Structural and biochemical insights into heterotetramer formation of oncogenic K-Ras4BG12V and Rgl2, a RalA/B activator

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Abstract

About a quarter of total human cancers carry mutations in Ras isoforms. Accumulating evidence suggests that small GTPases, RaA and RaB, and their activators, Ra guanine nucleotide exchange factors (RaGEFs), play an essential role in oncogenic Ras-induced signalling. We studied the interaction between human K-Ras4B and the Ras binding domain (RBD) of Rgl2 (Rgl2RBD), one of the RBD-containing RaGEFs. We show that the G12V oncogenic K-Ras4B mutation increases the affinity with Rgl2RBD. The crystal structure of the K-Ras4BG12V: Rgl2RBD complex shows a 2:2 heterotetramer where the Switch I and Switch II regions of each K-RasG12V interact with both Rgl2RBD molecules. This structural arrangement is highly similar to the H-RasE31K:RALGDS crystal structure and is distinct from the well-characterised Ras:RAF RBD complexes. Importantly, the G12V mutation was found at the dimer interface of K-Ras4BG12V with its partner. Solution state NMR and mass photometry analyses support the heterotetramer formation. Our study reveals a distinct mode of Ras:effector complex formation by RaGEFs, and offers a possible mechanistic explanation for how the oncogenic K-Ras4BG12V hyperactivates the RaA/B pathway.
**Main Text**

**Introduction**

Ras belongs to a family of small G protein that switches between two states, GDP-bound form ($\text{Ras}^{\text{GDP}}$) and GTP-bound form ($\text{Ras}^{\text{GTP}}$), and regulates a wide range of cellular activities (Cox and Der, 2010). Guanine nucleotide exchange factors (GEFs), typically activated by growth factor signalling, mediate the conversion of $\text{Ras}^{\text{GDP}}$ to $\text{Ras}^{\text{GTP}}$, whilst the GTP-bound status lasts only transiently as the intrinsic GTPase activity, aided by the GTPase activating Proteins (GAPs), hydrolyses the bound GTP into GDP (Vetter and Wittinghofer, 2001). Ras acts as a signalling hub where the $\text{Ras}^{\text{GTP}}$, but not $\text{Ras}^{\text{GDP}}$, physically interacts with multiple Ras effectors, which then transmit the signal to downstream molecules, including ERK, Akt and small G proteins RalA and RalB (Simanshu et al., 2017).

There are three human RAS genes, K-RAS, N-RAS and H-RAS, and as K-RAS is alternatively spliced at exon 4, the three RAS genes produce four Ras isoforms K-Ras4A, K-Ras4B, N-Ras and H-Ras (Castellano and Santos, 2011). Among them, K-RAS4B typically represents more than half of all RAS transcripts (Newlaczyl et al., 2017). The Catalogue Of Somatic Mutations In Cancer (COSMIC) shows that about 25% of all cancers carry mutations in RAS genes, and K-RAS is responsible for about 70% of these mutations (COSMIC, v.95). Hence, it is vital to obtain more insights into how the oncogenic K-Ras signalling leads to cancers.

Extensive earlier biochemical studies revealed that oncogenic RAS mutations cause a reduction of the GTP hydrolysis rate, generating an increased population of $\text{Ras}^{\text{GTP}}$ (Bollag and McCormick, 1991; Der et al., 1986; Gibbs et al., 1984; Manne et al., 1985; McGrath et al., 1984; Scheffzek et al., 1997). This likely leads to an overactivation of the downstream signalling pathways and therefore is considered the major cause of the K-Ras oncogenicity. In addition, recent biochemical, structural and molecular modelling studies indicate that oncogenic RAS mutations affect the interaction kinetics with the effector molecules and may trigger a biased overactivation of a set of effectors (Hunter et al., 2015; Mazhab-Jafari et al., 2015; Pantsar et al., 2018; Smith and Ikura, 2014). Therefore, obtaining more insights into the Ras-effector interaction mechanism is essential to understanding the oncogenic-Ras mediated tumorigenesis process.
Among Ras effectors, Raf kinase and PI3K, which ignite ERK and Akt signalling respectively, have attracted much attention, especially because they were found not only to be activated by oncogenic Ras but also to be able to carry oncogenic mutations themselves (Chalhoub and Baker, 2009; Maurer et al., 2011). However, accumulating evidence suggests that the misregulation of Ral GTPases, RalA and RalB, rather than ERK and Akt signalling pathways, may be the initial trigger of oncogenic-Ras induced tumorigenesis. For example, in the oncogenic K-ras(G12D) knock-in mouse model, although neoplastic and developmental defects were observed, hyper-activation of ERK or Akt was undetected (Tuveson et al., 2004). In humans, ERK hyper-activation is often missing in cancer cell lines and tissues with oncogenic Ras mutations, whereas RalA and RalB are essential in oncogenic-Ras induced cell proliferation, motility and tumorigenesis (Campbell et al., 2007; Lim et al., 2005; Lim et al., 2006; Luo and Sharif, 1999; Miller et al., 1997; Yip-Schneider et al., 1999; Yip-Schneider et al., 2001; Zago et al., 2018). Therefore, a biased misregulation of the Ral GTPases may be the critical feature behind Ras oncogenicity. Indeed, NMR-based effector competition assays suggested the intriguing possibility that the oncogenic Ras molecules may develop an altered effector preference leading to a biased hyperactivation of RalA (Smith and Ikura, 2014).

Activation of RalA and RalB is mediated by guanine nucleotide exchange factors (RalGEFs). There are eight RalGEFs reported for humans, and four of them, RALGDS, Rgl1, Rgl2 and Rgl3, have a Ras-association (RA) domain (in this work, we generically call it Ras Binding Domain (RBD)) responsible for Ras-binding (Apken and Oeckinghaus, 2021). RALGDS was the first to be identified as a Ras effector among these RalGEFs and is by far the most studied (Neel et al., 2011). RALGDS is one of the first effector molecules that was crystallised together with an active Ras. Rat RALGDS was co-crystalised with human H-Ras harbouring an E31K mutation, which helped complex formation (Huang et al., 1998). A recent modelling approach, integrating proteomic data of Ras, its 56 effectors and their interaction affinities, predicts that Rgl2 would generate the highest concentration of Ras:effector complex among RalGEFs in 28 out of 29 healthy human tissues (Catozzi et al., 2021). Furthermore, a critical contribution of Rgl2 in oncogenic Ras induced tumour phenotypes has been reported (Vigil et al., 2010). However, the Ras:Rgl2 complex interface has not been studied.
In this report, we examined the mode of interaction between human K-Ras4B and the RBD of Rgl2 (Rgl2\textsuperscript{RBD}). We observed striking affinity increase of the interaction of K-Ras4B with the Rgl2\textsuperscript{RBD} upon introduction of G12V oncogenic mutation. Our crystal structure of K-Ras4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} complex shows a heterotetramer formation, highly similar to the reported H-Ras\textsuperscript{E31K}:RALGDS\textsuperscript{RBD} complex, but distinct from other Ras:effector complexes including Ras:Raf1 kinase. Strikingly, the G12V oncogenic mutation is located at the dimer interface of K-Ras4B\textsuperscript{G12V} with its homodimeric partner. Solution NMR analyses support the heterotetramer formation and mass photometry confirms the presence of the heterotetramer. Our findings provide an interesting possibility that K-Ras4B\textsuperscript{G12V} oncogenicity might be due to its capability to form a heterotetramer with Rgl2.

Results

Active K-Ras4B\textsuperscript{G12V} has a stronger affinity to Rgl2\textsuperscript{RBD} than K-Ras4B\textsuperscript{WT}

Throughout this study, we used recombinant bacteria constructs of human K-Ras4B lacking the C-terminal hyper-variable region and the Rgl2 RBD (Rgl2\textsuperscript{RBD}) consisting of amino acids 648 – 735 of human Rgl2. The binding of Rgl2\textsuperscript{RBD} and K-Ras4B\textsuperscript{G12V} was confirmed for the active K-Ras4B\textsuperscript{G12V} loaded with a non-hydrolysable GTP analogue (Guanosine-5'-(\(\beta,\gamma\)-imido)triphosphate (GMPPNP)), by GST-pulldown assays (Figure 1-figure supplement 1A and 1B). To examine whether the binding mode of Rgl2\textsuperscript{RBD} differs between K-Ras4B\textsuperscript{WT} and K-Ras4B\textsuperscript{G12V}, the binding affinities were determined by biolayer interferometry (BLI). We noticed that both K-Ras4B\textsuperscript{WT} and K-Ras4B\textsuperscript{G12V}, purified at 4\textdegree C at all times as described in the Materials and Methods, retained GTP as the major bound guanine nucleotide, and we confirmed that the K-Ras4B\textsuperscript{WT} and K-Ras4B\textsuperscript{G12V} samples that were subjected to the incubation condition of the BLI assay were still associated with GTP (Figure 1A). Therefore, we used these GTP-bound samples without loading GMPPNP so that we could minimize possible artefacts caused by GMPPNP. The on/off kinetics between K-Ras4B and Rgl2 were fast for both wildtype and G12V mutant (Figure 1B lower panels), and the sensorgram curves could not be fitted to the pre-programmed curve fitting models (Data Analysis HT Software, Sartorius). Therefore, we deduced the equilibrium dissociation constant (K\textsubscript{D}) using steady-state analysis (Figure 1B upper panels). Strikingly, the affinity between Rgl2\textsuperscript{RBD} and K-Ras4B was substantially increased when K-Ras4B carried an oncogenic mutation, G12V,
where Glycine at position 12 is replaced with Valine. This is consistent with the size-exclusion chromatography analysis conducted under the same condition, showing that Rgl2\textsuperscript{RBD} does not form a stable complex with K-Ras4B\textsuperscript{WT} in contrast to K-Ras4B\textsuperscript{G12V} (Figure 1-figure supplement 1C and 1D). The circular dichroic (CD) spectra for K-Ras4B\textsuperscript{WT} and K-Ras4B\textsuperscript{G12V} samples across the increasing temperature from 20°C to 90°C also showed improved structural stability for K-Ras4B\textsuperscript{G12V}, which might contribute to the higher affinity towards Rgl2\textsuperscript{RBD} (Figure 1 C-E).

