Tyr912S (~3.2 Å). A hydrogen-bonded tyrosine bridge (3.2 Å) between the two phenols of Tyr912S and Tyr71R links HRas and SOS. Comparison of the unbound structure (dark green) with this ligand-bound Ras:SOS complex shows limited movement for the majority of protein side chains in this region, with some notable exceptions. Thr74R moves 2.4 Å, making room for the ligand phenylsulfonamide. A small movement in the associated α -helix (residues Gln70R-Thr74R), opens up the binding pocket slightly. Tyr71R is displaced by 1.1 Å, accommodating the cyclopropyl group of **5**, however, it remains within H-bonding distance of Tyr912S. In contrast to HRas, SOS residues are unperturbed upon binding of **5**.

Synthesis of analogues of **5** led to acylsulfonamide **6** (**Fig. 3b**). Similar interactions are seen with Leu6R, Asp54R, and His911S. Compared with **5**, the distance between Thr74R and the S=O group of **6** increases to a range no longer conducive to hydrogen bonding. Lys5R rotates and moves ~4 Å. However, most notable is the movement of Tyr71R, which flips from the position observed in the unbound structure, where it forms a tyrosine bridge with Tyr912S, to an alternative location at the back of the pocket. This likely results from the increased steric bulk of the piperidinone ring of **6** compared with the isopropyl of **5**. The Tyr71R phenol OH moves 8.1 Å, opening up a new cavity which is occupied by a number of water molecules. **Figure 3c** shows the wide range of movement observed for the Tyr71R side chain amongst Ras structures. In unbound KRas (brown) the phenol group overlaps with site B. In the HRas (dark green):SOS (lilac) complex, Tyr71R forms a tyrosine bridge with Tyr912S and creates a pocket

between the two proteins into which compounds such as **5** may bind. Increasing the size of the small molecule ligand, such as in **6** (grey), is accompanied by further movement of Tyr71R (light green), opening up a larger binding pocket between the two proteins. Disruption of the HRas:SOS tyrosine bridge does not significantly alter the binding affinity of the fragments (**Table 1**).

X-ray crystal structures of ligands binding solely to Ras have been published since the work described in this paper was completed.^{5,6} Comparison of published crystal structures with that of the HRas:SOS:**5** complex shows that site B corresponds with the relatively shallow ‘binding site 2’ of the published fragments **7** and **8** on KRas (**Fig. 3d**). Compound **5** interacts with HRas through its acylsulfonamide motif, while the corresponding space is left unoccupied by **7** and **8**, which instead extend beyond the confines of site B and place polar groups in the spaces occupied by Tyr912S and His911S in the HRas:SOS complex. The resulting steric clash with SOS seems likely to contribute to the inhibition of nucleotide exchange that has been reported for **8**.⁶

The challenge of obtaining biological activity

While we demonstrated binding of fragments at sites A and B by X-ray crystallography, and were able to quantify their binding affinities by NMR, this did not translate into a measurable effect on nucleotide exchange (*vide infra*). This was not surprising, since we anticipated more potent compounds would be required to show functional activity. We synthesized analogues of **1** and **5**, varying both substituents of the sulfonamide. The most interesting compound identified was **6**, where the site B binding pocket was expanded. We also made analogues of triazine **3**, varying the heteroatoms in the triazine ring, switching the thiomethyl group, and varying the piperazine side chain. We found that the basicity of the piperazine ring was critical to maintain the high levels of solubility required for screening and crystallization at high concentrations. However, after several rounds of chemistry optimization, we were unable to improve the affinity of these hits significantly or demonstrate that such small ligands were measurably able to stabilize the Ras:SOS complex.

A growing body of literature suggests that use of a ligand which binds covalently to a protein target may offer a way to permanently deactivate protein function.^{7,8,17,18,19} Considering the picomolar affinity of Ras for GDP, we hypothesised that an irreversible inhibitor may afford the only realistic chance of inhibiting GTPase activity. We identified Cys118R in the HRas:SOS complex as a potentially reactive protein side chain proximal to the GDP

binding site on HRas (site C in **Fig. 1**) and set out to discover fragments which would irreversibly bind to it. This cysteine residue is conserved between HRas and KRas.

