nature portfolio

https://doi.org/10.1038/s41594-021-00667-6

Supplementary information

A structural model of a Ras–Raf signalosome

In the format provided by the authors and unedited

Supplementary Information
A structural model of a Ras-Raf signalosome

Supplementary details of model construction

Preparation of simulations by type

Simulations of K-Ras monomer in solvent, on the membrane, and in a lattice

Wild-type K-Ras4B (residues 1–169) was prepared from the crystal structure PDB 4DSN with either GDP or GTP bound. Multiple simulations were launched, and the simulations in which GDP or GTP appeared unstable were discarded. For the membrane simulations, wild-type K-Ras4B (residues 1–169) was prepared from PDB 4DSN, loaded with either GTP or GDP, with the hypervariable region (HVR) residues 170–185 added to the structure in an initially extended conformation in which Cys185 was farnesylated (fCys185). The N terminus was charged. K-Ras was initially positioned in proximity to the membrane. This procedure was adopted for both the GDP- and GTP-bound K-Ras structures. For the crystal lattice simulation, GTP-bound K-Ras4B (residues 1–167) was arranged in an orthorhombic lattice, with 24 copies in the simulation cell, which was constructed based on PDB 3GFT.

Simulations of K-Ras dimerization in solvent and on the membrane

In solvent simulations, two copies of GTP-bound K-Ras proteins were placed in the simulation box without contact with one another and in arbitrary relative orientations, which differed in different simulations. In simulations of dimerization on the membrane, two copies of GTP-bound K-Ras were positioned on the membrane and not in contact with one another, with their HVR farnesyl groups buried in the membrane; the two K-Ras proteins were in the same orientation on the membrane initially. The initial membrane orientations were generated from simulations of monomeric GTP-bound K-Ras on the membrane (snapshots at 1 μs).

The GTP-mediated asymmetric (GMA) K-Ras dimer model was simulated in solvent. At the beginning of each simulation, the switch I regions in both the GTP donor and acceptor were adjusted to be consistent with the crystal structure PDB 4DSN.

Simulations of an RBD-bound K-Ras monomer and dimer

A model of monomeric GTP-bound K-Ras, bound in turn with the Ras-binding domain (RBD) of C-Raf, was constructed based on a crystal structure (PDB 4G0N) of H-Ras bound with the C-Raf RBD. This model was then simulated in solvent and on the membrane.

Based on the crystal structure PDB 4G0N, a C-Raf RBD was positioned on each protomer of the GMA K-Ras dimer and then simulated. At the beginning of each simulation, the switch I region of each K-Ras protein was adjusted to adopt the active conformation exhibited by the crystal structure PDB 4DSN. We simulated the RBD-bound K-Ras dimer both in solvent and on the membrane. In the setup of the simulations of RBD-bound K-Ras dimer on the membrane, the

K-Ras dimer was positioned in the membrane orientation from the simulation of K-Ras dimerization on the membrane (Figure 1A).

Unbiased binding simulations of Gal-3 and farnesyl

The Gal-3 carbohydrate-binding domain (residues 113–250) was prepared from the crystal structure PDB 3ZSM. Three copies of farnesylated cysteine, each capped on both ends (with N-terminal acetyl and C-terminal N-methyl amide residues), were initially placed in solvent at random positions inside the simulation box; the Gal-3 protein was positioned at the center. This simulation protocol is similar to one described previously.[1]

Simulations of a C-Raf RBD and CRD with K-Ras in solvent and on the membrane

Starting from the RBD-bound K-Ras structure, the nuclear magnetic resonance (NMR) structure of the cysteine-rich domain (CRD) of C-Raf (residues 136–187) (PDB 1FAR) was linked to the structure of the C-Raf RBD by a short linker (residues 132–135), and positioned in arbitrary orientations proximal to the K-Ras protein.

Simulations of monomeric and dimeric C-Raf linker in solvent

The C-Raf linker (residues 188–339) was constructed in an extended conformation and allowed to collapse in simulations. In simulations that included two copies of the C-Raf linker, a harmonic flat-bottom distance restraint was employed to restrict the distance between the Ser339 residues of the two strands to ~15 Å. This restraint was devised so that the linker dimer would be geometrically compatible with the C-Raf kinase domain (KD) dimer (residues 340–615), in

which the distance between the two Tyr340 residues is 11.5 Å according to the crystal structure PDB 3OMV.

Construction of the Ras-Raf signalosome model

Building the K-Ras octamer

The K-Ras octamer was constructed by repeating the GMA dimer interactions in a string of eight K-Ras proteins, starting from the K-Ras dimer on the membrane that was formed in the simulations of K-Ras dimerization, with the donor of the GMA dimer being the head of the octamer (Figure 1B). The switch I and II regions in each K-Ras protein were rebuilt to be consistent with the conformation in PDB 4DSN and to maintain the key interactions between GTP and Tyr32, Lys16, and Thr35. The interaction between the GTP γ-phosphate and either Arg135 or Lys128 that was observed in the GMA dimer model was conserved in constructing the octamer model. The switch II conformation was adjusted to create a groove in that region in the base-tier K-Ras proteins; this groove is important in the stacking interactions (to accommodate the β2–β3 hairpin of a K-Ras protein from the second tier). The series of polar interactions at the stacking interface (Lys165–Asp98, Lys172–Glu105, Arg161–Asp91, and Glu154–Arg88) were also maintained by minor adjustments to the side-chain conformations.

Adding a C-Raf RBD to each K-Ras protein

A C-Raf RBD (residues 54–131) was placed on each K-Ras unit, based on a structure of H-Ras in complex with the RBD (PDB 4G0N).

Adding a Galectin-3 protein to each K-Ras protein

The farnesylated cysteine (fCys185) of K-Ras was inserted into Gal-3 based on the complex structure of farnesylated cysteine-bound Gal-3, which was generated by unbiased binding simulations. The K-Ras HVR (residues 170–184) was built in with an extended conformation and allowed to relax in simulations. A Gal-3 protein at index *n* was positioned near K-Ras *n* such that it bound to the fCys185 and HVR of K-Ras, and its Lys210, Glu205, and Gln220 residues interacted with the Asp117, Arg100, and Asn74 residues of C-Raf RBD *n−1*, which itself made a primary interaction with K-Ras *n−1* (the GTP donor to K-Ras *n*). The Gal-3 protein bound to the fCys185 residue of K-Ras *1* was positioned in a similar orientation, although it did not interact with an RBD in the same manner. The four Gal-3 proteins that bound to the fCys185 residues of K-Ras *1–4* at the base tier were positioned on the membrane such that the first (residues 113–126) and last (residues 242–250) β strand of each Gal-3, and the K-Ras HVRs bound to each Gal-3 protein, were proximal to the membrane. The other Gal-3 proteins were positioned in an orientation similar to the Gal-3 proteins of the base tier: Gal-3 *n+4* stacked onto Gal-3 *n*, with β strands of the former (residues 242–250) and latter (residues 183–190) resembling an antiparallel β sheet. We considered only the structurally resolved carbohydrate-binding domain of Gal-3, not the unresolved N-terminal region.

Adding a C-Raf CRD to each K-Ras protein

We performed 24 simulations (totaling 122 μs) of the three-domain system in solvent (CRD tethered to a K-Ras–RBD complex), in which the CRD was initially in contact with neither the K-Ras protein nor the RBD. The simulations generated a set of structural models (Figure S7E). A CRD pose was considered only if, once positioned on the K-Ras helical assembly, it did not clash with any K-Ras proteins or the membrane. Based on the successful poses, we constructed

models of membrane-bound K-Ras bound to both the RBD and CRD for further simulation (31 simulations of 140 μs in aggregate). In one of these simulations, the K-Ras–CRD structure adopted a stable pose in which the Glu174, Arg143, Phe151, Lys157, and Leu160 residues of the CRD were positioned proximally to the Arg41, Asp54, Met170, Glu76, and Ile163 residues, respectively, of K-Ras, and a CRD Zn^{2+} interacts with Glu3 of K-Ras. The K-Ras/CRD interaction with this CRD pose exhibits electrostatic complementarity (Figure S7H).