A 2:2 tetramer of K-Ras4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} complex in the crystal structure

We conducted crystallization trials to obtain structural insights into the K-Ras4B:Rgl2\textsuperscript{RBD} complex. To purify the complex, a mixture of K-Ras4B and Rgl2\textsuperscript{RBD} was loaded onto a size-exclusion column. We could obtain the K-Ras4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} complex in a peak that eluted earlier than K-Ras4B\textsuperscript{G12V} alone (Figure 1-figure supplement 1C). However, we were unable to obtain an elution peak for K-Ras4B\textsuperscript{WT}:Rgl2\textsuperscript{RBD} complex (Figure 1-figure supplement 1D). The inability of K-Ras4B\textsuperscript{WT} to form a stable complex that survives the gel filtration chromatography is consistent with the relatively low affinity between K-Ras4B\textsuperscript{WT} and Rgl2\textsuperscript{RBD} seen in Figure 1B.

Crystals of the K-Ras4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} complex were obtained which diffracted at a resolution of 3.07Å (Table 1). After molecular replacement with the structure of the human H-Ras\textsuperscript{E31K}:rat RALGDS\textsuperscript{RBD} complex (PDB ID 1LFD) (Huang et al., 1998), the K-Ras4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} structure was manually built using Coot (Emsley et al., 2010) and was refined with PHENIX (Torices and Munoz-Pajares, 2015) (Figure 2A). The overall arrangement of K-Ras4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} crystal structure is similar to H-Ras\textsuperscript{E31K}:RALGDS\textsuperscript{RBD} crystal structure (PDB ID 1LFD (Huang et al., 1998))( Figure 2-figure supplement 1A-C). The complex forms a 2:2 heterotetramer where β2 (within Switch I) of Ras and β2 of Rgl2\textsuperscript{RBD} generate a continuously extended β-sheet, along with interaction at Switch II of the same Ras molecule with the second Rgl2\textsuperscript{RBD} (Figure 2B, Figure 2-figure supplement 2A). In both K-Ras4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} and H-Ras\textsuperscript{E31K}:RALGDS\textsuperscript{RBD} cases, the Ras-Ras interface is formed within the region spanning amino acids 1-90 where K-Ras4B\textsuperscript{G12V} and H-Ras\textsuperscript{E31K} share 100% amino acid sequence identity except for the point mutations G12V and E31K.

The K-Ras4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} heterotetramer complex is stabilized by a network of hydrogen bonds and hydrophobic interactions (Figure 2B, C and D, Figure 2-figure supplement 2A), leading to a complimentary surface charges (Figure 2-figure supplement 2B). K-Ras4B\textsuperscript{G12V} residues within the Switch I interact with residues in β1, β2 and α1 of Rgl2\textsuperscript{RBD}. These interactions include a salt...
bridge between E37 of K-Ras4B<sup>G12V</sup> Switch I and R653 of Rgl2<sup>RBD</sup> (Figure 2B and Figure 2-figure supplement 2A). The Switch II of the same K-Ras4B<sup>G12V</sup> molecule also contributes to complex formation by interacting with the second Rgl2<sup>RBD</sup> through residues in β2 and α1 (Figure 2B and Figure 2-figure supplement 2A). Meanwhile, two K-Ras4B<sup>G12V</sup> molecules have direct contact through the Switch I, Switch II and α3 (Figure 2C and Figure 2-figure supplement 2A). Strikingly, the V12 residue of the oncogenic G12V mutation is within 4 Å of the ring of Y32 of the neighboring K-Ras4B<sup>G12V</sup> contributing to the K-Ras4B<sup>G12V</sup>: K-Ras4B<sup>G12V</sup> interface (Figure 2C and Figure 2-figure supplement 2A). The β1 of both Rgl2<sup>RBD</sup> molecules run anti-parallel to each other, interacting through various hydrophobic interactions and hydrogen bonds at both side-chain and backbone levels (Figure 2D).

**Solution NMR analyses support the K-Ras4B<sup>G12V</sup>:Rgl2<sup>RBD</sup> heterotetramer formation**

The K-Ras4B<sup>G12V</sup>:Rgl2<sup>RBD</sup> complex was further analyzed in solution. First, the solution structure of the Rgl2<sup>RBD</sup> construct was determined by solution NMR (Figure 3A) (Table 2). The structure retained the ββαββ ubiquitin-fold structure, a common feature for the RBDs (Kiel and Serrano, 2006). Overall, it is similar to Rlf, the mouse homologue of human Rgl2 (PDB ID 1RLF)(Esser et al., 1998). However some differences can be observed, even though the primary sequences are similar (Figure 3-figure supplement 1A). The structures of the Rgl2<sup>RBD</sup> molecules in the K-Ras4B<sup>G12V</sup>:Rgl2<sup>RBD</sup> complex crystal structure are highly comparable with the structure of the NMR solution structure of free Rgl2<sup>RBD</sup> indicating that the complex formation causes relatively small structural changes (Figure 3B).

The K-Ras4B<sup>G12V</sup>:Rgl2<sup>RBD</sup> complex was next analyzed in solution by NMR chemical shift perturbation. Two-dimensional (2D) <sup>15</sup>N–<sup>1</sup>H-heteronuclear single quantum coherence (HSQC) spectrum were measured of <sup>15</sup>N-labelled Rgl2<sup>RBD</sup> sample, in the absence or presence of increasing amount of non-labelled K-Ras4B<sup>G12V</sup> (Figure 3C, Figure 3-figure supplement 1 B and C). Chemical shift perturbations were observed for most of the Rgl2<sup>RBD</sup> residues, in agreement with K-Ras4B<sup>G12V</sup>:Rgl2<sup>RBD</sup> complex formation in solution (Figure 3-figure supplement 1 B and C). Most of the Rgl2<sup>RBD</sup> residues at the K-Ras4B<sup>G12V</sup>:Rgl2<sup>RBD</sup> and Rgl2<sup>RBD</sup>:Rgl2<sup>RBD</sup> interfaces in the crystal structure showed a greater change (either decreased or increased) in their NMR signal intensities, indicating their participation to the complex formation (Figure 3C). Notably, signals from the Rgl2<sup>RBD</sup> residues in β1 at the N-terminal end made the largest increase. An explanation...
may be that the highly flexible N-terminal region of free Rgl2 (Figure 3A) becomes confined into
a more defined structure upon complex formation as observed in the crystal structure.

It has been shown that a lysine residue of RALGDS, that corresponds to K686 of Rgl2, plays an
essential role in H-Ras^{E31K}:RALGDS complex formation (Huang et al., 1998)(Figure 3-figure
supplement 2 A). In the K-Ras4B^{G12V}:Rgl2^{RBD} complex crystal structure, K686 forms a hydrogen
bond with D33 of K-Ras4B^{G12V} (Figure 3-figure supplement 2 B). K686 was also among the
residues that showed a substantial signal decrease in the NMR titration assays (Figure 3C). We
therefore mutated K686 into an alanine and as expected the K686A mutant lost the ability to
interact with K-Ras4B^{G12V} (Figure 3-figure supplement 2 C), confirming the important role of
K686 in the interaction.

We next examined the NMR chemical shift perturbation of K-Ras4B^{G12V} in solution by 2D \(^{15}\text{N}-^{1}\text{H-}
HSQC, in absence or presence of non-labelled Rgl2^{RBD}. We noticed that when loaded with
GMPPNP, the number of detectable peaks of the \(^{15}\text{N} \text{labelled K-Ras4B}^{G12V} \text{signal was}
substantially decreased compared to the number of signals obtained from the GDP-loaded
sample (Figure 3-figure supplement 3) as previous studies reported (Ito et al., 1997; Menyhard
et al., 2020). Consequently, most of the K-Ras4B^{G12V} residues at the K-Ras4B^{G12V}:Rgl2^{RBD} complex
interface could not be detected, except for K42 and K88, both of which were showing
substantially decreased signal intensities, in agreement with the expected participation in the
complex formation (Figure 3-figure supplement 4 C). Furthermore, upon Rgl2^{RBD} titration, most
of the \(^{15}\text{N} K\text{-Ras4B}^{G12V} \text{signals were reduced} \text{(Figure 3-figure supplement 4 A-C). This overall}
signal reduction was consistent with broadening arising as a result of the large complex size.

**Mass photometry confirms the presence of a heterotetramer of K-Ras4B^{G12V}:Rgl2^{RBD} complex**

To confirm that K-Ras4B^{G12V}:Rgl2^{RBD} forms a heterotetramer in solution, we analyzed the
complex by mass photometry, a label free technique that has recently been adapted to measure
the mass of biomolecules in solution (Young et al., 2018). Our commercially-available instrument
(see Materials and methods) can measure accurately masses in the range 30-5000 kDa, and is
suitable to differentiate between K-Ras4B^{G12V}:Rgl2^{RBD} heterodimer (31kDa) or heterotetramer
(62kDa). The K-Ras4B^{G12V}:Rgl2^{RBD} complex was freshly prepared by size exclusion
chromatography and the peak fraction that contained both proteins at 1:1 ratio was used for
the measurement (Figure 4A). The histogram of the frequency counts for this sample showed a
peak, that after calibration with the proper standard (Native Mark), corresponds to a biomolecule with molecular weight of 68 KDa (Figure 4B). As the expected molecular weight of the K-Ras4B\(^{G12V}\):Rgl2\(^{RBD}\) heterotetramer is about 62 KDa, the result strongly indicates that at least part of the complex population exists as the 2:2 heterotetramer in solution.

**Discussion**

We conducted interaction studies of human K-Ras4B and Rgl2\(^{RBD}\) to obtain structural insights into the oncogenic Ras-dependent activation of the RalA/B pathway. Using X-ray crystallography and the crystal structure of human H-Ras\(^{E31K}\) and rat RALGDS complex (PDB ID 1LFD) as a molecular replacement template (Huang et al., 1998), we reveal a heterotetramer formation stabilised by the oncogenic G12V mutation. The presence of heterotetramer in solution was further confirmed by mass photometry.