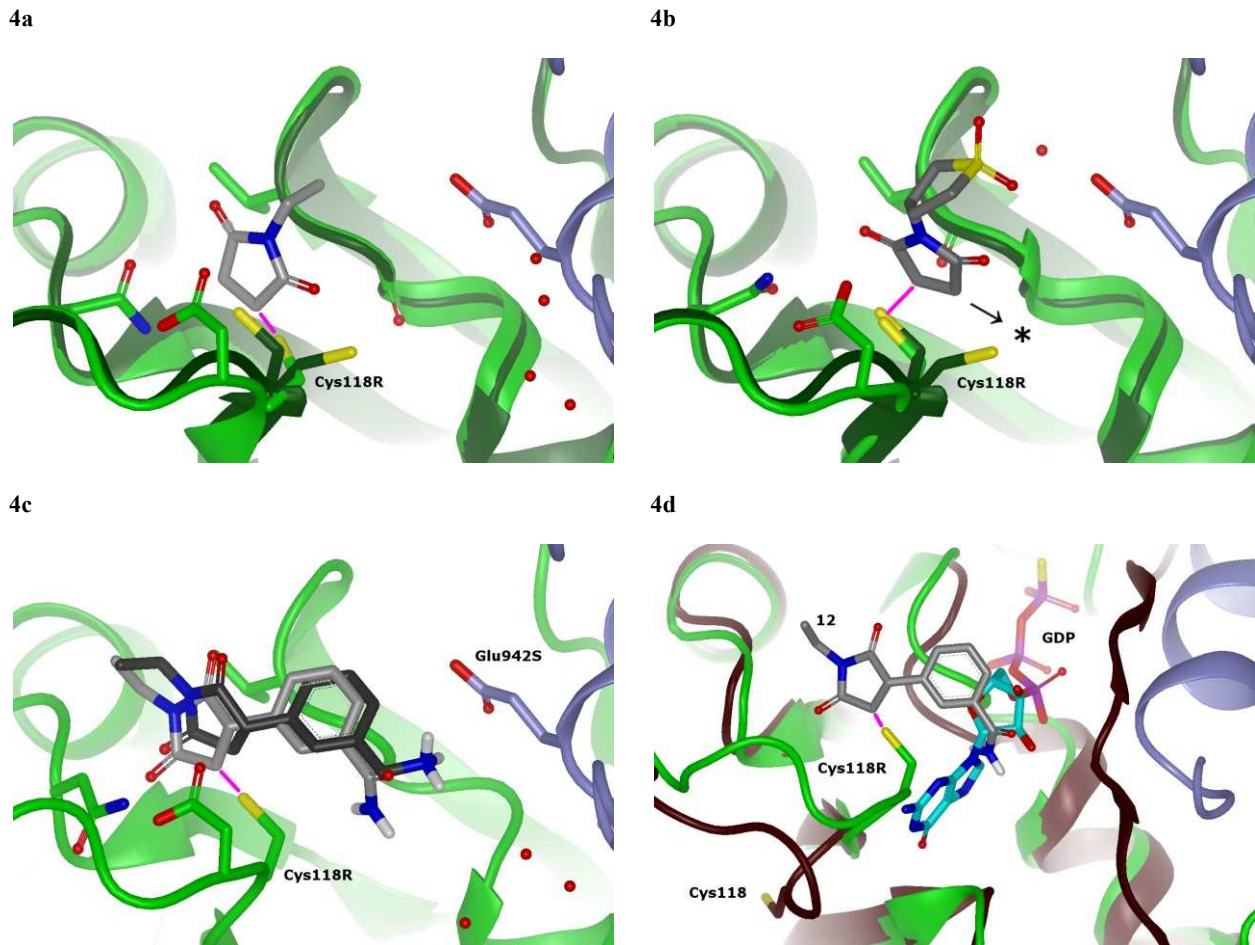


Figure 4 | Fragments binding at HRas:SOS site C. HRas colored green, SOS lilac. Where side chain movements occur on ligand binding, the relevant side chains from the HRas:SOS unbound structure *1BKD* are shown in dark green for comparison. **(a)** Covalent binding of **9** (represented by a magenta line) restrains the otherwise flexible side-chain of Cys118R into a single conformation. **(b)** Covalent binding of **10** causes Cys118R to adopt a different side-chain rotamer to that observed on binding of **9**, allowing the targeting of a new growth vector, marked by *. **(c)** The *meta*-substituents of **11** (dark grey) and **12** (light grey) follow a similar path down a lipophilic channel on Ras. **(d)** Overlay of HRas:SOS (green/lilac) in complex with **12** (grey) on the PDB KRas structure *4DSO* shows that **12** occludes the GDP (cyan) binding site on KRas (brown). A dramatic shift is seen in the position of Cys118 of KRas compared with Cys118R of the HRas:SOS:**12** complex.

Irreversible binders at HRas:SOS site C

A library of 400 compounds was selected, comprising members with fragment-like properties²⁰ (MWt \leq 300, cLogP 1-3, cLogD -1 to 3, \leq 2H-bond donors, \leq 6 H-bond acceptors, \leq 2rings, \leq 6 rotatable bonds and \leq 20 heavy atoms) and potentially reactive functional groups. These were identified from reports in the literature²¹ and by mining the data from an in-house glutathione reactivity assay,²² in which compounds with half-lives in the range of 15-3000 minutes were judged to be suitably reactive. Typical chemotypes represented in the library are shown in **Table 2**. Compounds were screened by mass spectrometry, by observing the mass increase of the HRas:SOS complex following compound addition.