In our signalosome model we used this K-Ras–CRD structure: The CRDs bound to the K-Ras proteins of the base tier also interact with the membrane, as do the Zn^{2+} ions bound to the CRDs. The stability of the Glu76-Lys157 and Glu3-Zn^{2+} interactions in CRD/K-Ras pairs, over the course of a nearly 100-μs long and restraint-free simulation of the complete signalosome model (Figure S7F), is indicative of the stability of the CRD/K-Ras pose in the model. In this model, the CRDs of the second tier interact with K-Ras molecules with the same pose, although they do not contact the membrane.

Adding the C-Raf linkers and kinase domains to the model

Our simulations of the C-Raf linker in solvent suggested that it may adopt a diverse set of conformations, with a large variation in the distance between the N and C termini. We selected eight different C-Raf linker conformations from these simulations. In the signalosome model, each linker was attached to a CRD (ending at Trp187), and the linker of C-Raf *n* (bound to K-Ras *n*) was positioned adjacent to the linker of C-Raf *n+4* (bound to K-Ras *n+4*). (Other C-Raf pairing patterns may also be possible, as discussed in the following section.) In the model, the KD of C-Raf *n* was arranged to dimerize with the KD of C-Raf *n+4*. The structure of the KD dimer (PDB 3OMV) was placed slightly above the membrane, with the Tyr340 residues facing inward toward the K-Ras assembly. An ATP and one Mg^{2+} ion were placed at the ATP binding

site of each KD. The C termini of the linkers were adjusted so that linker *n* and *n+4* were connected to a KD dimer. Steric clashes and interweaving of the linkers were avoided in the modeling. Phosphorylation of Ser338 and Tyr341 was also introduced. In subsequent simulations of the model, the two linkers developed extensive contact with one another (Figure 1F).

Constructing an 8-protomer Ras-Raf signalosome model with alternative C-Raf pairing

Although there are no clear experimental observations favoring a particular pattern of C-Raf pairing, given the length and flexibility of the C-Raf linker, it is structurally feasible for C-Raf *n* to pair with C-Raf *n*+1, C-Raf *n*+3, or C-Raf *n*+5, rather than with C-Raf *n*+4. C-Raf *n* pairing with C-Raf *n*+2 or CRD *n*+6 can be excluded, because the CRD C-termini separation for such a pair is too large (~150 Å). The separation between the CRD C termini is 80–100 Å for an *n/n+1*, *n/n+3*, or an *n/n+5* C-Raf pairing, while the separation is only 35–50 Å for an *n*/*n*+4 pairing. The signalosome model does not dictate a uniform pattern of Raf dimerization, but the *n/n+4* pairing (Figure 1F) would appear more likely if we consider that the other pairings require a significant part of a C-Raf linker to adopt an extended loop conformation to circumvent the Gal-3 proteins (Figure S9F, lower right panel). Further investigation is required to clarify whether C-Raf dimerization follows a uniform pattern and what that pattern might be. To demonstrate that other C-Raf dimerization patterns may be possible, we developed an alternative Ras-Raf signalosome model in which C-Raf *n* is paired with C-Raf *n*+1 (Figure S9F).

Adding the 14-3-3σ dimer to each C-Raf KD dimer

C-Raf was extended C-terminally to residue 625, with Ser621 phosphorylated. A 14-3-3σ dimer was attached to each KD dimer based on the crystal structure PDB 4IEA, in which each 14-3-3σ is bound with a C-Raf phospho-peptide (residues 618–625) including pSer621. The orientation of the 14-3-3σ dimer with respect to the KD dimer is unknown, but limited flexibility is afforded by the two residues (Ile616 and Asn617) that connect the KD and the phospho-peptide bound to 14-3-3σ. Under this constraint, together with an assumption of symmetry that ensures the two 14-3-3σ proteins interact with the two KDs in the same way, we constructed a model of the C-Raf KD dimer bound with the 14-3-3σ protein. Based on this hetero-tetrameric C-Raf KD–14-3-3σ model, 14-3-3σ proteins were incorporated into the signalosome model.

Adding MEK1 to each C-Raf KD

Based on a structure of a B-Raf KD bound with MEK1 (PDB 4MNE), we positioned a MEK1 KD (residues 66–382) abutting each C-Raf KD as a substrate protein in the signalosome model. This also positioned the N-lobe of MEK1 against 14-3-3σ. The missing residues of MEK1 (residues 275–305) were modeled as an extended loop, which collapsed towards the C-lobe of the MEK1 KD in simulations.

Simulating the eight-protomer Ras-Raf signalosome model

The simulated signalosome model consists of eight protomers, each of which includes a K-Ras, a Gal-3, a C-Raf, a 14-3-3σ, and a MEK1 protein. These components were incorporated into the model in a stepwise fashion: 1) by extending the GMA dimer, a K-Ras octamer was constructed and simulated. A model with consistent K-Ras/K-Ras interactions, and stability in a simulation

of ~10 μs, was used in further modeling; 2) 8 C-Raf RBD domains were added to the K-Ras octamer based on an RBD-Ras crystal structure, and the resulting model was simulated. The K-Ras/RBD model was stable in these μs-long simulations; 3) based on our simulation studies of Gal-3 interactions with the farnesylated K-Ras tail, with full-length K-Ras protein, and with another Gal-3, 8 Gal-3 proteins were added to the K-Ras/RBD octamer model, and the resulting model was simulated. At the end, a K-Ras/RBD/Gal-3 model, stable for 20 μs in a simulation, was chosen for further modeling; 4) after our simulation study of CRD interaction with an RBD-bound K-Ras, 8 C-Raf CRD domains were added to the model and simulated. In the event that a Gal-3 molecule or a CRD deviated from the initial modeled position in the simulation, we manually repositioned the Gal-3 or CRD, and then relaunched the simulation. A model with consistent CRD/K-Ras and Gal-3/K-Ras interactions, stable in μs-long simulations, was chosen; 5) after simulations of C-Raf linkers and their interaction with one another in isolation, and modeling of the two-protomer C-Raf/MEK/14-3-3σ complex, each C-Raf in the octamer model was extended to full length, with the linkers paired with one another, and with the kinase domains dimerized and interacting with 14-3-3σ dimers and MEK kinases. This model was then simulated to nearly 100 μs, and remained stable in the simulation without any artificial restraints (Figure S1B).

In step 5, the simulation of a complete signalosome model consisted of five phases. In the first phase, weak harmonic positional restraints (with a spring constant of 1 kcal mol^{-1} $Å^{-2}$) were applied to the backbone atoms of the CRD and the KD domain of each C-Raf for 1 μs. This allowed the disordered C-Raf linkers to equilibrate. In the second phase, weak harmonic flat-bottom distance restraints were employed for ~1 μs to facilitate structural relaxation at the predicted protein-protein interfaces, particularly the CRD interface with K-Ras and the Gal-3/Gal-3 interface. This was designed to allow these protein-protein interactions to equilibrate after more stringent harmonic positional restraints were removed. In the third phase, all

restraints were removed, and extensive equilibrium MD simulations were performed. In the fourth phase, the complex was inspected for overall stability and symmetry in the interaction surfaces. Any Gal-3 protein, C-Raf CRD, or MEK1 kinase that had deviated substantially from the initial model was remodeled, and then the simulation was relaunched. These four phases were repeated, until a stable signalosome model was achieved. In the fifth phase, the final signalosome model was simulated for nearly 100 μs without any restraint applied to any protein-protein interactions.