**Mode of interaction between Ras and Rgl2/RALGDS is distinct from other Ras:RBD interactions**

The key contributor of the heterotetramer formation seen in this Ras:RalGEF complex, K-Ras4B\(^{G12V}\):Rgl2\(^{RBD}\), and the previously published H-Ras\(^{E31K}\):RALGDS\(^{RBD}\) is the interaction between the Switch II region of Ras and the second RBD molecule (Figure 2B and Figure 2-figure supplement 3A). This feature is distinct from the previously reported Ras:RBD crystal structures, where the Switch II region is either not participating in the interaction (as seen in Ras:Raf1\(^{RBD}\) (PDB ID 6VJJ)(Tran et al., 2021)) or is interacting with an RBD that also interacts with the Switch I region of the same Ras molecule (as seen in Ras:PI3K\(^{RBD}\) (PDB ID 1HE8)(Pacold et al., 2000), Ras:PLCe\(^{RBD}\) (PDB ID 2CSL)(Bunney et al., 2006), Ras:RASSF5\(^{RBD}\) (PDB ID 3DDC)(Stieglitz et al., 2008)), hence forming a Ras:RBD heterodimer, rather than a heterotetramer, in each crystal lattice (Figure 2-figure supplement 3B). One interesting case is a recently reported structural study of Rgl1, yet another RalGEF (Eves et al., 2022). Crystal structures of Rgl1 in complex with either K-Ras\(^{WT}\) or K-Ras\(^{G12V}\) (PDB ID 7SCW and 7SCX, respectively), showed that one Rgl1\(^{RBD}\) interacts with one K-Ras molecule through both Switch I and Switch II, as other heterodimer examples shown in Figure S8B. It will be interesting to further examine whether the K-Ras:Rgl1\(^{RBD}\) may have the capacity to form a heterotetramer as a common feature for Ras:RalGEF complexes.

**Oligomerisation of K-Ras:effector complexes**
Accumulating evidence suggests that K-Ras is capable of forming a dimer in solution even in the absence of effectors (Andreadelis et al., 2022; Ingolfsson et al., 2022; Jang et al., 2016; Lee et al., 2021; Lee et al., 2020; Muratcioglu et al., 2015; Ozdemir et al., 2022; Packer et al., 2021; Prakash et al., 2017; Sarkar-Banerjee et al., 2017). The physiological importance of Ras dimerisation and its potential to be a therapeutic target was highlighted by a G12D-specific inhibitor, BI2852, which may cause artificial dimerisation and block the protein function (Kessler et al., 2019; Tran et al., 2020). Furthermore, Ras forms nanoclusters in the membrane in vivo and in vitro (Lakshman et al., 2019; Plowman et al., 2005; Prior et al., 2003; Weise et al., 2011; Zhou et al., 2014). The modes of these Ras oligomer formation are dependent on various parameters including membrane lipid compositions, Ras nucleotide binding status, availability of Ras effectors and actin cytoskeleton and are expected to be context dependent.

Previously it has been suggested that dimerisation status may be categorised into four classes based on the utilized α helices and β sheets as follows; (i) α4/α5 (Andreadelis et al., 2022; Jang et al., 2016; Lee et al., 2021; Lee et al., 2020; Packer et al., 2021; Prakash et al., 2017), (ii) α3/α4 (Jang et al., 2016; Muratcioglu et al., 2015; Prakash et al., 2017), (iii) α/β (Lee et al., 2021), and (iv) β/β (Jang et al., 2016; Muratcioglu et al., 2015) (Figure 2-figure supplement 4). Importantly, the K-Ras4B^G12V::K-Ras4B^G12V interface that we observe in the K-Ras4B^G12V::Rgl2^RBD heterotetramer does not belong to any of these categories and uses unstructured regions of Switch I, Switch II, V12 in the P-loop and K88 (Figure 2, Figure 2-figure supplement 1 and 4). Interestingly, this K-Ras4B^G12V::K-Ras4B^G12V interface is similar to the spatial arrangement of symmetry mates of human H-Ras^WT crystal (PDB ID 5P21) (Pai et al., 1990) (Figure 2-figure supplement 5), indicating that this Ras::Ras contact is likely stabilized by Rgl2^RBD.

Currently, we do not have information as to the orientation of the K-Ras4B^G12V::Rgl2^RBD in relation to the plasma membrane (PM). The Rgl2 molecules could be away from the plasma membrane (PM-away conformation), or could be in direct contact with the plasma membrane (PM-proximity conformation) (Figure 5). In either case, the complex status is distinct from the case for Ras::Raf1 kinase complex which can be formed onto the existing Ras::Ras homodimer of the α4/α5 interface (Packer et al., 2021). If the majority of the cellular K-Ras4B^G12V molecules exist as the α4/α5 homo-dimer at the PM, what drives the K-Ras4B^G12V::Rgl2^RBD heterotetramer formation by causing a major structural change is an important future question.
Direct involvement of V12 in the K-Ras4B:Rgl2\textsuperscript{RBD} complex formation and oncogenicity

Strikingly, the K-Ras4B\textsuperscript{G12V}:K-Ras4B\textsuperscript{G12V} interface of the K-Ras4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} tetramer complex involves the residue V12, an oncogenic amino-acid substitution occurring in about 28% of mutated KRAS cases (COSMIC). This G12V substitution substantially increases the affinity towards Rgl2\textsuperscript{RBD} compared to the wildtype (Figure 1B). In agreement with this observation, our attempt to biochemically purify the K-Ras4B\textsuperscript{WT}:Rgl2\textsuperscript{RBD} complex has been unsuccessful as K-Ras4B\textsuperscript{WT} and Rgl2\textsuperscript{RBD} eluted in separate fractions when subjected to size exclusion chromatography (data not shown). In the K-Ras4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} tetramer complex, the presence of V12 creates a larger hydrophobic pocket together with Y32, compared to the wildtype case of G12, seen in the H-Ras\textsuperscript{WT} crystal (PDB ID 5P21) or in the H-Ras\textsuperscript{E31K}:RALGDS\textsuperscript{RBD} crystal (PDB ID 1LFD) (Figure 2-figure supplement 5 and 6). To our knowledge, the K-Ras4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} complex serves as the first example where a structural explanation is provided for an oncogenic-Ras driven stabilization of a Ras:effector complex. Interestingly, the E31K substitution, used in the H-Ras\textsuperscript{E31K}:RALGDS crystal structure analysis to stabilize the complex (Huang et al., 1998) (Figure 2-figure supplement 6E), has been reported in cancer samples with HRAS mutation (COSMIC). Therefore, the capability of Ras to form a stable complex with RalGEFs may be directly linked with Ras oncogenicity. It is interesting to note that in the previously proposed Ras:Raf1 kinase heterotetramer complex derived from SAXS data (Packer et al., 2021), the position of the amino acid residue 12 is relatively distant from the interacting surfaces. An interesting speculation could be that the oncogenic substitution mutations at glycine 12 might have a greater impact on RalGEF-mediated signalling than Raf kinase-mediated signalling (Figure 5).

Conclusion

To summarize, our work demonstrates an increased affinity of K-Ras4B\textsuperscript{G12V} oncogenic mutant with a RalGEF, Rgl2, and provides a structural explanation by revealing the complex interface. The information will open the way to target oncogenic-KRAS-induced tumorigenesis by novel strategies, including interfering molecules for the newly identified interfaces.
**Materials and Methods**

**Plasmid constructs**

The RBD of human Rgl2 was obtained by amplifying a cDNA fragment encoding the position 643 – 740 of the human Rgl2 by PCR with a pair of primers (5’ TACTTCCAATCCATGGGGCCAGGGGCCTCTGATTGCGG3’) and (5’ TATCCACCTTTACTGTCA)

TGTAGCAGTAGGACCTTCCGCGCTGC 3’) using human cDNA prepared from hTERT RPE-1 cells using GoScript Reverse Transcription System (Promega) following the manufacturer’s instruction. The amplified Rgl2RBD fragment was cloned into pLEICS2 vector (PROTEX, University of Leicester), which contains a glutathione S-transferase (GST) affinity tag and a Tobacco Etch virus (TEV) potease cleavage site at the N-terminal end of the Rgl2RBD, using In-Fusion HD EcoDry enzyme (Takara Bio, #638915), following the manufacturer’s instruction. A point mutation, K686A, was introduced by site-directed mutagenesis using a pair of primers (5’ ATCAGTCGTGTCCTTAAGGCAAACAATCGTGACTCT 3’) and (5’ CAGAGTCACGATTGTTGCTTAAAGGACAGACTGAT 3’) by following the QuikChange™ method (Agilent Technologies, La Jolla, CA, USA).

A cDNA fragment encoding the position 1 – 169 (C-terminal truncated) of the human wildtype K-Ras4B isoform was amplified by PCR with a pair of primers (5’ TACTTCCAATCCAGTACTGAATATAAACTTGTGGTAGTTGGAGCTG’) and (5’ TATCCACCTTTACTGTCACTTTTCTTTATGTGTTTCTCAGAAATTCTGATAAGTAGGACTC 3’) using human cDNA prepared from hTERT RPE-1 cells as described above. The produced fragment was cloned in pLeics1 plasmid (PROTEX, University of Leicester), which introduces a His6 tag and a TEV cleavage site at the N-terminus. Site directed mutagenesis was conducted to introduce the oncogenic G12V mutation using a pair of DNA oligos (5’ AGTTGGAGCTTGGCGTAGGCAAGAGTGCC 3’) and (5’ GTCAAGGGCCTTGCTAGGGCGTAGCAAGGTGCC 3’) by following the QuikChange™ method (Agilent Technologies, La Jolla, CA, USA).

**Protein expression**

DE3 Rosetta cells (Novagen) carrying expression plasmids were grown at 37°C in TY media until OD600 reached about 0.6. Then protein expression was induced by adding Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM and keeping the culture at 18°C overnight in a shaking incubator. Cells were collected by centrifugation and resuspended in
either SL1 buffer (Tris 20mM (pH 7.65), NaCl 150 mM, 5 mM imidazole) for His-tagged KRas or in PBS-NaCl buffer (237 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, and 2 mM KH₂PO₄, pH 7.4) for Rgl2 and RalGDS. The cell suspensions were then stored at -80°C.