We selected the reactive *N*-ethylmaleimide (NEM) group as an ideal electrophilic ‘warhead’ to bind to Cys118R. Other warheads were deemed either insufficiently reactive (giving incomplete cysteine adduction) or too reactive (reaction with multiple cysteine or histidine residues on the HRas:SOS complex) to progress. Using X-ray crystal structures of the HRas:SOS:inhibitor complex to guide design, we anticipated that building constructive non-covalent binding interactions with the protein (*i.e.* increasing K_i) might allow replacement of the maleimide with a less reactive motif (*i.e.* reducing K_{inact}) whilst retaining overall inhibitory activity.²³ Substitution of the maleimide double bond offered the possibility of modulating the electrophilicity of the Michael acceptor warhead, once additional non-covalent interactions had been identified.

Promising hits from the mass spectrometry screen of reactive fragments included *N*-substituted maleimides **9** and **10**. X-ray crystal structures obtained subsequently showed **9** (**Fig. 4a**) and **10** (**Fig. 4b**) binding with a distance of 1.8 Å between the ligand C-3 and the Cys118R sulfur atom, and continuous electron density, confirming that a covalent bond is formed between ligand and protein (**Supplementary Fig. 1**). Interestingly, the pyrrolidine-2,5-dione rings in **9** and **10** were observed to adopt different orientations. The C-4 atom in **10** presented an ideal vector for growing the fragment into a lipophilic channel (indicated with * in **Fig. 4b**), and a set of analogues was synthesized to vary at this position. Analogues **11** and **12**, which carry a *meta*-substituted phenyl ring at C-4, were successfully crystallized with HRas:SOS, adopting similar binding modes in which the C-4 substituent extends down a lipophilic groove adjacent to Glu942S (**Fig. 4c**). With a *meta*-substituent effectively anchoring the pendant phenyl group in one orientation, compounds **13** and **14** were designed to probe interactions with Glu942S, *via* a *bis*-3,5-substituted ring.

Comparison of the published KRas:GDP crystal structure *4DSO* and our HRas:SOS structures shows reorganization of the GDP binding pocket following association of Ras and SOS (**Fig. 4d**). This reconfiguration involves Cys118R, which is otherwise buried in the interior of the KRas:GDP complex where it may be somewhat protected from electrophiles. When SOS binds to Ras and displaces GDP, the Cys118R residue is exposed to solvent, rendering it more vulnerable to reaction with **9-14**. Once such a reaction has occurred, the covalently-bound fragment partly occludes the nucleotide binding site and could potentially also prevent reorganization of the Cys118R loop of Ras, thus locking the protein into the catalytically inactive Ras:SOS complex.

As we had hoped, fragments which covalently modified HRas in the crystal environment also demonstrated functional activity in preventing wild type KRas activation. Due to the spectral properties of these compounds it was not always possible to use previously-reported methods for monitoring nucleotide exchange with MANT-labeled GDP⁶ as many of the compounds interfered with the excitation/emission spectra. Therefore, a novel homogeneous time-resolved fluorescence (HTRF) assay was designed to profile this series. In this homogenous assay, biotin-KRas:GDP was labeled with streptavidin europium and GST-Raf was labeled with anti-GST XL665. Activation of KRas was initiated with the addition of SOS and GTP γ S. Upon binding of the active GTP γ S-bound streptavidin:biotin-KRas to Raf-GST:Anti-GST XL665, the europium donor and XL665 acceptor are brought into close proximity resulting in an increased acceptor emission at 665 nm (**Supplementary Fig. 2**).

Compounds **10-14** were pre-incubated with either KRas:GDP, SOS alone or a KRas:GDP:SOS mixture for 2.5 hours to allow the compound to react, then inactivated by the addition of dithiothreitol (DTT). All samples were then made up to contain the same concentrations of KRas:GDP and SOS, and incubated with streptavidin-europium for 4 hours. In a separate reaction GST-Raf was incubated with anti-GST XL665. The exchange reaction was initiated by the addition of GTP γ S and GST-Raf. The activation of KRas was then monitored via HTRF between the streptavidin-europium and XL665 upon binding of active KRas to GST-Raf.

Complete inhibition of KRas activation was achieved only when **10-14** were pre-incubated with KRas:GDP:SOS (**Fig. 5**), which supports the hypothesis that Cys118R becomes more accessible during the conformational changes which occur during SOS-mediated nucleotide exchange. Neither pre-incubation of **11-13** with KRas:GDP with subsequent addition of SOS nor pre-incubation with SOS followed by addition of KRas:GDP led to significant inhibition of the Ras-Raf interaction. A modest degree of KRas inhibition (~40-50%) was observed when **10** and **14** were pre-mixed with KRas alone. This may be due to the increased reactivity of the Michael

acceptors in these molecules causing them to behave in a less specific manner, as we had observed during the initial irreversible fragment screening campaign; **10** is an unsubstituted maleimide and **14** has two strong electron withdrawing groups on its phenyl ring. The inhibitory effects of **10-14** could be eliminated by the addition of DTT (1 mM) during the pre-incubation step, presumably because DTT reacted with the maleimide warhead before it was able to irreversibly modify the KRas:SOS complex.