Supplementary analyses

Simulations of GTP-bound K-Ras in a crystal packing

In X-ray crystal structures of wild-type Ras, although binding to GTP analogs appears to be more stabilizing to the active conformation of the switch regions than GDP binding, conformational heterogeneity remains (Figure S3E and S3F). In crystal structures of the GTP-bound Ras mutant T35S, the heterogeneity is more pronounced (Figure S3G), consistent with the fact that T35S is an inactivating mutation. The conformation of Ras bound to GTP analogs in crystals at the switch regions (Figure S3E) appears much less varied than in our simulations of GTP-bound K-Ras (Figure S3B). To reconcile this apparent discrepancy, we set up a crystal lattice with 24 copies of wild-type GTP-bound K-Ras in a simulation box, based on the crystal lattice underlying PDB 3GFT, and simulated this for 4 μs. From the 24 copies, we took a conformational snapshot every 1 μs. We found that K-Ras was significantly less flexible in the lattice than in solvent; in the lattice the relative population for the active conformation at the switch one region was 45%, as opposed to 11% in the solvent (Figure S3A and S3H). This result

suggests that crystal packing may mask the inherent conformational fluctuation of GTP-bound K-Ras. We also simulated RBD-bound K-Ras, and found that the switch regions were most stable in the active conformation upon RBD binding (Figure S3D).

Timescale of protein dimerization and the length of our simulations

In the absence of a favorable electrostatic steering force, an upper bound for the "on" rate of protein-protein binding is ~10^6 $M^{-1}s^{-1}$.[2] At the millimolar protein concentration relevant for our simulations, the average time required for a binding event is thus ~1 ms. Since the timescale of our individual simulations of K-Ras dimerization (~10 μs) is significantly shorter than that of protein-protein, we would not expect most of the simulations to converge to a single dimer model. Of the tens of K-Ras/K-Ras binding simulations we conducted, indeed only two (one in solvent and one with membrane) arrived at the GMA K-Ras dimer structure. (We did not obtain any other dimer from these simulations more than once, although this itself is not sufficient evidence to support the GMA dimer.) To obtain a Boltzmann ensemble of K-Ras dimer conformations, the simulations need to sample both the formation and the dissociation of the GMA dimer adequately. The former event has only been sampled twice, and the latter has not been sampled. We thus do not expect that the snapshot from all the simulations combined can generate the Boltzmann ensemble of K-Ras dimers, or a correct relative population of the dimers.

Analogous Arg-GTP interaction in Toc33-Toc34 dimers

A trans interaction similar to that in the GMA K-Ras dimer model has been reported[3] between arginine and GTP phosphate in GTP-mediated dimers of Toc33 and Toc34 (Figure S2H, PDB

3BB1), two isoforms of a GTPase receptor protein in the outer envelope of chloroplasts; indeed, these homodimers do not promote GTP hydrolysis.[4]

^{15}N-HSQC broadening profile of K-Ras and K-Ras dimerization

Twelve residues exhibited broadening of ^{15}N-HSQC spectra upon sample dilution of GTP-γS-bound truncated K-Ras-4B (Residue 1–166).[5] Eight of the twelve residues (Lys16, Asp30, Glu31, Asn86, Asp119, Gln131, Arg135, Lys147) are located at the GMA dimer interface, and four are not (Ile24, Glu37, Ser39, Val44) (Figure 2E). The broadening of the eight residues can be easily explained by the dimerization. The broadening of Ile24 (immediately N-terminal to switch I) and Glu37 and Ser39 (immediately C-terminal to the switch I) is consistent with the notion that GMA dimerization stabilizes the donor's active conformation of switch I. Val44 is adjacent to the α5 helix of the acceptor interface, and the broadening may be a result of the altered α5 dynamics in the GMA dimer.

NMR chemical shift perturbations (CSPs) were also observed upon sample dilution of full-length GTP-γS-bound K-Ras4B for nine residues. The CSP profile of the full-length construct is not identical to that of the tail-truncated construct (residues 1–166), potentially because in solution the HVR tail interacts with the catalytic domain and affects K-Ras dimerization.[6] The CSPs are broadly consistent with the GMA dimer as well. Of the nine CSP residues, six residues are part of the GTP-acceptor interface (Ile84, Val125, Ala130, and Glu143) or immediately adjacent to the interface (Val114 and Tyr157); Glu91 is adjacent the GTP-donor interface; Asp38 is immediately C-terminal to the switch I; Ala18 is secluded and interacts intimately with the mediating GTP, and may be indirectly affected by the GTP-mediated dimerization.

The GMA K-Ras dimer has not been captured by X-ray crystallography

Notably, in our survey of crystal structures we did not find a Ras GMA dimer. We believe that this could be attributable to the fact that GMPPNP is a poor mimic of GTP with respect to mediating Ras dimerization as shown by NMR analysis.[5] To understand why GMPPNP is a poor mimic of GTP in Ras dimerization, we simulated the GMA K-Ras dimer with GMPPNP replacing GTP for 8 μs. We observed that the interactions at the GMPNP-mediated interface resembled those in the GTP-mediated interface, with one important exception: In the case of GTP, the amide group of the receiver Gln131 side chain was favorably positioned next to the GTP ether group linking the β and γ phosphates. In the simulation of GMPPNP, the Gln131 amide was similarly positioned next to the linking nitrogen atom of GMPPNP, but the polar hydrogen bonded to this linking nitrogen is separated by only 2.5 Å from a polar hydrogen on Gln131, a collision making this conformation clearly unfavorable (Extended Data Fig. 2D). This observation is also consistent with the finding[5] that GTPγS, which like GTP bears an ether linker, is a better mediator of Ras dimerization. Another potential explanation for the absence of the Ras GMA dimer in crystal structures is that the constraint of membrane association, which is missing in crystal structures, may be required to stably form this interaction.

Intriguing questions with regard to G13D and G13R oncogenic mutations

Unlike the other oncogenic mutations we tested, in our simulations G13R and G13D disrupted the GMA K-Ras dimer (Figure S2K). This is corroborated by our preliminary BRET data, which showed that G13D disrupts K-Ras assembly (Figure S5F). Further investigation is required to

address whether these two mutations indeed are incompatible with the GMA dimer and if so, what might be the biological implications.

Complicating factors associated with the R135A mutation in K-Ras

In light of the interaction between Arg135 and GTP predicted by the GMA dimer model, we tested the R135A mutation, with the expectation that its effect would be similar to the other four mutations we tested. We did not, however, observe increased CFP emission after YFP bleaching for R135A (Figure S5E), and its effect on *K-Ras*^{lox}*/K-RAS*^{MUT} cell growth was moderate (Figure 3F and Figure S4B). R135A also did not disrupt ERK phosphorylation, although it boosted AKT phosphorylation (Figure 3G and Figure S4C). To reconcile these results, we considered the possibility that R135A might alter the biochemical properties of K-Ras. Indeed, although Arg135 is distal from the nucleotide binding site, we found that in the absence of any GEF protein, the inherent nucleotide exchange rate of R135A was significantly higher than that of the wild-type (Figure S5D). This may have increased the population of GTP-bound K-Ras in the cells and masked the effect of R135A on K-Ras dimerization.

R135A/R128A double mutation

The double mutation R135A/R128A attenuates H-Ras nanoclustering.[7] In addition to reducing effector recruitment and MAPK signaling, the double mutation is thought to affect nanoclustering primarily by altering the orientation of Ras on the membrane.[8] If similar helical assemblies of H-Ras underlie the nanoclustering, our model suggests that the R135A/R128A mutation would attenuate H-Ras nanoclustering and signaling by disrupting GMA H-Ras dimers. Given the connection between the GMA dimerization and the orientation of Ras on the

membrane (Figure 2G), the observed change in Ras orientation may in part be associated with a double mutation–induced change in Ras dimerization.