**Protein expression of stable isotope labelling K-Ras4BG12V and RBD of Rgl2 for NMR measurement**

The gene encoding human K-Ras4BG12V and Rgl2RBD were constructed into the expression vector pGHL9 and pLEICS2 over-expressed in *Escherichia coli* (E.coli) strain BL21 (DE3) and Rosetta, respectively. Uniformly $^{13}$C, $^{15}$N-labeled protein was obtained by growing bacteria at 37 °C in M9 minimal media, containing $[^{13}$C₆]-glucose and $^{15}$NH₄Cl (Isotec) as the sole carbon and nitrogen source, supplemented with 20 mM MgSO₄, 0.1 mM CaCl₂, 0.4 mg/ml thiamin, 20 µM FeCl₃, salt mix [4 µM ZnSO₄, 0.7 µM CuSO₄, 1 µM MnSO₄, 4.7 µM H₃BO₃], and 50 mg/L ampicilin. K-Ras4BG₁₂V NMR sample was prepared essentially as described previously (Ito et al., 1997). Protein expression of Rgl2RBD was induced by adding 119mg/L IPTG at an OD 600 nm of 0.5. After 18 h of further growth, cells were harvested, and washed with a pH 7.5 lysis buffer [50 mM Tris-HCl, 25% sucrose, and 0.01% NP-40]. Uniformly $^{15}$N-labeled K-Ras4BG₁₂V and Rgl2RBD were produced by the identical steps unless growing cells in M9 medium containing $[^{12}$C₆]-glucose and $^{15}$NH₄Cl (Isotec).

**Purification of GST-tagged Rgl2RBD**

Bacteria cell suspensions were thawed and supplemented with Triton X-100 to a final concentration of 0.1 % (v/v). Cells were broken by a probe sonicator and insoluble materials were removed by centrifugation. The supernatant was mixed with glutathione (GSH) beads (GE Healthcare, #17-0756-01) and incubated for 20 min at 4°C. The GSH beads were washed three times with the PBS-NaCl buffer. the GST-Rgl2RBD fusion protein was either eluted by the elution buffer (50mM Tris-Cl (pH 8.0), 100mM NaCl, 5mM GSH) for GST pulldown experiments or the Rgl2RBD was separated from the GST tag through cleavage by tobacco etch virus (TEV) protease, which was prepared as previously described (Kapust et al., 2001; Tropea et al., 2009), for structural analysis. The obtained Rgl2RBD or GST-Rgl2RBD fusion proteins were concentrated using a concentrator (10 kDa MWCO, Merck, Amicon Ultra centrifugal filters, #UFC901024) and filtrated using a centrifugal filter unit (Milipore, Ultrafree, #UFC30GV00) before conducting size exclusion chromatography (SEC) with a gel-filtration column (GE Healthcare, HiLoad Superdex 30...
attached to an FPLC (Akta) system. SEC was carried out in a gel filtration buffer (20 mM Tris-Cl (pH 7.65), 100 mM NaCl, 5mM MgCl2, 1mM tris (2-carboxyethyl) phosphine (TCEP)).

Purification of His-tagged K-Ras4B wildtype and oncogenic G12V mutant proteins

Bacteria cell suspensions were supplemented with Triton X-100 to a final concentration of 0.1 % (v/v). Cells were broken by a probe sonicator and insoluble materials were removed by centrifugation. The soluble cell lysates were applied on a Ni-sepharose excel (GE Healthcare, #17-3712-01), packed in a column of 4 ml bed volume with SI1 buffer buffer (20mM Tris, pH6.5, 150mM NaCl, 5 mM imidazole). The column was washed with 20 ml of SL1 Buffer, then with 20 ml of SI3 Buffer (20mM Tris, pH6.5, 150mM NaCl, 6mM Imidazole) and finally with 15 ml of SI4 Buffer (20mM Tris, pH6.5, 150mM NaCl, 10mM Imidazole). The His-tagged Kras protein was eluted from the column by applying 10 ml of Elution buffer (50mM Tris, pH 7.65, 150mM NaCl, 200mM Imidazole), followed by 10 ml of 1 M imidazole. In order to remove the His6-tag at the N-terminal end, TEV protease, prepared as previously described (Tropea et al., 2009), was added to the elution fraction to about a 2 % molar ratio of the His6-K-Ras4B preparation and incubated overnight at 4°C. Nucleotide exchange of the purified K-Ras4B wildtype or G12V proteins was carried out essentially as previously described (Ito et al., 1997). The proteins were diluted 10 times by the exchange buffer (20mM Tris-Cl (pH7.5) 1mM EDTA, 1mM TECP) and denatured by the addition of EDTA to a final concentration of 5 mM. The sample was mixed with about 10 time molar excess of guanosine 5’-β,γ-imido]triphosphate (GMMPPNP, SIGMA G0635) or guanosine 5’-diphosphate (GDP, SIGMA G7127). The reaction was incubated at 37°C for 20 minutes then put it on ice for 20 minutes. The protein was renatured by adding ice cold MgCl2 to a final concentration of 20 mM.

Purification of isotope labelling K-Ras4B<sup>G12V</sup>

All the procedures described below were carried out at 4 °C unless otherwise stated. All the isotope-labelled K-Ras4B<sup>G12V</sup> samples were purified by the same step. The cells dispersed in the lysis buffer was disrupted by sonication for 30 min on ice with hen egg lysozyme (0.1 mg/mL). The cell debris was clarified by centrifugation at 14,000 g for 1 h. The supernatant was loaded onto a 25 mL of DEAE-Sepharose Fast Flow (Cytiva) anion exchange equilibrated with buffer A [50 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 1 mM dithiothreitol (DTT), 0.1 mM APMSF (FUJIFILM Wako)]. After washing the column with buffer A until sufficiently low of UV absorption at 280
The K-Ras4B\textsuperscript{G12V} protein was eluted by linearly increasing the concentration of KCl from 0 to 350 mM with a flow rate of 0.5 mL/min in buffer A. The fractions containing the target protein were concentrated to 5 mL with Amicon Ultra-15 10 kDa (Merck). The concentrated sample was loaded onto a 320 mL of HiLoad Superdex 75 (GE Healthcare Life Science) gel filtration with a flow rate of 0.8 mL/min using FPLC systems (AKTA pure 25, GE Healthcare Life Science). The 5 mL sample concentrated from the fractions involving the target proteins with Amicon Ultra-15 10 kDa was loaded on Resource Q (GE Healthcare Life Science) anion-exchange column equilibrated with buffer A using the FPLC systems. After washing the column with 30 mL of buffer A, K-Ras4B\textsuperscript{G12V} was eluted by an KCl, the K-Ras4B\textsuperscript{G12V} protein was eluted by linearly increasing the concentration of KCl from 0 mM to 350 mM with a flow rate of 1 mL/min in buffer A. The purity of the K-Ras4B\textsuperscript{G12V} and Rgl2\textsuperscript{RBD} samples in each step was confirmed by SDS-PAGE. Protein concentrations were determined by NanoDrop 2000 (ThermoFisher) measuring UV absorption at 280 nm. K-Ras4B\textsuperscript{G12V} samples for NMR measurements were concentrated and dissolved in NMR buffer A (90% \textsuperscript{1}H\textsubscript{2}O/ 10% \textsuperscript{2}H\textsubscript{2}O containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM MgCl\textsubscript{2}, 1 mM \(\beta\)-mercaptoethanol). Rgl2\textsuperscript{RBD} samples for NMR measurements were NMR buffer B (90% \textsuperscript{1}H\textsubscript{2}O/ 10% \textsuperscript{2}H\textsubscript{2}O containing 1 mM Na\textsubscript{2}HPO\textsubscript{4}-NaH\textsubscript{2}PO\textsubscript{4} (pH 7.4), 150 mM NaCl).

**K-Ras4B-Rgl2\textsuperscript{RBD} binding measurement using BLI**

Octet Red\textsuperscript{96} (Sartorius) was used for Biolayer interferometry assays of KRas4B (G12V or WT) and Rgl2\textsuperscript{RBD} interactions. Anti-GST biosensors (Sartorius #18-5096) were used to immobilise GST-Rgl2\textsuperscript{RBD} (provided as a 13 \(\mu\)M solution in the binding reservoir well), and the baseline was stabilised in BLI buffer (20mM Tris pH7.65, 0.5mM NaCl, 5mM MgCl\textsubscript{2}, 1mM TCEP) for 200sec. As a negative control, GST only (provided as a 13 \(\mu\)M solution in the binding reservoir well) was used. Protein concentrations were determined by absorbance at 280 nm using the following values; the extinction coefficient (\(\epsilon\)) for KRas (WT) and KRas(GV) was estimated to be 19685 cm\(^{-1}\) M\(^{-1}\), by taking into account that the bound GTP adds 7765 cm\(^{-1}\) M\(^{-1}\) (Smith and Rittinger, 2002), and the molecular weights (including GTP) were estimated to be 19856 and 19898, respectively. The association of KRas4B was measured for 300sec in 6 serial dilutions concentrations. For KRas (WT) the concentrations used ranged from 625 nm - 10 \(\mu\)M and for KRas4B (G12V) the concentrations ranged from 200 nM – 6.4 \(\mu\)M. The dissociation steps were
measured in fresh BLI buffer for 300sec. For each assay a biosensor immobilised with GST only
and a sample well with only buffer instead of KRas4B (WT/G12V) was set up for double
referencing. The experiments were conducted at 23°C. The resulting data were processed using
Data Analysis HT Software (ver. 12.0.2.59)(FORTE BIO) and reference biosensor and reference
wells were subtracted from sample wells (double reference). Because of the fast on- and off-
rates, full kinetic analysis using a global 1:1 model resulted in low quality fitting. Therefore,
steady state curves were used to determine the $K_D$ values.

**Analysis of bound nucleotide**

The nucleotide-binding status of K-Ras4B$^{WT}$ and K-Ras4B$^{G12V}$ were examined by denaturing the
proteins and detecting the released nucleotides (Smith and Rittinger, 2002). About 2 nmoles of
K-Ras4B molecules were adjusted to the volume of 200 µl with AKTA buffer. Add 12.5 µl of 10%
perchloric acid to denature the protein. Add 8.75 µl of 4M CH₃COONa, pH 4.0, to neutralise the
sample and centrifuge it to precipitate the denatured protein. The supernatant was analysed by
High-performance liquid chromatography (HPLC) using an ion-exchange column (Partisil 10 SAX
column, Whatman) equilibrated with 10 mM NH₄H₂PO₄ (buffer A). The column was run with the
following gradient condition with 0.6 M NH₄H₂PO₄ (buffer B) at a flow rate of 0.8 ml/min. Step1:
100% buffer A (0% buffer B) for 11 min, Step2: a gradient increase to 40% buffer B over 6 min,
Step3: a gradient increase to 50% buffer B over 23 min, Step4: a gradient increase to 100 %
buffer B over 1 min, Step 5: 100 % buffer B for 19 min, Step 6: a gradient decrease to 0% buffer
B (100 % buffer A) over 1 min, and Step 7: 100% Buffer A for 14 min. The nucleotides were
detected by 254 nm absorption. As a reference control, 1 µl of 10 mM GTP or GDP was diluted
to 200 µl AKTA buffer and was processed in the same manner as protein samples.