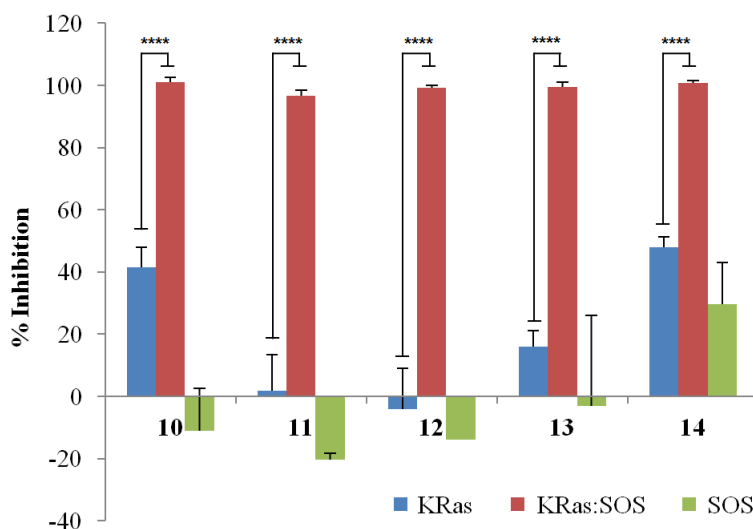


Figure 5 | Inhibition of KRas:SOS functional activity by irreversible inhibitors. Compounds **10-14** (200 μ M) were pre-incubated with either GDP-loaded Biotin-KRas alone (blue), GDP-loaded biotin-KRas plus SOS (red) or SOS alone (green). All samples were then made up to contain the same concentrations of KRas:GDP and SOS. **10-14** caused complete inhibition of functional activity when pre-mixed with KRas:GDP and SOS together, but modest or no inhibition when pre-mixed with KRas:GDP or SOS alone. No inhibition was observed with non-covalent compounds **1**, **3** or **5** (data not shown). Error bars show one standard deviation. Comparison of KRas and KRas:SOS data using the unpaired T-test shows all results to be statistically significant, $p < 0.0001$ (****). Full data are shown in **Supplementary Table 2**.

Site C irreversible binders show time-dependent inhibitory activity against both wild type and mutant KRas

The inactivation of wild type KRas, KRas(G12V) and KRas(G12C) was monitored using a MANT-dGDP assay and a fluorescence assay-compatible compound, **12**. Initially, the KRas constructs were pre-equilibrated with MANT-dGDP to replace the bound unlabelled nucleotide which co-purifies with KRas. All three constructs then underwent SOS-mediated nucleotide exchange which resulted in an increase in fluorescence upon MANT-dGDP binding. At

equilibrium, the reaction cycled between the MANT-dGDP and the unlabelled nucleotide. Upon the addition of **12** (200 μ M), time dependent inactivation of each of the KRas constructs was observed (**Fig. 6**). An excess amount of unlabelled GDP was included as a control to compete with the MANT-dGDP resulting in a loss of fluorescence. Since the rate of reduction of fluorescence is similar with the addition of either excess GDP or irreversible inhibitor **12**, this supports the hypothesis that nucleotide exchange is driven in both cases by the rate of association of Ras:SOS. If excess GDP is present, SOS associates with Ras, the MANT-dGDP is displaced, and is then replaced with unlabelled GDP when SOS dissociates. If **12** is present when SOS associates with Ras and displaces MANT-dGDP, the resulting KRas:SOS, KRas(G12V):SOS or KRas(G12C):SOS complex reacts with the irreversible inhibitor at Cys118R, forming a dead-end complex which prevents the subsequent re-binding of MANT-dGDP.