The E62R mutation abolishes AKT phosphorylation

The E62R mutation is unique among the mutations we tested in this study, in that it abolishes AKT phosphorylation (Figure 3G) and is located at the switch II region. Raf primarily binds at the switch I region, but PI3K kinase binds with both the switch I and II regions.[9] Although E62R may disrupt the GMA dimerization and hinder ERK phosphorylation, it may more effectively disrupt Ras-PI3K binding and affect AKT phosphorylation in the PI3K-AKT pathway. Further investigation is needed to understand the effect of the E62R mutation on this pathway. Basal expression levels of the E62R mutant were lower than those of the other mutants, in agreement with previous reports that demonstrated that some mutations in the switch II region of K-Ras are hypomorphic.[10,11]

Simulations investigating membrane orientations of K-Ras

In the NMR-identified α orientation, a bound GTP tends to have the guanine moiety closer than the phosphates to the membrane, but in the NMR-identified β orientation the opposite is the case, and the GTP is also further removed from the membrane. We thus used the guanine and phosphate distances to the membrane to represent a K-Ras orientation (Figures S2E and S2F). We simulated a GTP-bound K-Ras monomer on the membrane for 67 μs in total and a (GTP-bound) K-Ras monomer bound with a C-Raf RBD on the membrane for 50 μs in total, and compared the distributions of K-Ras orientations in these simulations with the distribution in a simulation of the GMA dimer. The RBD-free monomer adopted two clusters of orientations, one

in the RBD-compatible α region, and one in the RBD-occluding β region (Figure 2G). In contrast, the RBD-bound monomer only adopted orientations of the α region, as did the two K-Ras proteins in the simulations of a GMA dimer. In the α orientation, as suggested by a previous study,[12] Met170 at the C terminus of the α5 helix tended to be partially buried in the membrane in our simulations. This analysis indicates that the membrane interaction of K-Ras may promote GMA dimerization by restraining the K-Ras orientation, and that GMA dimerization may promote K-Ras recruitment of Raf proteins by imposing membrane orientations favorable to RBD binding.

Comparison to previously reported Ras dimer and oligomer models

A number of Ras dimer structures have been proposed,[13–16] including symmetric models that use either the α4-α5 helices or the α3-α4 helices as the dimer interface. A pentamer model has also been proposed[17] that uses both interfaces. Because our signalosome model also involves Ras-Ras interfaces at these two locations, the mutagenesis data that support those models are also consistent with the signalosome model. Specific mutations have included K101E, E107K, K101A/R102A, H94A/H95A,[17] E98K/D105K, K165E/K172D,[14] K147, D154, and R161.[13] These mutations were found to disrupt Ras nanoclustering. Combinations of mutations that recover nanoclustering include K101E/E107K[17] and the E98K/D105K/K165E/K172D quadruple mutation.[14] In our analysis of the GMA dimer interface we found that K147 and D154 participate in acceptor interactions, H94, H95, E98, K101, K102, D105, E107, K165, and K172 participate in stacking interaction (Figure S6A), and R161 participates in both. Because of potential interface overlaps with the previous models, it is difficult to use a single mutation to discriminate between the GMA and other models. The combination of K101E and E107K single mutations, and the combination of E98K/D105K and K165E/K172D double mutations, are

shown to restore Ras nanoclustering.[17] This was explained by the pentamer model, but it can also be explained by the helical assembly model, since the residues belonging to these two sets of mutations participate the stacking interaction (Figure S6A).

One important distinction is that previously reported models do not place switches I and II, which are modulated by GTP binding, at Ras-Ras interfaces. This makes it difficult to explain the effects of GTP on K-Ras dimerization, whereas the GMA dimer provides a straightforward explanation. In the previous dimer and pentamer models, each Ras-bound Raf RBD is in contact with one Ras. By contrast, in our model each RBD is in contact with at least two K-Ras protomers, offering a structural explanation for the finding that RBD binding promotes K-Ras dimerization.[18] Our GMA model and the previous models also differ in that they impose different membrane orientations on the Ras molecules. The α3–α5 helices, which are parallel or anti-parallel to one another, are parallel to the membrane surface in the GMA model (α orientation), but are nearly perpendicular (β orientation) in the previous models. As a result, the switch III region,[8] which regulates Ras membrane orientation, is in contact with the membrane in the GMA dimer but not in earlier models. The previous models leave no room for involvement of Gal-3 in the dimerization. We think that this connection between K-Ras orientation, dimerization structure, and Gal-3 involvement is important, and suggests that the presence of Gal-3 may regulate the membrane orientation and dimerization structure of K-Ras. Because experimental data suggest that Gal-3 is crucial to Raf signaling,[19] it is not likely that the Ras assembly underlying Raf signaling is incompatible with Ras/Gal-3 interaction, as the previously reported dimer and oligomer models of K-Ras are.

Estimate of the local concentration of constituent proteins in the Ras-Raf signalosome

Our modeled eight-protomer signalosome is approximately 140 Å in radius and 80 Å in height, and thus about 5×10^6 Å^3 in volume. This translates to a local concentration of approximately 2.7 mM for each of the eight proteins constituent to the signalosome.

Additional details of the secondary and tertiary Ras-Ras interactions

In the secondary Ras-Ras (stacking) interaction, the α3 helix and N-terminal part of the switch II region of a K-Ras protein at position *n* packs with the α5 helix and β2-β3 hairpin of K-Ras *n*+4 (Figure 4C). The stacking interface of K-Ras *n*+4 largely overlaps with the membrane interface of a K-Ras protein in the base tier (Figure 1B). The stacking interaction of two K-Ras proteins involves a buried interface with an area of about ~1450 Å^2 and a number of salt bridges at the interface, including Glu98-Lys165 and Asp105-Lys172 (Figure S6A). This may explain a recent report that E98K/D105K and K165E/K172D mutations individually disrupt K-Ras nanoclustering but together rescue it.[17] The stacking is also consistent with K-Ras nanoclustering being disrupted by K101A/R102A and H94A/H95A mutations,[14] as these residues are also located at the stacking interface in the helical assembly. The tertiary interface is smaller, with a buried interface area of ~660 Å^2, and involves the α4 helix of K-Ras *n* and the α1 helix and switch I region of K-Ras *n*+3 (Figures 4C, right panel, and Figure S6B).

The locations of various reported Ras mutations in the signalosome model

The surface of a K-Ras protein in the signalosome model is almost entirely covered by interactions with other proteins, and so it is not surprising that many mutations affecting the

function of K-Ras are located at the interfaces between Ras and other proteins in the signalosome model. We did not consider these mutations in the modeling. Both the K-Ras helical assembly and interactions of K-Ras with the membrane were determined by the membrane-anchored GMA K-Ras dimer model generated by simulations, and the RBD interactions with the Ras assembly were determined by the resolved crystal structure of the Ras-RBD complex.