**Crystallography**

The purified and GMPPNP-loaded KRas4B (G12V) and Rgl2$^{RBD}$ were mixed in gel filtration buffer
and the complex was purified on SEC using a gel-filtration column (GE Healthcare, HiLoad
Superdex 75). The peak fractions containing both proteins in the 1:1 ratio were collected and
concentrated to set up crystallization screenings. Crystals of KRas4B (G12V) and Rgl2$^{RBD}$ were
obtained using sitting-drop vapour diffusion at room temperature, with 100nl of protein
(11.6mg/ml) against 100nl of crystallisation buffer (0.2M sodium/potassium phosphate ph 7.5,
0.1M HEPES pH 7.5, 22.5% v/v PEG smear Medium (Molecular Dimensions MD2-100-259), 10%
v/v glycerol. The crystals were frozen in liquid nitrogen with 20% glycerol as cryoprotectant. Data were collected at Diamond beamline I04. AIMLESS (Evans and Murshudov, 2013) was used for data reduction before obtaining phaser solution using the HRas-RALGDSRBD complex structure (PDB ID 1LFD) as search model with PhaserMR (McCoy et al., 2007). The structure was built using multiple rounds of refinements using PDBredo, REFMAC, PHENIX and COOT (Emsley et al., 2010; Joosten et al., 2014; Murshudov et al., 2011; Torices and Munoz-Pajares, 2015). The coordinates of the complex have been deposited to the Protein Data Bank (PDB) under access code 8B69.

**Circular dichroism (CD) spectroscopy**

K-Ras4B(WT) and (G12V) proteins at a concentration of 20 μM were prepared in the CD buffer (50 mM phosphate (pH 7.6), 1.5 mM Tris (pH 7.6), 5mM MgSO4, 7.5 mM NaCl, 0.375 mM MgCl2), placed in a quartz cuvette of 0.1 cm path length and CD spectra were recorded at wavelengths ranging from 195 to 250 nm using a Chirascan™-plus CD spectrometer (Applied Photophysics) at 20 °C. The melting curves of these proteins were examined at 220 nm at temperatures ranging from 20°C to 90°C. Measurements were conducted at 1 °C increment.

**NMR spectroscopy**

K-Ras4B(G12V) NMR sample was prepared essentially as described previously (Ito et al., 1997). The bacteria expression plasmid for H-Ras was modified to encode K-Ras4B(G12V) by site-directed mutagenesis and used to produce K-Ras4B(G12V). Loading of a GTP analogue, GMPPNP (Jena Bioscience) was conducted essentially as previously described (Ito et al., 1997). Rgl2RD sample was prepared as described above. All NMR samples were measured in 20 mM Tris-Cl (pH 7.65), 100 mM NaCl, 5mM MgCl2, and 0.1% β-mercaptoethanol at 303 K. All spectra were analysed with the CcpNmr Analysis 2.5.1 software. Backbone chemical shifts of K-Ras4B(G12V) and Rgl2 have been deposited to the BioMagResBank (BMRB) with accession ID 34754 and PDB with accession ID 8AU4, respectively.

All NMR experiments were performed at 25°C probe temperature in a triple-resonance cryoprobe fitted with a z-axis pulsed field gradient coil, using Bruker AVANCE-III HD 600 MHz spectrometers. All spectra were processed with the Azara software package (Boucher). For the 3D data, the two-dimensional maximum entropy method (2D MEM) or Quantitative Maximum
Entropy (QME) (Hamatsu et al., 2013) were applied to obtain resolution enhancement for the indirect dimensions. All NMR spectra were visualized and analyzed using the CcpNmr Analysis 2.5.0 software (Vranken et al., 2005). All of the 3D triple-resonance experiments used for the assignments of K-Ras4B and Rgl2RBD were performed on 13C/15N samples in NMR buffer A and B, respectively. The backbone 1H, 13C, and 15N for K-Ras4B and Rgl2RBD, and side-chain 13Cα and 13Cβ resonance assignments for Rgl2RBD were achieved by analyzing six types of 3D triple-resonance experiments, HNCO, HN(CA)CO, HNCA, HN(CO)CA, CBCANNH, and CBCA(CO)NNH. 3D HBHA(CBCACO)NH, H(CCCO)NH, (H)CC(CO)NH, HCCH-COSY, and HCCH-TOCSY experiments on the 12C/15N-labeled Rgl2RBD were performed for side-chain 1H and 13C resonance assignments. A 15 ms 13C isotropic mixing time was employed for the (H)CC(CO)NH, H(CCCO)NH and HCCH-TOCSY experiments. For the collection of NOE-derived distance restraints of Rgl2RBD, 3D 15N-separated and 3D 13C-separated NOESY-HSQC spectra were measured on the 13C/15N-labeled Rgl2RBD. A 100 ms NOE mixing period was employed for the 3D NOESY experiments. All 2D and 3D NMR data were recorded using the States-TPPI protocol for quadrature detection in indirectly observed dimensions. Water flip-back 1H pulses and the WATERGATE pulse sequence were used for solvent suppression in the experiments performed on 15N-labeled, and 13C/15N-labeled samples, whereas presaturation and gradient-spoil pulses were used for 13C-labeled samples.

A series of 2D 1H-15N HSQC spectra were measured for titration experiments of 15N-labelled K-Ras4B<sup>G12V</sup> with in the presence of non-labelled Rgl2RBD. The experiments were performed in the NMR buffer at 25 °C and the peptide concentration was increased stepwise (for the 15N-K-Ras4B<sup>G12V</sup> / Rgl2RBD, its molar ratio of 1:0.25, 1:0.5, 1:0.75, 1:1, 1:1.5, and 1:2 were used, while for the 15N-Rgl2RBD / K-Ras4B<sup>G12V</sup>, its molar ratio of 1:0.25, 1:0.5, 1:1, 1:1.5, 1:2, and 1:3 were used). The mean chemical shift difference $\Delta\delta_{\text{ave}}$ for each amino acid was calculated as $[(\Delta\delta^{1H})^2 + (\Delta\delta^{15N})^2]^{1/2}$ where $\Delta\delta^{1H}$ and $\Delta\delta^{15N}$ are the chemical shift differences (Hz) between K-Ras4B<sup>G12V</sup> or Rgl2RBD on their own and the proteins in the presence of the other side.

**NMR structure calculation**

Intra-residual and long-range NOEs were automatically assigned by the program CYANA with the use of automated NOE assignment and torsion angle dynamics (Güntert and Buchner, 2015). The peak position tolerance was set to 0.03 ppm for the 1H dimension and to 0.3 ppm for the
13C and 15N dimensions. Hydrogen-bond and dihedral angle restraints were not used. CYANA calculations were started from 100 conformers with random torsion angle values, simulated annealing with 50,000 torsion angle dynamics steps was applied. The 20 conformers with the lowest final target-function values of CYANA were selected and optimised with OPALp 2.1 [22,23] using the AMBER force field [24,25]

**Mass photometry measurement**

Freshly purified KRas4B (G12V) and Rgl2RBD were mixed in AKTA buffer, concentrated to about 500 µl using a concentrator (Merck, Amicon Ultra centrifugal filters, #UFC901024), and loaded to a gel-filtration column (GE Healthcare, HiLoad Superdex 75). The peak fractions containing both proteins in the 1:1 ratio were collected and the concentration was estimated by OD280 and the predicted extinction coefficient of 24155 M⁻¹cm⁻¹. The sample was diluted to 40 nM in gel filtration buffer and a 20 µL aliquot was subjected to mass photometry (OneMP, Refeyn). The measurement was done in conventional microscope cover glass (Marienfeld, nº 1.5H) cleaned by rinsing with deionized water (×5) and isopropanol (×5) followed by drying under a N₂ flow, using a silicon gasket (Grace Bio-labs) in order to confine the sample. Adsorption of individual molecules of complex was detected across an imaging area of 10.8 µm by 2.9 µm.

Video recordings from interferometric scattering microscopy for a duration of 60s were obtained and the single events corresponding to surface adsorption of the complex were identified using AcquireMP software (Refeyn). Data analysis was performed using DiscoverMP software (Refeyn) and OriginPro 2021 (OriginLab).

**Graphical representation of protein structures**

Protein structure images were generated using Pymol (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC). Electrostatic surface charge potential images were produced using Pymol vacuum electrostatics function. Amino acid residues in the interaction surfaces of protein complexes in a PDB format were predicted using LIGPLOT (Wallace et al., 1995).

**Materials availability statement**
Bacteria expression plasmids to generate His$_6$-K-Ras4B$_{G12V}$(1-169), untagged K-Ras4B$_{G12V}$(1-169) and GST-Rgl2$^{RBD}$(643-740) are freely available upon request.

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References


**Figures and Tables**

![Diagram](image)

**Figure 1.** K-Ras4B<sup>G12V</sup> is structurally more stable and has a stronger affinity towards Rgl2<sup>RBD</sup> than K-Ras4B<sup>WT</sup>.