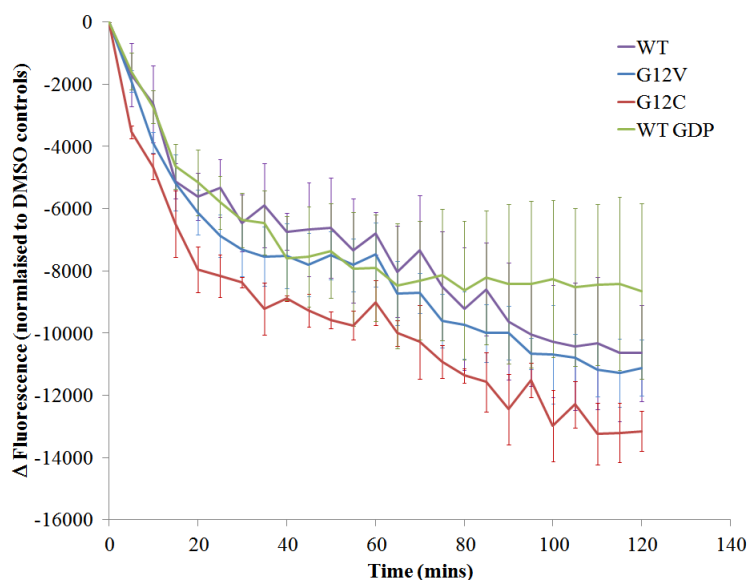


Figure 6 | Inhibition of SOS-mediated MANT-labelled nucleotide exchange by **12 in a range of Ras mutants.** KRas wild type, KRas(G12V) and KRas(G12C) were equilibrated with MANT-dGDP which is environmentally sensitive and significantly increases in fluorescent quantum yield when protein bound. **12** was added (200 μ M) and inactivation was monitored over time. A reduction in fluorescence was observed against all three KRas constructs (red, blue and purple lines). An excess of GDP was used as a control to compete off the MANT-dGDP (green line). Color-coded error bars for measurements are also shown. Full data are shown in **Supplementary Table 3**.

DISCUSSION AND CONCLUSIONS

Our work with non-covalent fragments showed that it was possible to characterize compounds from multiple series binding on SOS and at the HRas:SOS interface, however, none of these compounds were sufficiently potent to show

functional activity in the Ras:Raf HTRF assay. Synthesis of analogues of **1**, **3**, **5** and **6** led to compounds with similar binding affinities to the parent fragments and unproductive SAR. The report by Burns *et al.* that small molecules which bind at SOS site A cause an increase in the rate of SOS-mediated nucleotide exchange¹¹ was not corroborated by experiments with our fragments, suggesting that more dramatic changes or hybridization of series may be required to achieve sufficient binding affinity. While we were not able to detect functional biological activity for the probes identified at sites A and B, we believe that our observations of specific ligandable²⁴ interaction points may suggest a potential way forward using larger molecules such as macrocycles²⁵ or constrained peptides.²⁶ The distance between the closest atoms on non-covalent fragments bound in sites A and B is 9.1 Å. Precedent for linking distant fragment sites suggests that connecting two weak fragments can bring very large increases in potency, but is most likely to succeed when fragments are significantly closer together than sites A and B. If fragment ligands for sites A and B could be iteratively grown towards each other, however, this strategy may be more likely to succeed.²⁷

While our efforts to inhibit Ras by stabilization of the Ras:SOS complex by fragments binding in a reversible manner were unsuccessful, we showed that reactive fragments which covalently modify site C near to the nucleotide binding site were effective Ras inhibitors. We demonstrated complete inhibition of KRas:SOS functional activity following reaction of maleimides **10-14** with Cys118R, a previously undescribed method of inhibition of Ras. A synthetic chemistry campaign led to fragments which showed a consistent binding mode at site C, and resulted in inhibition of wild type KRas in a novel HTRF assay measuring the Ras-GTP:Raf interaction. Several potential vectors were identified for further growth of these inhibitors; for example, along the lipophilic channel from the *meta* position of phenyl groups in **11-14**, or from the maleimide *N*-substituent towards SOS.

An important objective for a covalent approach to Ras:SOS inhibition is to reconcile the need to measure variations in potency of molecules – and hence structure-activity relationships – with the requirement for a warhead of high reactivity necessary to observe any inhibitory activity. With the fragment series based on maleimides, although it was possible to measure potency and inhibition of nucleotide exchange, this measure was driven largely by K_{inact} , and small changes in K_i were difficult to detect. The determination of K_i changes is important to assist the design of more potent, but less reactive inhibitors. Tuning the reactivity of the covalent modifier by modulating the electrophilicity of the Michael acceptor warhead could potentially be achieved by using an electron-donating linker such as ether or amine. Kathman *et al.* recently published an example of this approach being used successfully against a cysteine protease.²⁸

Our work has explored three small molecule binding sites on the Ras:SOS complex, and details synthetically accessible small molecule probes that bind at each site. We demonstrated inhibition of KRas functional activity with compounds which bind irreversibly at site C, and showed that covalent binding with HRas in a protein crystal environment translates to nucleotide exchange inhibition in the more oncology disease-relevant isoform KRas. Furthermore, this mechanism of KRas inactivation appears to be applicable to mutant forms of KRas which also share a Cys118 residue, such as KRas(G12C) and KRas(G12V). Although significant work remains to optimize the biological activity of the compounds we have described at site C, our discoveries open the way to a new strategy to target aberrant Ras signalling by intervening in the SOS-mediated activation of Ras.