Many cancer mutations and RASopathy mutations, including T50I, C51Y, R164Q, G48R, E49K, D47A/E49A, E153V, and F156L, are located at the β2-β2 turn and the α4 and α5 helices (the so-called "switch III" region[8]) and alter Ras nanoclustering.[20] At the base tier of the signalosome model, these mutations are involved in the Ras-membrane interaction (Figure S8A). This is consistent with the possibility that the adverse effect of these mutations comes from altering the interactions of Ras with the membrane.[20,21] Additionally, in the signalosome model these mutations are located at the upper interface of the Ras-Ras stacking (i.e., the interface of Ras *n+4* stacked with Ras *n*), which primarily involves the β2-β2 turn and α5 helix (Figure 4C, right panel). The functional effects of these mutations and their connections with RASopathy and cancer thus may partially arise from their effects on Ras-Ras stacking in addition to Ras-membrane interactions. Two H-Ras mutations, G48R and D92N, are found together in spitzoid tumor samples and are known to elevate H-Ras nanoclustering.[20] Assuming the signalosome model is broadly applicable to H-Ras, we suggest that Gly48 and Asp92 may be adjacent to one another in *trans* in Ras stacking (Gly48 at the upper stacking interface of Ras *n+4* and Asp92 at the lower stacking interface of Ras *n* (Figure S6A)), and these two mutations may interact with one another, potentially strengthening the stacking and promoting the formation of the Ras signalosome.

Modeling of Gal-3 and Raf-CRD

In the modeling, we attempted to accommodate the findings that the RBD mutations D117A and D117R weaken the nanoclustering and RBD-galectin binding;[22] that the Gal-3 mutation V126A does not appear to affect the farnesyl binding, but does affect Gal-3 colocalization with K-Ras at the membrane;[19] and that a thiodigalactoside-derived Gal-1 inhibitor does not affect the interaction between Gal-1 and the C-Raf RBD.[22] In the resulting signalosome model, the Asp117 residue of the RBD is located at the Gal-3–RBD interface, the thiodigalactoside inhibitor binding site of Gal-3 is positioned away from that interface, and Val126 is proximal to the membrane (Figure S7A). At the base tier, the Gal-3 position relative to K-Ras is broadly consistent with the position of similar farnesyl capping proteins (such as PDE6δ) relative to small G proteins (Figure S7C). The unstructured N-terminal segment of Gal-3 may be crucial to Gal-3 oligomerization;[23] although we did not include this in our model, ample space is available to accommodate it.

A number of Gal-3 mutations have been reported to disrupt Gal-3 dimerization (W181L),[24] Gal-3 co-localization with K-Ras (G182A),[23] and K-Ras nanoclustering (V126A).[19] Additionally, the Gal-1 quadruple mutation C3S/L5Q/V6D/A7S—corresponding to Gal-3 residues Pro113, Ile115, Val116, and Pro117—has been reported to disrupt Gal-1 dimerization and H-Ras nanoclustering.[22] We accommodated these findings in our modeling of Gal-3–Gal-3 interactions, and in the signalosome model these Gal-3 residues are located at the Gal-3 stacking interfaces (Figure S7B).

A CRD is connected with an RBD by a linker of only four residues, and limited exposed K-Ras surface area is available for interacts with a CRD in the helical assembly after RBD binding. The CRD pose is largely constrained by RBD-CRD connectivity, the interactions of the two zinc

ions in contact with the membrane, and the limited available space. CRD mutations at Ser177, Thr182, Met183,[25] Phe151, Leu149,[26] and Ras mutation at Val45[27] have been shown to affect CRD-Ras binding. In the Ras-CRD pose adopted in the model, these mutations are located at or near the Ras-CRD interface (Figure S7F) or at the CRD interface with the membrane. Further, in the model the CRD directly interacts with the switch II region of Ras, which is consistent with the finding that mutations in the switch II regions (G60A and Y64W) may disrupt CRD binding.[28] Although the Ras-CRD pose in the model is consistent with existing experimental data, it remains to be experimentally validated; we note, however, that this pose is not an essential element of the overall structure of the Ras-Raf signalosome model.

Comparison with the recently reported Ras-CRD structures

We compared our Ras-CRD model with the recently reported crystal and NMR Ras-CRD structures.[29–31] In these experimentally determined structures the CRD is positioned between the β2-β3 strands and the N-terminal half of the α5 helix, whereas in our model the CRD is positioned between the same strands and the C-terminal half of the helix. Although the CRD positions are similar, the CRD pose in the NMR structure on lipid nanodiscs differs substantially from the crystal structures. It is worth noting that the NMR structure was solved in the presence of lipids, whereas the crystal samples were not. In our simulations, analysis of K-Ras/CRD interactions showed populations of both CRD positions, although the poses varied. The NMR and crystal conformation corresponds to cluster 2 in Figure S7E. This variability in CRD positions and poses seen in a crystal lattice (crystal structure), solvent (NMR), and in simulations is consistent with the notion that CRD interactions with Ras are very weak and likely malleable, depending on Ras dimerization and membrane orientation. The CRD position in the crystal structures, for example, is incompatible with the α membrane orientation of Ras, and thus not

adopted in our signalosome model, as GMA dimer formation imposes the α orientation. Such a CRD position should be possible in potential assemblies involving K-Ras dimers and oligomers that impose a β orientation.[13,14–16]

Opposing role of Gal-3 and PDEδ with respect to K-Ras membrane localization

Both PDEδ and Gal-3 are known to cap the farnesyl group of K-Ras, and our modeling of K-Ras interactions with Gal-3 are informed by the crystal structures of K-Ras/PDEδ.[32] We are not, however, aware of any evidence that PDEδ plays a similar role to Gal-3 or Gal-1 in promoting Ras nanoclustering. Whereas Gal-3 is found to be localized to membrane rafts[33] and mediate membrane localization of K-Ras,[34] PDEδ was found to return K-Ras from the cell membrane to the cytosol,[35] and likewise the K-Ras/PDEδ complex tends to dissociate upon contact with membrane.[36] It thus appears that PDEδ and Gal-3 play opposing roles with respect to K-Ras membrane localization.

Modeling of the C-Raf kinase dimer in complex with a 14-3-3σ dimer

A dimer structure of 14-3-3σ, in which each monomer is bound to a peptide representing the phosphorylated C-terminal loop (residues 618–625) of C-Raf, has previously been resolved.[37] We modeled a complex of the 14-3-3σ dimer and C-Raf kinase domain dimer (PDB 4IEA).[38] This was largely constrained by the fact that only two unresolved C-Raf residues connect the kinase domain and the resolved 14-3-3σ–bound C-terminal loop. Based on a crystal structure of MEK1-bound C-Raf kinase domain,[39] we added two MEK1 kinases to the complex structure as substrates of C-Raf (Figure 1F). (A Raf/MEK/14-3-3 complex structure was reported by Eck and colleagues[40] while this manuscript was under revision. Our model, at a high level, is largely

consistent with this cryo-EM structure (Figure S9A), although in our model the Raf kinase domains make more direct contact with 14-3-3 proteins than in the cryo-EM structure.)

The signalosome model may not be applicable to other Ras effectors

It is far from clear whether the Ras-Raf signalosome model is applicable to other effectors beyond Raf kinases. This model is almost certainly not applicable to PI3K signaling: A PI3K protein would clash with the membrane at the base tier of the Ras helical assembly and lose access to its phosphatidylinositol 4,5-bisphosphate (PIP2) substrate lipid at any higher tiers of the helical assembly. Raf and PI3K are laterally segregated on the membrane in nanoclusters.[41] An increase in the concentration of Gal-1 has been shown to lead to an increase in C-Raf activation by H-Ras[42] but a decrease in PI3K activation.[43] In light of PI3K preferentially binding to Ras nanoclusters in which Ser181 is phosphorylated,[44] we conjecture that distinct complex structures underlie Ras-Raf and Ras-PI3K nanoclusters. Consistent with this notion, with the exception of E62R, the mutations we designed to disrupt GMA K-Ras dimerization do not reduce phosphorylation of AKT in the PI3K pathway (Figures 3G and S4C).