Analyses of binding kinetics of GST-Rgl2<sup>RBD</sup> and K-Ras4B<sup>WT</sup> or K-Ras4B<sup>G12V</sup>. Biolayer Interferometry (BLI) was used to directly measure the steady state binding of K-Ras4B (analyte) to immobilized GST-Rgl2<sup>RBD</sup> (ligand). (A and C) K-Ras4B<sup>WT</sup> and K-Ras4B<sup>G12V</sup> samples used for BLI and Circular Dichroism (CD) were confirmed to be fully loaded with GTP. The nucleotide binding
status of K-Ras4B\textsuperscript{WT} and K-Ras4B\textsuperscript{G12V} were examined by denaturing the proteins and detecting
the released nucleotides by anion exchange chromatography. Samples of pure GDP or GTP were
used as references. (B) BLI results of binding between K-Ras4B\textsuperscript{WT}/K-Ras4B\textsuperscript{G12V} and Rgl2\textsuperscript{RBD}. GST-Rgl2\textsuperscript{RBD} was immobilised on the bio-sensors, and varying concentrations of free K-Ras4B\textsuperscript{WT} (left panels) and K-Ras4B\textsuperscript{G12V} (right panels) were provided and the interactions were measured at
23°C. The kinetics diagrams (lower panels, association at t=0(s), dissociation at t=300(s)) revealed a fast on/off binding. Therefore, steady state curves (upper panels) were used to
estimate the K\textsubscript{D} values; about 6.1 µM for K-Ras4B\textsuperscript{WT}:Rgl2\textsuperscript{RBD} and 1.4 µM for K-Ras4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD}, respectively. (D) Circular dichroism (CD) spectra of K-Ras4B\textsuperscript{WT} and K-Ras4B\textsuperscript{G12V} (20 µM ) at 20°C. (E) CD signal intensity at 220 nm as a function of temperature from 20°C to 90°C.
**Figure 1-figure supplement 1.** Rgl2 Ras binding domain interacts with active K-Ras4B<sup>G12V</sup>.

(A) A schematic diagram of domains organization of human Rgl2 as defined in the UniProt database (Uniprot number: O15211). A domain spanning amino acid residues 648-735 is annotated as “Ras-associating” by PROSITE annotation rule PRU00166. In this work, we...
generically call this domain Rgl2 Ras Binding Domain (Rgl2\textsuperscript{RBD}). (B) Rgl2\textsuperscript{RBD} interacts with active K-Ras4B\textsuperscript{G12V}. Bacteria recombinant Rgl2\textsuperscript{RBD} fragment spanning the amino acid residues 643-740 of Rgl2 was fused with GST and the fusion protein was fixed on glutathione beads. Recombinant K-Ras4B\textsuperscript{G12V} 1-169, loaded with either GDP or non-hydrolysable GTP analogue, GMPPNP, was applied on the beads to examine the protein-protein interaction. GST-Rgl2\textsuperscript{RBD} interacted only with the GMPPNP-loaded K-Ras4B\textsuperscript{G12V}. (C) K-Ras4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} complex was purified using size exclusion chromatography. The fractions were analyzed by 15% or 12% SDS-PAGE gel (lower panel) according to the elution profile (upper panel). Fractions indicated by the green double-arrow line were used for crystallization trials. (D) K-Ras4B\textsuperscript{WT} and Rgl2\textsuperscript{RBD} preparations were mixed and applied to size exclusion chromatography. The elution profile (upper panel) shows little complex formation. Peak fractions were analyzed by 15% SDS-PAGE gel (lower panel).
Figure 2. Crystal structure of the K-Ras4B<sup>G12V</sup>:Rgl2<sup>RBD</sup> 2:2 heterotetramer
(A) Cartoon representation of the structure of the heterotetramer complex of K-Ras4B\(^{G12V}\) and Rgl2\(^{RBD}\) with top view and side view; the two K-Ras4B\(^{G12V}\) molecules are shown in dark and pale cyan and the two molecules of Rgl2\(^{RBD}\) in pink and violet. Switch-I and Switch-II regions of K-Ras4B\(^{G12V}\) are shown in green and yellow, respectively, and the \(\alpha\)-helix and \(\beta\)-sheets are numbered for each chain. The Mg\(^{2+}\) is shown as a grey sphere. The structure shows that each K-Ras4B\(^{G12V}\) molecule interacts with two Rgl2\(^{RBD}\) molecules (referred to as 1 and 2) at Switch-I and Switch-II individually. (B) The interacting interface of K-Ras4B\(^{G12V}\) and Rgl2\(^{RBD}\), highlighting the residues involved in hydrogen and hydrophobic interactions between \(\beta1\), \(\beta2\) and \(\alpha1\) of Rgl2\(^{RBD}\) (violet sticks) and K-Ras4B\(^{G12V}\) Switch-I (green sticks, enlarged in the green box) or Switch-II (yellow sticks, enlarged in the yellow box). An orange dotted line shows a salt bridge formed between E37 of K-Ras4B\(^{G12V}\) and R653 of Rgl2\(^{RBD}\). K686 of Rgl2\(^{RBD}\), which plays an essential role in the complex formation as shown in Figure 3-figure supplement 2B, is annotated in purple letters. (C) The interacting interface of K-Ras4B\(^{G12V}\): K-Ras4B\(^{G12V}\), highlighting the residues involved in hydrogen and hydrophobic interactions between two K-Ras4B\(^{G12V}\) molecules. The interface comprises Switch-I, Switch-II and \(\alpha3\) of the two K-Ras4B\(^{G12V}\) molecules. The oncogenic mutation, V12, is annotated in red letters. (D) The interacting interface of Rgl2\(^{RBD}\): Rgl2\(^{RBD}\), highlighting the residues involved in hydrogen and hydrophobic interactions between two Rgl2\(^{RBD}\) molecules, shaded in pink and violet. The interaction occurs at the N-terminal between the two anti-parallel \(\beta1\).
Figure 2-figure supplement 1. K-Ras4B<sup>G12V</sup>:Rgl2<sup>RBD</sup> complex is highly similar to H-Ras<sup>E31K</sup>:RALGDS<sup>RBD</sup> complex

(A) The structures of the K-Ras4B<sup>G12V</sup>:Rgl2<sup>RBD</sup> complex and the H-Ras<sup>E31K</sup>:RALGDS<sup>RBD</sup> complex (PDB: 1LFD) were superimposed by aligning the RBD chains. (B) and (C) Superimposition of each
corresponding chain of the K-Ras4B<sup>G12V</sup>: Rgl2<sup>RBD</sup> complex and H-Ras<sup>E31K</sup>:RALGDS<sup>RBD</sup> complex individually shows that each chain is similar to its respective counterpart structure. (B) K-Ras4B<sup>G12V</sup> and H-Ras<sup>E31K</sup> chains are in cyan and grey. (C) Rgl2<sup>RBD</sup> and RALGDS<sup>RBD</sup> are in purple and light pink. Amino acid sequence similarities of human Rgl2<sup>RBD</sup> and rat RALGDS<sup>RBD</sup> are shown below the RBD cartoon representations, and the conserved amino acids in Rgl2<sup>RBD</sup> and RALGDS<sup>RBD</sup> are shaded in light green and dark green, respectively. The structural similarity between Rgl2<sup>RBD</sup> and RALGDS<sup>RBD</sup> was seen beyond the regions composed of the conserved amino acids. The RALGDS<sup>RBD</sup> sequence is derived from PDB 1LFD, which is rat RALGDS<sup>RBD</sup>, and the numbering was done according to Uniprot Q03386. Root-mean-square deviation of atomic positions (RMSD) values were generated using Pymol.
Figure 2-figure supplement 2. Schematic representation of the intermolecular contacts observed in the X-ray structure
(A) The residues in K-Ras4B\textsuperscript{G12V} Switch-I region are shown in green, in the Switch-II region are shown in yellow background and those for Rgl2\textsuperscript{RBD} molecules 1 and 2 are shown in pink. V12 oncogenic mutation is in red. Hydrogen bonds, salt bridges and hydrophobic interactions were predicted by LIGPLOT and represented by green dashed lines, solid orange lines and blue dotted lines, respectively. (B) The electrostatic surface charge shows that the negatively charged Switch-I and Switch-II regions of KRas4B\textsuperscript{G12V} interact with the positively charged surface of Rgl2\textsuperscript{RBD}-1 and Rgl2\textsuperscript{RBD}-2. Surface charge potential was computed using the Pymol vacuum electrostatics function, and the negatively charged and positively charged areas are shown in red and blue, respectively.
Figure 2-figure supplement 3. Ras:RalGEF<sup>RB</sup> complex formation is distinct from other Ras:effector complexes.

K-Ras<sup>G12V</sup>:Rgl<sup>2RB</sup> complex was compared with representative Ras:effector complexes. The spectrum colour feature illustrates the orientation and ubiquitin super-fold status for one of the RBD(s). When two RBDs interact with one Ras molecule, the second RBD is coloured pink (Rgl<sup>2RB</sup>) or blue (RALGDS<sup>RBD</sup>). The K-Ras<sup>G12V</sup> in the Rgl<sup>2RB</sup> complex is in cyan, and other Ras molecules from previously published structures are in grey. The interacting residues involved in Ras Switch-II and RBDs are depicted with magenta sticks and are labelled. (A) The two Ras:RalGEF complexes (K-Ras<sup>G12V</sup>:Rgl<sup>2RB</sup> complex and H-Ras<sup>E31K</sup>:RALGDS<sup>RBD</sup> complex PDB: 1LFD).
1. LFD) show similar structural features, including the orientation of the RBDs, the Switch-I contact and the mode of usage of Switch-II. Two RBDs (RBD-1 in the spectrum colour and RBD-2 in either pink or in blue) interact separately with a single Ras molecule at Switch-I and Switch-II regions. (B) Examples of Ras:effector complex crystal structures. K-Ras4B\textsuperscript{WT}:Raf1 complex (PDB: 6VJJ) shows that although Raf1\textsuperscript{RBD} has similar orientations of the ubiquitin fold structure to Rgl2\textsuperscript{RBD-1}, it does not utilise Ras Switch-II in complex formation. Superimposition of the K-Ras4B\textsuperscript{G12V} of the Rgl2\textsuperscript{RBD} complex onto the K-Ras4B\textsuperscript{WT} of the Raf1\textsuperscript{RBD} complex shows some differences in Switch-II, β1 and α3 (arrows). PI3K\textsubscript{γ} \textsuperscript{RBD} (PDB:1HE8) and PLC\textsubscript{ε} \textsuperscript{RBD} (PDB:2C5L) complexed with Ras utilise Switch-II region differently to Ras:RalGEF complexes despite a similar orientation of the ubiquitin fold structure of RBDs. A single RBD interacts at both Switch-I and Switch-II regions of one Ras molecule. Superimposed H-Ras molecules in these complexes to K-Ras4B\textsuperscript{G12V} in the Rgl2\textsuperscript{RBD} complex gave lower RMSD values compared to K-Ras4B\textsuperscript{WT} in the Raf1\textsuperscript{RBD} complex. H-Ras\textsuperscript{D30E/E31K}\textsuperscript{RASSF5RBD} complex (3DDC) shows yet another unique interaction where the additional α-helix at the N-terminal of the RBD interacts with the Switch-II region of the Ras molecule, instead of the RBD region itself. Rgl1 is one of the RalGEFs, as Rgl2 and RALGDS are. However, unlike Rgl2 or RALGDS, K-Ras4B\textsuperscript{G12V}:Rgl1\textsuperscript{RBD} complex (7SCX) and K-Ras4B\textsuperscript{WT}:Rgl1\textsuperscript{RBD} complex (7SCW) show one Rgl1\textsuperscript{RBD} to interact with both Switch-I and Switch-II of one Ras molecule.
Figure 2-figure supplement 4. Various Ras:Ras interfaces proposed by experimental and computational prediction studies.