ANCILLARY INFORMATION

Supporting Information Available: Experimental methods and any associated references are available in the online version of this paper. This material is available free of charge via the Internet at <http://pubs.acs.org>.

PDB ID codes: 4URU, 4URV, 4URW, 4URX, 4URY, 4URZ, 4US0, 4US1, 4US2.

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Author Contributions: J.J.G.W. designed the fragment libraries and compounds, analyzed data, supervised the chemistry team and wrote the manuscript. M.A. designed and performed mass spectrometry experiments and analyzed data. A.L.B. designed the research plan and wrote the manuscript. K.B., G.F., P.P. & G.W. designed synthetic chemistry routes, synthesized and characterized compounds. C.B. designed and performed protein crystallization experiments. C.C. designed biological experiments. K.E. performed K_D determinations via biomolecular NMR. P.F. & L.S. provided computational support for fragment library and compound design. M.R.V.F. analyzed data, designed compounds, supervised the chemistry team and wrote the manuscript. J.G.K. conceived the target hypothesis, oversaw the project team and wrote the manuscript. T.N. analyzed data and designed compounds. R.O. designed, expressed and purified protein constructs. S.J.P. purified proteins, designed,

performed and interpreted X-ray crystallography experiments and designed compounds. J.T. designed and performed biological experiments. J.A.T. designed, performed and interpreted X-ray crystallography experiments and designed compounds.

REFERENCES

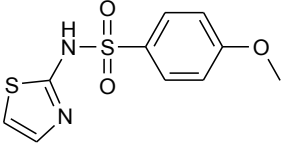
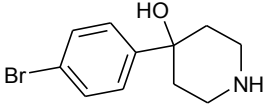
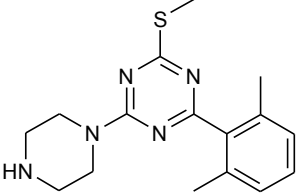
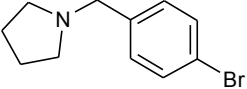
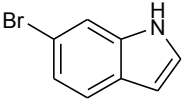
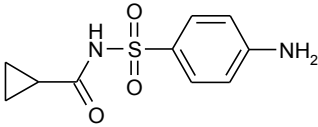
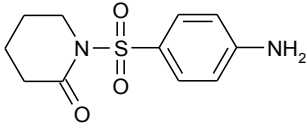
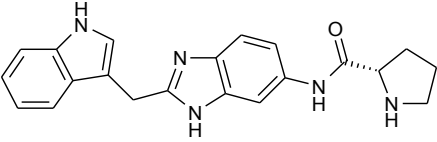
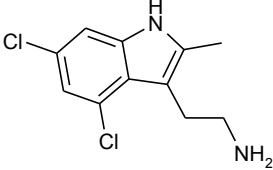
1. Karnoub, A. E.; Weinberg, R. A. Ras oncogenes: split personalities. *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 517-531.
2. Spiegel, J.; Cromm, P. M.; Zimmermann, G.; Grossmann, T. N.; Waldmann, H. Small-molecule modulation of Ras signalling. *Nat. Chem. Biol.* **2014**, *10*, 613-622.
3. Rusconi, P.; Caiola, E.; Broggin, M. RAS/RAF/MEK inhibitors in oncology. *Curr. Med. Chem.* **2012**, *19*, 1164-1176.
4. Shutes, A.; Onesto, C.; Picard, V.; Leblond, B.; Schweighoffer, F.; Der, C. J. Specificity and mechanism of action of EHT 1864, a novel small molecule inhibitor of Rac family small GTPases. *J. Biol. Chem.* **2007**, *282*, 35666-35678.
5. Sun, Q.; Burke, J. P.; Phan, J.; Burns, M. C.; Olejniczak, E. T.; Waterson, A. G.; Lee, T.; Rossanese, O. W.; Fesik, S. W. Discovery of small molecules that bind to K-Ras and inhibit Sos-mediated activation. *Angew. Chem. Int. Ed.* **2012**, *51*, 6140-6143.
6. Maurer, T.; Garrenton, L. S.; Oh, A.; Pitts, K.; Anderson, D. J.; Skelton, N. J.; Fauber, B. P.; Pan, B.; Malek, S.; Stokoe, D.; Ludlam, M. J.; Bowman, K. K.; Wu, J.; Giannetti, A. M.; Starovasnik, M. A.; Mellman, I.; Jackson, P. K.; Rudolph, J.; Wang, W.; Fang, G. Small-molecule ligands bind to a distinct pocket in Ras and inhibit SOS-mediated nucleotide exchange activity. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 5299-5304.
7. Ostrem, J. M.; Peters, U.; Sos, M. L.; Wells, J. A.; Shokat, K. M. K-Ras(G12C) inhibitors allosterically control GTP affinity and effector interactions. *Nature* **2013**, *503*, 548-551.
8. Lim, S. M.; Westover, K. D.; Ficarro, S. B.; Harrison, R. A.; Choi, H. G.; Pacold, M. E.; Carrasco, M.; Hunter, J.; Kim, N. D.; Xie, T.; Sim, T.; Jänne, P. A.; Meyerson, M.; Marto, J. A.; Engen, J. R.; Gray, N. S. Therapeutic targeting of oncogenic K-Ras by a covalent catalytic site inhibitor. *Angew. Chem. Int. Ed. Engl.* **2014**, *53*, 199-204.
9. Renault, L.; Guibert, B.; Cherfils, J. Structural snapshots of the mechanism and inhibition of a guanine nucleotide exchange factor. *Nature* **2003**, *426*, 525-530.