Lys128 and Arg135, two key residues at the putative GMA dimer interface, are conserved in H-Ras and N-Ras, and in 6 of the 13 human Ras isoforms.[4] Homology analysis (Figure S2L) shows that these two residues are not well conserved evolutionarily, suggesting that the signalosome structure is not general to small G-proteins. Lys128 and Arg135, however, are conserved in mammals, suggesting that the signalosome model may be broadly relevant to mammalian MAPK signaling. In many respects, findings on H-Ras nanoclustering echo those on K-Ras, and the signalosome model is consistent with the structures of other Ras and Raf proteins (such as N-Ras, H-Ras, A-Raf, and B-Raf) involved in MAPK signaling. With local

alterations, the model may be extendable to those Ras and Raf proteins and provide a framework for understanding their overlapping yet distinct roles in MAPK signaling.

Interactions involving the switch II pocket of K-Ras

SOS1 carries four basic residues (Lys949, Arg950, His951, and Lys953) at the turn of the helical hairpin that are not conserved beyond the SOS family. The helical hairpin is inserted into the switch II pocket of K-Ras in the putative secondary SOS1 interaction with K-Ras in the helical assembly. This could potentially explain the specificity of SOS1 in up-regulating MAPK signaling.

The effect of the D154Q/R161E double mutation

Ambrogio et al. reported[23] that D154Q and R161E single mutations disrupt K-Ras dimerization. These findings were used to support a symmetric K-Ras dimer model that uses the α4-α5 helices as the dimer interface. Because the GTP acceptor in the GMA dimer model uses the same interface, and D154 and R161 are involved in salt bridges at the GMA dimer interface (Figure 3B), the findings are highly consistent the GMA dimer model. The previous study also showed that D154Q/R161E recovers K-Ras nanoclustering, and this was interpreted as supporting evidence for the symmetric K-Ras dimer model. Our simulation of an octameric D154Q/R161E helical assembly showed that these two mutations may be compensatory in the K-Ras helical assembly. The simulation showed that D154Q disrupts the D154-K147 salt bridges at the primary interfaces, but repositions the α5 helix, enabling the formation a compensatory E161-R102 salt bridge at the secondary interface (Figure S6G).

SOS1 and GAP access to K-Ras molecules beyond the base tier

Our structural analysis of SOS1 suggests that the linker connecting the GEF and the PH domains of SOS1 (residues 546–579) is sufficiently long and flexible to allow the SOS1-GEF domain to dock to any K-Ras of at least the second tier of the helical assembly while the SOS1-PH domain is anchored to the membrane in a favorable membrane orientation[45] (Figure S9G). This analysis suggests that the helical assembly cannot grow beyond the second tier, which is consistent with a previous estimate that a K-Ras nanocluster contains 5–8 K-Ras proteins.[46] It is conceivable, however, that the helix portion of the PH-GEF linker (PDB entry 1XD4) could unwind, allowing greater reach for the GEF domain and thereby larger K-Ras assemblies. Similar analysis also suggested that the linker connecting the GAP domain and the membrane-anchoring C2 domain of Ras-GAP protein (residues 677–747) is amply long to allow the GAP domain access to K-Ras proteins in the second (and possibly higher) tiers of the helical assembly.

Supplementary References

1. Shan, Y., Kim, E.T., Eastwood, M.P., Dror, R.O., Seeliger, M.A., and Shaw, D.E. (2011). How does a drug molecule find its target binding site? J. Am. Chem. Soc. *133(24)*, 9181−9183.

2. Schreiber, G., Haran, G., and Zhou, H.-X. (2009). Fundamental aspects of protein-protein association kinetics. Chem. Rev. *109(3)*, 839–860.

3. Gasper, R., Meyer, S., Gotthardt, K., Sirajuddin, M., and Wittinghofer, A. (2009). It takes two to tango: regulation of G proteins by dimerization. Nat. Rev. Mol. Cell Biol. *10(6)*, 423–429.

4. Koenig, P., Oreb, M., Rippe, K., Muhle-Goll, C., Sinning, I., Schleiff, E., and Tews, I. (2008). On the significance of Toc-GTPase homodimers. J. Biol. Chem. *283(34)*, 23104–23112.

5. Muratcioglu, S., Chavan, T.S., Freed, B.C., Jang, H., Khavrutskii, L., Freed, R.N., Dyba, M.A., Stefanisko, K., Tarasov, S.G., Gursoy, A., Keskin, O., Tarasova, N.I., Gaponenko, V., and Nussinov, R. (2015). GTP-dependent K-Ras dimerization. Structure 23(7), 1325–1335.

6. Chavan, T.S., Jang, H., Khavrutskii, L., Abraham, S.J., Banerjee, A., Freed, B.C., Johannessen, L., Tarasov, S.G., Gaponenko, V., Nussinov, R. and Tarasova, N.I. (2015). High-affinity interaction of the K-Ras4B hypervariable region with the Ras active site. Biophys J, *109(12)* 2602–2613.

7. Guzmán, C., Šolman, M., Ligabue, A., Blaževitš, O., Andrade, D.M., Reymond, L., Eggeling, C., and Abankwa, D. (2014). The efficacy of Raf-recruitment to H-ras depends on H-ras membrane conformer specific nanoclustering. J. Biol. Chem. *289(14)*, 9510−99533.

8. Abankwa, D., Hanzal-Bayer, M., Ariotti, N., Plowman, S.J., Gorfe, A.A., Parton, R.G., McCammon, J.A., and Hancock, J.F. (2008). A novel switch region regulates H-ras membrane orientation and signal output. EMBO J. *27(5)*, 727–735.

9. Pacold, M.E., Suire, S., Perisic, O., Lara-Gonzalez, S., Davis, C.T., Walker, E.H., Hawkins, P.T., Stephens, L., Eccleston, J.F., and Williams, R.L. (2000). Crystal structure and functional analysis of Ras binding to its effector phosphoinositide 3-kinase γ. Cell *103(6)*, 931–944.

10. Shieh, A., Ward, A.F., Donlan, K.L., Harding-Theobald, E.R., Xu, J., Mullinghan, C.G., Zhang, C., Chen, S.-C., Su, X., Downing, J.R., Bollag, G.E., and Shannon, K.M. (2013). Defective K-Ras oncoproteins overcome impaired effector activation to initiate leukemia in vivo. Blood *121(24)*, 4884–4893.

11. Shankar, S., Pitchiava, S., Malik, R., Kothari, V., Hosono, Y., Yocum, A.K., Gundlapalli, H., White, Y., Firestone, A., Cao, X., Dhanasekaran, S.M., Stuckey, J.A., Bollag, G., Shannon, K., Walter, N.G., Kumar-Sinha, C., and Chinnaiyan, A.M. (2016). KRAS engages AGO2 to enhance cellular transformation. Cell Rep. *14(6)*, 1448–1461.

12. Neale, C. and García, A.E. (2018). Methionine 170 is an environmentally sensitive membrane anchor in the disordered HVR of K-Ras4B. J. Phys. Chem. B *122(44)*, 10086–10096.

13. Ambrogio, C., Köhler, J., Zhou, Z.-W., Wang, H., Paranal, R., Li, J., Capelletti, M., Caffarra, C., Li, S., Lv, Q., Gondi, S., Hunter, J.C., Lu, J., Chiarle, R., Santamaría, D., Westover, K.D., and Jänne, P.A. (2018). KRAS dimerization impacts MEK inhibitor sensitivity and oncogenic activity of mutant KRAS. Cell *172(4)*, 857–868.

14. Prakash, P., Sayyed-Ahmad, A., Cho, K.-J., Dolino, D.M., Chen, W., Li, H., Grant, B.J., Hancock, J.F., and Gorfe, A.A. (2017). Computational and biochemical characterization of two partially overlapping interfaces and multiple weak-affinity K-Ras dimers. Sci. Rep. *7*, 40109.