The top panel on the left shows the K-Ras4B$^{G12V}$:K-Ras4B$^{G12V}$ interface of the K-Ras4B$^{G12V}$:Rgl2$^{RBD}$ heterotetramer complex (this study). The K-Ras4B$^{G12V}$:K-Ras4B$^{G12V}$ contacts (shaded in purple) are located in loop regions of Switch-I, Switch-II and $\alpha$3. The K-Ras4B$^{G12V}$:K-Ras4B$^{G12V}$ interface is...
distinct from previously proposed dimerization interfaces, which can be classified into four categories based on the relevant structural elements; α5-α4, α4-α3, α-β and β-β, as listed in (a)-(n). Images of a single Ras molecule at the Ras:Ras interface are presented with the interacting residues highlighted in purple, and the involved α-helices and β-sheets are annotated. PDB IDs of the image templates are indicated next to each image. (a)-(f) Examples of Ras:Ras interfaces that involve α4-α5. (g)-(i) Examples of Ras:Ras interfaces that involve α3-α4. (j) A K-Ras4B<sup>G12D</sup>:K-Ras4B<sup>G12D</sup> interface that lies in α4-β2 and α1-β6. The two K-Ras4B<sup>G12D</sup> in the dimer are in two different orientations for this interaction to occur. (k)-(m) Examples of Ras:Ras interfaces that involve β-β. The interfaces overlap with the effector binding region and are thought to impair effector interaction. (n) The dimer formation mediated by the β-β contact was aided by a small molecule inhibitor BI2852 (shown in purple). The Mg<sup>2+</sup> is depicted as a dark-grey sphere and the bound nucleotide is shown as a ball-and-stick model.
Figure 2-figure supplement 5. Spatial Ras:Ras arrangements of K-Ras4B\textsuperscript{G12V}:K-Ras4B\textsuperscript{G12V} in the K-Ras4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} crystal complex and H-Ras\textsuperscript{WT}:H-Ras\textsuperscript{WT} interface in the H-Ras\textsuperscript{WT} crystal (5P21)
Neighboring Ras molecules are shown to reveal comparable spatial arrangements. The side view (top panel), top view (middle panel) and the blow-up of the area Y32 (bottom panel) are shown. Switch I and Switch II regions are indicated in green and yellow. (A) K-Ras4B\textsuperscript{G12V}:K-Ras4B\textsuperscript{G12V} in the K-Ras4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} complex. The Rgl2\textsuperscript{RBD} is shown as a ribbon in light magenta. (B) H-Ras\textsuperscript{WT}:H-Ras\textsuperscript{WT} in the H-Ras\textsuperscript{WT} crystal (5p21). Spatial molecular arrangements around Y32 are similar in both cases, but the oncogenic V12 provides a larger hydrophobic pocket.
Figure 2-figure supplement 6. V12 contributes to Ras:Ras interaction
Ras residues at position 12 and Y32 are shown to demonstrate the V12 contribution to stabilise the K-Ras4B\textsuperscript{G12V}:K-Ras4B\textsuperscript{G12V} interface of the K-Ras4B\textsuperscript{G12V}:Rgl\textsuperscript{2RBD} heterotetramer. The Ras:Ras interface is indicated by a grey dotted line in (A)-(C). (A) A cartoon diagram of the K-Ras4B\textsuperscript{G12V}:K-Ras4B\textsuperscript{G12V} interface of the K-Ras4B\textsuperscript{G12V}:Rgl\textsuperscript{2RBD} crystal structure where V12 generates a hydrophobic pocket together with Y32. (B) A simulation of V12 -> G12 replacement in the structure shown in (A). The hydrophobic pocket is reduced by the G12 replacement. (C) A cartoon diagram of the H-Ras\textsuperscript{E31K}:H-Ras\textsuperscript{E31K} interface of H-Ras\textsuperscript{E31K}:RALGDS\textsuperscript{RBD} crystal structure (PDB ID 1LFD). Y32 orientation is different from (A). Nonetheless, with G12, the hydrophobic area around Y32 looks less robust compared to the G12 case shown in (A). (D and E) Extended views of (A) and (C) where the neighbouring E31 (A) and K31 (D) are shown. (D) E31 of K-Ras4B\textsuperscript{G12V} interacts with K-Ras4B\textsuperscript{G12V} K88 and Rgl\textsuperscript{2RBD} K685. (E) The hydrophobic pocket created by Y32 is not reinforced by G12, but K31 of H-Ras\textsuperscript{E31K} interacts with rat RALGDS\textsuperscript{RBD} D815, N818 and D820 (the numbering was done according to Uniprot Q03386), facilitating the complex formation.
Figure 3. NMR analysis of K-Ras4B{G12V}.Rgl2{RBD} complex formation in solution

(A) Rgl2{RBD} solution structure determined by NMR. The 2D $^1$H-$^1$5N HSQC spectrum of the assigned residues of the Rgl2{RBD} is shown (left). The Rgl2{RBD} retains the $\beta\beta\alpha\beta\beta\alpha\beta$ ubiquitin-fold structure (right). 20 Rgl2 NMR structures are superimposed. (B) Comparison between the Rgl2{RBD}
structures identified in solution NMR (shown in magenta) and in crystal complex with K-
Ras4B<sup>G12V</sup> (shown in cyan). (C) NMR signal intensity changes of <sup>15</sup>N-labelled Rgl2<sup>RBD</sup> upon addition
of non-labelled K-Ras4B<sup>G12V</sup> as a function of Rgl2<sup>RBD</sup> amino acid sequence. The original HSQC
spectra of the titration experiment are presented in Figure 3-figure supplement 1B. The signal
intensities of Rgl2<sup>RBD</sup> residues in the presence of three times molar excess of K-Ras4B<sup>G12V</sup> were
divided by the signal intensities in the absence of K-Ras4B<sup>G12V</sup> and plotted as a bar-chart graph.
Proline and unassigned Rgl2<sup>RBD</sup> residues are shaded in grey. Red-dotted lines are drawn at the
fold-change values of 0.5 and 1.5 to highlight the residues that show substantial increase or
decrease of the signals upon addition of K-Ras4B<sup>G12V</sup>. Rgl2<sup>RBD</sup> residues involved in the
Rgl2<sup>RBD</sup>:Rgl2<sup>RBD</sup> interface of the K-Ras4B<sup>G12V</sup>:Rgl2<sup>RBD</sup> crystal structure are highlighted in pink on
the amino acid sequence, and residues involved in the K-Ras4B<sup>G12V</sup>:Rgl2<sup>RBD</sup> interface are
highlighted in blue. Rgl2<sup>RBD</sup> residue position numbers according to the UniProt are indicated at
the bottom of the diagram. K686, which plays a crucial role in the K-Ras4B<sup>G12V</sup> complex
formation, is annotated in orange.
Figure 3-figure supplement 1. K-Ras4B^{G12V}:Rgl2^{RBD} complex formation in solution
(A) Comparison between the human Rgl2\textsuperscript{RBD} and mouse Rlf\textsuperscript{RBD} (PDB 1RLF) solution structures. Rgl2\textsuperscript{RBD} is presented in magenta, and 20 NMR structures are superimposed. Rlf\textsuperscript{RBD} is in cyan, and 10 NMR structures are superimposed. The primary structures of the two constructs are listed below. (B) and (C) \textsuperscript{1}H-\textsuperscript{15}N HSQC titration analysis of \textsuperscript{15}N-labelled Rgl2\textsuperscript{RBD} upon addition of non-labelled K-Ras4B\textsuperscript{G12V} supports the K-Ras4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} tetramer formation. (B) Overlays of \textsuperscript{2D} \textsuperscript{1}H-\textsuperscript{15}N HSQC NMR spectra from multipoint titrations of \textsuperscript{15}N-labelled Rgl2\textsuperscript{RBD} with non-labelled K-Ras4B\textsuperscript{G12V}. Rgl2\textsuperscript{RBD}:K-Ras4B\textsuperscript{G12V} molar ratios of the titration samples are colour-coded as follows; 1:0 - black, 1:0.25 - blue, 1:0.5 – cyan, 1:1 – green, 1:2 – orange and 1:3 – red. (C) The chemical shift perturbation of backbone \textsuperscript{1}HN and \textsuperscript{15}N nuclei of Rgl2\textsuperscript{RBD} with non-labelled K-Ras4B\textsuperscript{G12V}, presented in (B), is summarised as a column diagram. The mean shift difference \(\Delta\delta_{\text{ave}}\) was calculated as \(\sqrt{\left(\Delta\delta_{1\text{HN}}\right)^2 + \left(\Delta\delta_{1\text{5N}/10}\right)^2}\) where \(\Delta\delta_{1\text{HN}}\) and \(\Delta\delta_{1\text{5N}}\) are the chemical shift differences between Rgl2\textsuperscript{RBD} on its own and in the presence of non-labelled K-Ras4B\textsuperscript{G12V}. The bar graphs are colour-coded according to the Rgl2\textsuperscript{RBD}-K-Ras4B\textsuperscript{G12V} concentration ratio and are overlaid. Proline and unassigned residues are shaded in grey. Rgl2\textsuperscript{RBD} residues in the Rgl2\textsuperscript{RBD}:Rgl2\textsuperscript{RBD} interface of the K-Ras4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} crystal structure is highlighted in pink and residues in the K-Ras4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} interface of the crystal structure is highlighted in blue. Rgl2\textsuperscript{RBD} residue positions according to the UniProt are indicated at the bottom of the diagram.
Figure 3-figure supplement 2. Rgl2 K686 plays a key role in the complex formation

(A) Multiple sequence alignment of Ras binding domain (RBD) of human BRAF (residues 155-227) and Ras Associating (RA) domains of human RALGDS (residues 798-885), Rgl1 (residues 47-134), and Rgl2 (residues 9-180).