10. Peyroche, A.; Antony, B.; Robineau, S.; Acker, J.; Cherfils, J.; Jackson C. L. Brefeldin A acts to stabilize an abortive ARF-GDP-Sec7 domain protein complex: Involvement of specific residues of the Sec7 domain. *Mol. Cell* **1999**, *3*, 275–285.
11. Burns, M. C.; Sun, Q.; Daniels, R. N.; Camper, D.; Kennedy, J. P.; Phan, J.; Ojejniczak, E. T.; Lee, T.; Waterson, A. G.; Rossanese, O. W.; Fesik, S. W. Approach for targeting Ras with small molecules that activate SOS-mediated nucleotide exchange. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 3401-3406.
12. Boriack-Sjodin, P. A.; Margarit, S. M.; Bar-Sagi, D.; Kuriyan, J. The structural basis of the activation of Ras by Sos. *Nature* **1998**, *394*, 337-343.
13. Bosch, J.; Robien, M. A.; Mehlin, C.; Boni, E.; Riechers, A.; Buckner, F. S.; Van Voorhis, W. C.; Myler, P. J.; Worthey, E. A.; DeTitta, G.; Luft, J. R.; Lauricella, A.; Gulde, S.; Anderson, L. A.; Kalyuzhniy, O.; Neely, H. M.; Ross, J.; Earnest, T. N.; Soltis, M.; Schoenfeld, L.; Zucker F.; Merritt E. A.; Fan E.; Verlinde C. L.; Hol W. G. Using fragment cocktail crystallography to assist inhibitor design of *Trypanosoma brucei* nucleoside 2-deoxyribosyltransferase. *J. Med. Chem.* **2006**, *49*, 5939-5946.
14. Margarit, M. S.; Sondermann H.; Hall, B. E.; Nagar, B.; Hoelz, A.; Pirruccello, M.; Bar-Sagi, D.; Kuriyan, J. Structural evidence for feedback activation by Ras-GTP of the Ras-specific nucleotide exchange factor SOS. *Cell* **2003**, *112*, 685-695.
15. Haigh, J. A.; Pickup, B. T.; Grant, J. A.; Nicholls, A. Small molecule shape-fingerprints. *J. Chem. Inf. Model.* **2005**, *45*, 673-684.
16. Protein subunit nomenclature: Leu6R refers to leucine 6 on the Ras protein chain, and His911S refers to histidine 911 on SOS.
17. Kalgutkar, A. S.; Dalvie, D. K. Drug discovery for a new generation of covalent drugs. *Expert Opin. Drug Discov.* **2012**, *7*, 561-581.
18. Johnson, D. S.; Weerapana, E.; Cravatt, B. F. Strategies for discovering and derisking covalent, irreversible enzyme inhibitors. *Future Med. Chem.* **2010**, *2*, 949-964.
19. Mah, R.; Thomas, J. R.; Shafer, C. M. Drug discovery considerations in the development of covalent inhibitors. *Bioorg. Med. Chem. Lett.* **2014**, *24*, 33-39.
20. Congreve, M.; Carr, R.; Murray, C.; Jhoti, H. A 'rule of three' for fragment-based lead discovery? *Drug Discov. Today* **2003**, *8*, 876-877.