15. Jang, H., Muratcioglu, S., Gursoy, A., Keskin, O., and Nussinov R. (2016) Membrane-associated Ras dimers are isoform-specific: K-Ras dimers differ from H-Ras dimers. Biochem. J. *473(12)*, 1719–1732.

16. Lee, K.Y., Fang, Z., Enomoto, M., Gasmi-Seabrook, G., Zheng, L., Koide, S., Ikura, M. and Marshall, C.B. (2020). Two distinct structures of membrane-associated homodimers of GTP- and GDP-bound KRAS4B revealed by paramagnetic relaxation enhancement. Angew. Chem. Int. Ed. Engl. *59(27)*, 11037–11045.

17. Sarkar-Banerjee, S., Sayyed-Ahmad, A., Prakash, P., Cho, K.-J., Waxham, M.N., Hancock, J.F., and Gorfe, A.A. (2017). Spatiotemporal analysis of K-Ras plasma membrane interactions reveals multiple high order homo-oligomeric complexes. J. Am. Chem. Soc. *139(38)*, 13466–13475.

18. Packer, M.R., Parker, J.A., Chung, J.K., Li, Z., Lee, Y.K., Guterres, H., Cookis, T., Hossain, A., Donnelly, D.P., Agar, J.N., Makowski, L., Buck, M., Groves, J.T. and Mattos, C. (2020). Raf promotes dimerization of the Ras G-domain. FASEB J. *34*, 1.

19. Shalom-Feuerstein, R., Plowman, S.J., Rotblat, B., Ariotti, N., Tian, T., Hancock, J.F., and Kloog, Y. (2008). K-Ras nanoclustering is subverted by overexpression of the scaffold protein Galectin-3. Cancer Res. *68(16)*, 6608–6616.

20. Šolman, M., Ligabue, A., Blaževitš, O., Jaiswal, A., Zhou, Y., Liang, H., Lectez, B., Kopra, K., Guzmán, C., Härmä, H., Hancock, J.F., Aittokallio, T., and Abankwa, D. (2015). Specific cancer-associated mutations in the switch III region of Ras increase tumorigenicity by nanocluster augmentation. eLife *4*, e08905.

21. Mazhab-Jafari, M.T., Marshall, C.B., Smith, M.J., Gasmi-Seabrook, G.M., Stathopulos, P.B., Inagaki, F., Kay, L.E., Neel, B.G., and Ikura, M. (2015). Oncogenic and RASopathy-associated K-RAS mutations relieve membrane-dependent occlusion of the effector-binding site. Proc. Natl. Acad. Sci. U.S.A. *112(21)*, 6625–6630.

22. Blaževitš, O., Mideksa, Y.G., Šolman, M., Ligabue, A., Ariotti, N., Nakhaeizadeh, H., Fansa, E.K., Papageorgiou, A.C., Wittinghofer, A., Ahmadian, M.R., and Abankwa, D. (2016). Galectin-1 dimers can scaffold Raf-effectors to increase H-ras nanoclustering. Sci. Rep. *6*, 24165.

23. Lin, Y.-H., Qiu, D.-C., Chang, W.-H., Yeh, Y.-Q., Jeng, U.-S., Liu, F.-T., and Huang, J.-R. (2017). The intrinsically disordered N-terminal domain of galectin-3 dynamically mediates multisite self-association of the protein through fuzzy interactions. J. Biol. Chem. *292(43)*, 17845–17856.

24. Yang, R.-Y., Hill, P.N., Hsu, D.K., and Liu, F.-T. (1998). Role of the carboxyl-terminal lectin domain in self-association of galectin-3. Biochemistry *37(12)*, 4086–4092.

25. Daub, M., Jöckel, J., Quack, T., Weber, C.K., Schmitz, F., Rapp, U.R., Wittinghofer, A., and Block, C. (1998). The RafC1 cysteine-rich domain contains multiple distinct regulatory epitopes which control Ras-dependent Raf activation. Mol. Cell Biol. *18(11)*, 6698–6710.

26. Williams, J.G., Drugan, J.K., Yi, G.-S., Clark, G.J., Der, C.J., and Campbell, S.L. (2000). Elucidation of binding determinants and functional consequences of Ras/Raf-cysteine-rich domain interactions. J. Biol. Chem. *275(29)*, 22172–22179.

27. Hu, C.-D., Kariya, K.-I., Tamada, M., Akasaka, K., Shirouzu, M., Yokoyama, S., and Kataoka, T. (1995). Cysteine-rich region of Raf-1 interacts with activator domain of post-translationally modified Ha-Ras. J. Biol. Chem. *270(51)*, 30274–30277.

28. Drugan, J.K., Khosravi-Far, R., White, M.A., Der, C.J., Sung, Y.-J., Hwang, Y.-W., and Campbell, S.L. (1996). Ras interaction with two distinct binding domains in Raf-1 may be required for Ras transformation. J. Biol. Chem. *271(1)*, 233–237.

29. Cookis, T. and Mattos, C. (2020). Crystal structure reveals the full Ras:Raf interface and advances mechanistic understanding of Raf activation. *bioRxiv*, doi:10.1101/2020.07.28.225938.

30. Tran, T.H., Chan, A.H., Young, L. C., Bindu, L., Neale, C., Messing, S., Dharmaiah, S., Taylor, T., Denson, J.-P., Esposito, D., Nissley, D.V., Stephen, A.G., McCormick, F., and Simanshu, D.K. (2020). KRAS interaction with RAF1 RAS-binding domain and cysteine-rich domain provides insights into RAS-mediated RAF activation. *bioRxiv*, doi:10.1101/2020.07.31.231134.

31. Fang, Z., Lee, K.Y., Huo, K.G., Gasmi-Seabrook, G., Zheng, L., Moghal, N., Tsao, M.S., Ikura, M., and Marshall, C.B. (2020). Multivalent assembly of KRAS with the RAS-binding and cysteine-rich domains of CRAF on the membrane. Proc. Natl. Acad. Sci. U.S.A. *117(22)*, 12101–12108.

32. Dharmaiah, S., Bindu, L., Tran, T.H., Gillette, W.K., Frank, P.H., Ghirlando, R., Nissley, D.V., Esposito, D., McCormick, F., Stephen, A.G., and Simanshu, D.K. (2016). Structural basis of recognition of farnesylated and methylated KRAS4b by PDEδ. Proc. Natl. Acad. Sci. U.S.A. *113(44)*, E6766–E6775.

33. Hsu, D.K., Chernyavsky, A.I., Chen, H.Y., Yu, L., Grando, S.A., and Liu, F.T. (2008). Endogenous galectin-3 is localized in membrane lipid rafts and regulates migration of dendritic cells. J. Invest. Dermatol. *129(3)*, 573–583.

34. Levy, R., Biran, A., Poirier, F., Raz, A., and Kloog, Y. (2011). Galectin-3 mediates cross-talk between K-Ras and Let-7c tumor suppressor microRNA. PLoS One. *6(11)*, e27490.

35. Schmick, M., Vartak, N., Papke, B., Kovacevic, M., Truxius, D.C., Rossmannek, L., and Bastiaens, PIH. (2014). KRas localizes to the plasma membrane by spatial cycles of solubilization, trapping and vesicular transport. Cell. *157(2)*, 459–471.

36. Weise, K., Kapoor, S., Werkmüller, A., Möbitz, S., Zimmermann, G., Triola, G., Waldmann, H., and Winter, R. (2012). Dissociation of the K-Ras4B/PDEδ complex upon contact with lipid membranes: membrane delivery instead of extraction. J. Am. Chem. Soc. *134(28)*, 11503–11510.

37. Molzan, M., Kasper, S., Röglin, L., Skwarczynska, M., Sassa, T., Inoue, T., Breitenbuecher, F., Ohkanda, J., Kato, N., Schuler, M., and Ottmann, C. (2013). Stabilization of physical RAF/14-3-3 interaction by cotylenin A as treatment strategy for RAS mutant cancers. ACS Chem. Biol. *8(9)*, 1869–1875.