(B) Model showing the interaction sites of K-Ras4B(G12V) with Switch I (green) and Switch II (yellow) regions of Rgl2 RBD WT.

(C) Kinetic analysis showing the response (pm) over time (sec) for Rgl2 RBD WT x K-Ras4B(G12V) and Rgl2 RBD K686A x K-Ras4B(G12V).
648-735) and Rgl2 (residues 648-735), as defined by UniProt. The alignment reveals that
sequences are relatively divergent between BRaf\textsuperscript{RBD} and RalGDS family, although all RBD/RA
domains share the common ubiquitin fold $\beta\beta\alpha\beta\alpha\beta$ structure. The RalGDS family shares a high
degree of sequence homology, including the red-arrowhead-indicated key lysine 686 residue
(highlighted in red, K686 in Rgl2\textsuperscript{RBD}), which is involved in the Ras:RA complex interface. (B) The
conserved K686 residue in Rgl2\textsuperscript{RBD} interacts via a hydrogen bond and hydrophobic interaction
with D33 in the Switch-I region of KRas4B\textsuperscript{G12V}. (D) Rgl2\textsuperscript{RBD}-K686A mutant hinders the interaction
between Rgl2\textsuperscript{RBD} and KRas4B. The Biolayer interferometry analysis of Rgl2\textsuperscript{RBD}-K686A mutant and
KRas4B\textsuperscript{G12V} did not give a conclusive Kd, likely due limitation of the technique in measuring low
binding affinity. The reduced affinity of the K686A mutant can be illustrated by a comparison
between Rgl2\textsuperscript{RBD}-K686A mutant and Rgl2\textsuperscript{RBD}-WT amplitudes during the association step with
KRas4B\textsuperscript{G12V}, which shows a decrease in the case of K686A mutant.
Figure 3—figure supplement 3. Backbone resonance assignment of GDP-bound and GMPPNP-bound K-Ras4B<sup>G12V</sup>

The 2D $^1$H-$^1$N-HSQC spectra of GDP-Bound and GMPPNP-bound K-Ras4B<sup>G12V</sup>. Cross peaks are labelled with their corresponding backbone assignments. Residues which could not be assigned are shaded in grey in the primary sequence shown at the bottom.
Figure 3-figure supplement 4. K-Ras4B<sup>G12V</sup>:Rgl2<sup>RBD</sup> complex formation in solution
\[ ^1\text{H}-^{15}\text{N}-\text{HSQC titration analysis of}^{15}\text{N}-\text{labelled K-Ras4B}^{G12V} \text{ upon addition of non-labelled Rgl2}^{RBD} \]
supports the K-Ras4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} tetramer formation. (A) and (B) The 2D \[ ^1\text{H}-^{15}\text{N}-\text{HSQC NMR spectra of} \]
K-Ras4B\textsuperscript{G12V}. \[ ^{15}\text{N}-\text{labelled K-Ras4B}^{G12V} \text{ was titrated with non-labelled Rgl2}^{RBD}. \]
(A) The 2D \[ ^1\text{H}-^{15}\text{N}-\text{HSQC spectra of} \]
K-Ras4B\textsuperscript{G12V}: Rgl2\textsuperscript{RBD} complex mixed with the molar ratio of 1:0 (black, left panel) and 1:2 (red, right panel) are shown. Many signals from \[ ^{15}\text{K-Ras4B}^{G12V} \text{ residues}
disappeared upon the addition of Rgl2\textsuperscript{RBD}. (B) Superimposed 2D \[ ^1\text{H}-^{15}\text{N}-\text{HSQC NMR spectra of} \]
\[ ^{15}\text{N}-\text{labelled K-Ras4B}^{G12V}:\text{Rgl2}^{RBD} \text{ titration experiments. The titration samples are colour-coded as follows; 1:0-black, 1:0.25 - blue, 1:0.5 – cyan, 1:0.75 –green, 1:1 – yellow, 1:1.5 – orange and 1:2 – red}. \]
(C) Fold changes of the signal intensities of \[ ^{15}\text{N}-\text{labelled K-Ras4B}^{G12V} \text{ upon addition of non-}
labled Rgl2\textsuperscript{RBD}. The signal intensities of K-Ras4B\textsuperscript{G12V} residues in the presence of two times
molar excess of Rgl2\textsuperscript{RBD} were divided by the signal intensities in the absence of Rgl2\textsuperscript{RBD}, and the obtained values were plotted as a column graph. Undetectable residues are shaded in grey. The residue T2 was also shaded grey, as the chemical shift after the addition of Rgl2\textsuperscript{RBD} overlapped with other signals. A red-dotted line is drawn at the fold-change values of 0.5 to indicate that the most residues show substantial decrease of the signals upon addition of Rgl2\textsuperscript{RBD}. K-Ras4B\textsuperscript{G12V} residues in the K-Ras4B\textsuperscript{G12V}: K-Ras4B\textsuperscript{G12V} interface of the K-Ras4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} crystal structure is highlighted in orange, and residues in the K-Ras4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} interface of the crystal structure is highlighted in blue. K-Ras4B\textsuperscript{G12V} residue positions according to the UniProt are indicated at the bottom of the diagram.
Figure 4. K-Ras4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} complex can form a heterotetramer in solution

Mass photometry analysis of KRas4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} complex in solution. (A) K-Ras4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} complex was purified using size exclusion chromatography. The fractions were analysed by 15% SDS-PAGE gel (upper panel) according to the elution profile (lower panel). Fraction 5 was chosen and diluted to a concentration of 40nM for measurement using mass photometry (OneMP, Refeyn). (B) Cartoon representing the different complexes configurations possibilities indicating that only heterodimers (~31kDa) and heterotetramers (~62kDa) can be detected the system. Histogram of the frequency counts against of the purified KRas4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} complex with fitting for Gaussian distribution (red). The duration of the video analysed was 60s and it shows that the population identified has an average mass of ~68kDa, which is in good agreement with the expected MW of the heterotetramer (61.7kDa).
Figure 5. Possible K-Ras4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} complex orientations relative to the plasma membrane

Possible conformational arrangements of the K-Ras4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} complex at the plasma membrane (PM). The Rgl2\textsuperscript{RBD} molecules may be facing the cytoplasm (PM-away conformation) or the PM (PM-proximity conformation). Cartoons are prepared based on the previously proposed $\alpha$4-$\alpha$5 mediated K-Ras4B\textsuperscript{G12D}:K-Ras4B\textsuperscript{G12D} homodimer at the PM (PDB ID 7RSC) (Lee et al., 2021). The Ras:Raf1 kinase heterotetramer cartoon was generated by merging the K-Ras4B\textsuperscript{G12D}:K-Ras4B\textsuperscript{G12D} homodimer (PDB ID 7RSC) and K-Ras4B\textsuperscript{WT}:Raf1 crystal structure (PDB ID 6Vjj) (Tran et al., 2021; Packer et al., 2020). K-Ras4B is depicted in cyan, and domains are color coded as follows: Switch-I in yellow, Switch-II in green, $\alpha$4 and $\alpha$5 helices in blue, C-terminal hypervariable region (HVR) in purple and the residue at position 12 in red. For the K-Ras4B\textsuperscript{G12V}:Rgl2\textsuperscript{RBD} structure, the HVR is in grey, as the K-Ras4B\textsuperscript{G12V} in the original crystal structure lacked the HVR.
Table 1. Data collection and refinement statistics for human K-Ras4B^{G12V}:Rgl2^{RBD} complex.

Statistics for the highest-resolution shell are shown in parentheses.

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Table 2. NMR structure statistics for Rgl2

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<tbody>
<tr>
<td>Assigned $^1$H/$^{13}$C/$^{15}$N chemical shifts</td>
<td>305/256/56</td>
</tr>
<tr>
<td>NOE distance restraints $^b$</td>
<td>866/281/777</td>
</tr>
<tr>
<td>Max. distance restraint violation (Å)</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>Deviations from idealised geometry:</td>
<td></td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>0.0138 ± 0.0001</td>
</tr>
<tr>
<td>Bond angles (º)</td>
<td>1.81 ± 0.03</td>
</tr>
<tr>
<td>AMBER energy (kcal/mol)</td>
<td>–3624.25 ± 78.09</td>
</tr>
<tr>
<td>AMBER vdW energy (kcal/mol)</td>
<td>–279.71 ± 12.49</td>
</tr>
<tr>
<td>Ramachandran plot statistics $^c$ (%)</td>
<td>89.5/10.5/0.1/0</td>
</tr>
<tr>
<td>Backbone RMSD (Å)$^d$</td>
<td>0.42 ± 0.05</td>
</tr>
<tr>
<td>All heavy atom RMSD (Å)$^d$</td>
<td>0.84 ± 0.06</td>
</tr>
<tr>
<td>Backbone RMSD to the reference (Å)$^e$</td>
<td>0.91</td>
</tr>
<tr>
<td>All heavy atom RMSD to the reference (Å)$^e$</td>
<td>1.68</td>
</tr>
</tbody>
</table>

$^a$Where applicable, the average value and the standard deviation over the 20 energy-refined conformers obtained by the program OPALp 2.1 in the presence of the experimental restraints. CYANA calculations were started from 100 conformers with random torsion angle values, simulated annealing with 10,000 torsion angle dynamics steps was applied.

$^b$Short/medium/long-range distance restraints derived from NOESY spectra.

$^c$Percentage of residues in the most favoured/additionally allowed/generously allowed/disallowed regions of the Ramachandran plot according the program PROCHECK.

$^d$RMSD to the mean structure for residues 649-657, and 665–732.

$^e$RMSD between the closest structure to the reference among the ensemble conformations and the reference structure (the crystal structure of the K-Ras4B$^{G12V}$:Rgl2 complex).