21. Potashman, M. H.; Duggan, M. E. Covalent modifiers: An orthogonal approach to drug design. *J. Med. Chem.* **2009**, *52*, 1231-1246.
22. MacFaul, P. A.; Morley, A. D.; Crawford, J. J. A simple in vitro assay for assessing the reactivity of nitrile containing compounds. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 1136-1138.
23. Ward, R. A.; Anderton, M. J.; Ashton, S.; Bethel, P. A.; Box, M.; Butterworth, S.; Colclough, N.; Chorley, C. G.; Chuaqui, C.; Cross, D. A. E.; Dakin, L. A.; Debreczeni, J. É.; Eberlein, C.; Finlay, M. R. V.; Hill, G. B.; Grist, M.; Klinowska, T. C. M.; Lane, C.; Martin, S.; Orme, J. P.; Smith, P.; Wang, F.; Waring, M. J. Structure- and reactivity-based development of covalent inhibitors of the activating and gatekeeper mutant forms of the Epidermal Growth Factor Receptor (EGFR). *J. Med. Chem.* **2013**, *56*, 7025-7048.
24. Edfeldt, F. N.; Folmer, R. H. A.; Breeze, A. L. Fragment screening to predict druggability (ligandability) and lead discovery success. *Drug Discov. Today* **2011**, *16*, 284-287.
25. Giordanetto, F.; Kihlberg, J. Macrocyclic drugs and clinical candidates: What can medicinal chemists learn from their properties? *J. Med. Chem.* **2014**, *57*, 278-295.
26. Henchey, L. K.; Jochim, A. L.; Arora, P. S. Contemporary strategies for the stabilization of peptides in the α -helical conformation. *Curr. Opin. Chem. Biol.* **2008**, *12*, 692-697.
27. Ward, R. A.; Brassington, C.; Breeze, A. L.; Caputo, A.; Critchlow, S.; Davies, G.; Goodwin, L.; Hassall, G.; Greenwood, R.; Holdgate, G. A.; Mrosek, M.; Norman, R. A.; Pearson, S.; Tart, J.; Tucker, J. A.; Vogtherr, M.; Whittaker, D.; Wingfield, J.; Winter, J.; Hudson, K. Design and synthesis of novel lactate dehydrogenase A inhibitors by fragment-based lead generation. *J. Med. Chem.* **2012**, *55*, 3285-3306.
28. Kathman, S. G.; Xu, Z.; Statsyuk, A. V. A fragment-based method to discover irreversible covalent inhibitors of cysteine proteases. *J. Med. Chem.* **2014**, *57*, 4969-4974.

TABLES

Compound	Structure	HRas:SOS NMR binding K _D mM (std.err.)	Binding site	Protein Data Bank Accession Code

1		6.0 (± 6.0) ^a	A	4URU
2		1.7 (± 0.2)	A	4URV
3		1.6 (± 0.1)	A	4URW
4a		1.3 (± 0.2)	A ^b	4URX
4b		6.0 (± 3.0) ^a	A ^b	4URX
5		6.0 (± 2.0)	B	4URY
6		3.0 (± 0.4)	B	4URZ
7		nd ^c	KRas site 2 ^d	4EPY
8		nd ^c	KRas site 2 ^e	4DST

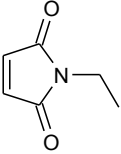
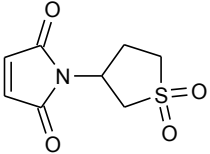
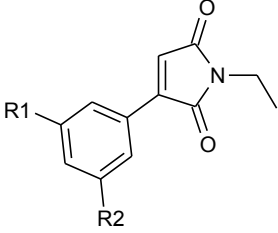
9		nd ^c	C	4US0
10		nd ^c	C	
11-14				
11	R1 = CH ₂ NH ₂ R2 = H	nd ^c	C	4US1
12	R1 = CONH ₂ R2 = H	nd ^c	C	4US2
13	R1 = COOH R2 = CN	nd ^c	C	
14	R1 = CONH ₂ R2 = Cl	nd ^c	C	

Table 1 | Structure and NMR binding K_D of HRas:SOS ligands. The crystal structures for HRas:SOS:compound complexes are deposited in the Protein Data Bank (PDB, <http://www.rcsb.org>) under the accession codes above. Values for K_D determined by NMR chemical shift mapping are shown with the standard error of the mean for each measurement. ^a Significant precipitation was observed. ^b **4a** and **4b** only bound when both ligands were present in the same cocktail. ^c K_D measurements were not generated for the literature compounds **7** & **8**, which bind to Ras alone, or covalently bound compounds **9** – **14**. ^d See reference 5. ^e See reference 6.

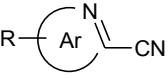
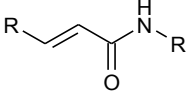
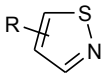
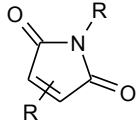

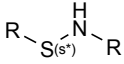
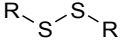
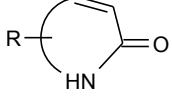
 <p>Heteroaryl nitrile^a</p>	 <p>Vinyl amide^a</p>	 <p>Isothiazole^b</p>	 <p>Maleimide^b</p>
 <p>Thiol^a</p>	 <p>Sulfanylamino^b</p>	 <p>Disulfide^a</p>	 <p>Vinyl lactam^b</p>

Table 2 | Examples of reactive functional groups represented in the covalent fragment library. R indicates any functional group.^a See reference 21. ^b See reference 22.

Table of contents graphic