38. Hatzivassiliou, G., Song, K., Yen, I., Brandhuber, B.J., Anderson, D.J., Alvarado, R., Ludlam, M.J.C., Stokoe, D., Gloor, S.L., Vigers, G., Morales, T., Aliagas, I., Liu, B., Sideris, S., Hoeflich, K.P., Jaiswal, B.S., Seshagiri, S., Koeppen, H., Belvin, M., Friedman, L.S., and Malek, S. (2010). RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. Nature *464(7287)*, 431–435.

39. Haling, J.R., Sudhamsu, J., Yen, I., Sideris, S., Sandoval, W., Phung, W., Bravo, B.J., Giannetti, A.M., Peck, A., Masselot, A., Morales, T., Smith, D., Brandhuber, B.J., Hymowitz, S.G., and Malek, S. (2014). Structure of the BRAF-MEK complex reveals a kinase activity independent role for BRAF in MAPK signaling. Cancer Cell *26(3)*, 402–413.

40. Park, E., Rawson, S., Li, K., Kim, B.W., Ficarro, S.B., Pino, G.G., Sharif, H., Marto, J.A., Jeon, H., Eck, M.J. (2019) Architecture of autoinhibited and active BRAF-MEK1-14-3-3 complexes. Nature *575(7783)*, 545–550.

41. Cho, K.-J., Kasai, R.S., Park, J.-H., Chigurupati, S., Heidorn, S.J., van der Hoeven, D., Plowman, S.J., Kusumi, A., Marais, R., and Hancock, J.F. (2012). Raf inhibitors target Ras spatiotemporal dynamics. Curr. Biol. *22(11)*, 945–955.

42. Belanis, L., Plowman, S.J., Rotblat, B., Hancock, J.F., and Kloog, Y. (2008). Galectin-1 is a novel structural component and a major regulator of H-Ras nanoclusters. Mol. Biol. Cell *19(4)*, 1404–1414.

43. Elad-Sfadia, G., Haklai, R., Ballan, E., Gabius, H.-J., and Kloog, Y. (2002). Galectin-1 augments Ras activation and diverts Ras signals to Raf-1 at the expense of phosphoinositide 3-kinase. J. Biol. Chem. *277(40)*, 37169–37175.

44. Barceló, C., Paco, N., Morell, M., Alvarez-Moya, B., Bota-Rabassedas, N., Jaumot, M., Vilardell, F., Capella, G., and Agell, N. (2014). Phosphorylation at Ser-181 of oncogenic KRAS is required for tumor growth. Cancer Res. *74(4)*, 1190–1199.

45. Wang, Q., Pechersky, Y., Sagawa, S., Pan, A. C., Shaw, D. E. (2019). Structural mechanism for Bruton's tyrosine kinase activation at the cell membrane. Proceedings of the National Academy of Sciences, *116(19)*, 9390–9399.

46. Zhou, Y. and Hancock, J.F. (2015). Ras nanoclusters: versatile lipid-based signaling platforms. Biochim. Biophys. Acta 1853(4), 841–849.

Supplementary Table

Table S1. List of simulations.

Description	Number of atoms	Total Simulation Time (μs)
K-Ras monomer		
K-Ras (1–169) – GDP in solvent	32k	75.4 (7 Simulations)
K-Ras (1–185) – GDP on membrane	189k	68.4 (12 Simulations)
K-Ras (1–169) – GTP in solvent	32k	75 (5 Simulations)
K-Ras (1–185) – GTP on membrane	189k	67.2 (13 Simulations)
K-Ras (1–167) – GTP in a crystal lattice with 24 copies	126k	10
K-Ras (1–169) – GTP with C-Raf RBD (54–131)	28k	50
Dimerization of GTP-bound K-Ras		
Two GTP-bound K-Ras (1–169) monomers separated in solvent initially	166k	700 (20 Simulations)
Two GTP-bound K-Ras (1–185) monomers separated on membrane initially	191k	363.3 (40 Simulations)
GTP-bound K-Ras (1–169) dimer in solvent	86k	100
GTP-bound K-Ras (1–169) dimer bound to C-Raf RBD (54–131) in solvent	153k	10
GTP-bound K-Ras (1–185) dimer bound to C-Raf RBD (54–131) on membrane	201k	11.7 (2 Simulations)
Gal-3 and Farnesylated Cysteine		
Gal-3 (113–250) binding with capped farnesylated cysteine in solvent	28k	30
GTP-bound K-Ras with C-Raf RBD and CRD		
GTP-bound K-Ras (1–169), C-Raf RBD and CRD (54–187) in solvent	94k	122.1 (25 simulations)
K-Ras (1–185) – GTP, C-Raf (54–187) on membrane	101k	139.9 (32 Simulations)
C-Raf Linker in Solvent		
One copy of C-Raf linker (188–339)	124k; 217k; 1,017k	53 (8 Simulations)
Two copies of C-Raf (188–339)	426k; 427k; 1,014k	84.1 (7 Simulations)

GTP-bound K-Ras, C-Raf, Gal-3, 14-3-3σ and MEK1 Octamer on the Membrane		
Eight units of K-Ras (1–185) – GTP, C-Raf (54–625) – ATP, Gal3 (113–250), MEK1 (66–382), 14-3-3σ (2–231), with C-Raf *n, n+4* dimerized	1,708k–2,854k	104.1 (13 Simulations)
Eight units of K-Ras (1–185) – GTP, C-Raf (54–625) – ATP, Gal3 (113–250), MEK1 (66–382), 14-3-3σ (2–231), with C-Raf *n, n+1* dimerized	1,741k	114.4 (3 Simulations)

Supplementary Movie Legends

Movie S1, related to Figure 1. The architecture of the Ras-Raf signalosome model. Illustration of the 16-protomer signalosome, in which a 16-member helical assembly of K-Ras on the membrane was assembled, followed by sequential addition of Gal-3, the RBD, CRD, the linker and KD of C-Raf, the 14-3-3σ dimer, and finally MEK1 kinase. Colors are consistent with those in Figure 1D.

Movie S2, related to Extended Data Figure 2A (left panel). Simulation of K-Ras dimer formation in solvent. An unbiased simulation starting with two spatially separated GTP-bound K-Ras proteins in solvent, in which the GTP-mediated asymmetric dimer formed spontaneously at approximately 16 μs. The simulation time is marked. The GTP donor is colored in cyan and the GTP acceptor in pink. At the end of the movie, this dimer is superimposed with the GTP-mediated asymmetric dimer that was generated by simulating K-Ras dimerization on a membrane.

Movie S3, related to Figure 2A. Simulation of K-Ras dimer formation on a membrane. An unbiased simulation starting with two spatially separated GTP-bound K-Ras proteins on a membrane, in which the GTP-mediated asymmetric dimer formed spontaneously at approximately 6.5 μs. The simulation time is marked. The GTP donor is colored in cyan and the GTP acceptor in pink. The membrane is shown in gray in the background; GTP molecules are also shown.

Supplementary Dataset Legends

Dataset S1, related to Figure 1D. Atomic coordinates of the structural model of the eight-protomer Ras-Raf signalosome with K-Ras (chains A, B, P, Q, W, Y, E, G), C-Raf (chains C, D, R, S, X, Z, F, H), Gal-3 (chains N, O, V, U, I, J, K, T), 14-3-3σ (chains n, o, v, u, i, j, k, t), and MEK1 (chains c, d, r, s, x, z, f, h).

Dataset S2, related to Figure 1A. Atomic coordinates of the GMA K-Ras dimer on a membrane.