Supporting Information:
A “Tug of War” maintains a dynamic protein-membrane complex: MD simulations of C-Raf RBD-CRD bound to K-Ras4B at an anionic membrane

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Supplemental Method
Starting model: C-RafRBD-CRD in solution
The structure of the C-RafRBD-CRD complex was modeled by assembling the separately available crystal structures of the C-Raf RBD (PDB, 4G0N) and C-Raf CRD (PDB, 1FAQ) using a flexible linker with the protein sequence of residues 133-136 that links these two domains. The C-Raf RBD and C-Raf CRD are connected using the webserver AIDA, which links the protein subdomains together by addressing the relative orientation between RBD and CRD.¹ The predicted structure was then used as starting configuration of C-Raf (denoted, SC0). There are two zinc fingers in C-Raf CRD. Each zinc finger has one zinc ion surrounded by three Cysteine residues and one Histidine residue. Histidine is in the uncharged HSE form and Cysteine is patched with CYN.² The ion coordination within the Zinc finger was stable in all simulations. In total, three independent simulations (U1, U2 and U3; Table S1) were performed for 1.0, 0.5 and 0.5 µs respectively.

C-RafRBD-CRD: K-Ras4B complex at the membrane
The modeled C-RafRBD-CRD structure (above) was further combined with K-Ras4B (PDB, 4DSO) to construct the initial C-RafRBD-CRD: K-Ras4B complex, guided by a crystal structure of H-Ras bound with RBD (PDB, 4G0N) (Ref. 18 in main text). We carried out 6 independent simulations for the complex, named as Simulation #1 to #6 (see Table S1). In Simulation #1 to #4, the center of mass of the catalytic domain of K-Ras4B was placed ~6 nm away from the center of x-y membrane bilayer plane in the Z direction. In Simulation #5, an effector-interacting orientation of K-Ras (where helix 3 and helix 4 are in direct contact with the membrane) was used as the starting structure (Refs. 30,31 in main text). In Simulation #1, #2
and #5, the starting configurations of C-Raf^{RBD-CRD} was that of SC0 (see section above). In Simulation #3 and #4, two other different configurations extracted from the 1 µs simulation of C-Raf in solution were adopted. In this way, the initial displacement of CRD relative to the membrane could be larger (~8.5 nm, Simulation #3) or smaller (~5 nm, Simulation #4) than in Simulation #1 and Simulation #2 (~6 nm). In addition, one simulation, #6, was carried out by placing the CRD near switch II of K-Ras to test whether this proximity leads to the formation of stable contacts. This simulation was performed for ~240 ns. In all simulations the K-Ras4B catalytic-domain was linked to the HVR and anchored to the membrane with a C-terminal farnesyl group pre-inserted into the membrane following previous studies (Refs. 30, 31 in main text). The parameters for the farnesyl group were generated by the CHARMM generalized force field (CGenFF). The model membrane was composed of 360 POPC (Palmitoyloleoyl-phosphatidyl-choline) and 90 POPS (1-Palmitoyl-2-oleoylphosphatidylserine) lipid molecules (20 % POPS). The POPS are equally distributed in each leaflet of the membrane. All model membranes were created by the CHARMM-GUI and equilibrated for 100 ns.

**Simulation Conditions**

The modeled structure of the C-Raf^{RBD-CRD}: K-Ras4B complex was solvated in a box of TIP3P water. A number of water molecules were replaced randomly by Sodium and Chloride ions to obtain a neutral charge system with a near-physiological ion concentration of 150 mM. The simulation systems were energy minimized for 2000 steps. Sequentially, a short simulation of 0.5 ns was performed with a harmonic restraint on protein heavy atoms and lipid phosphate groups, followed by another 0.5 ns restraint simulation on only the Ca atoms of the proteins. The initial 50 ns of all simulations were performed using the NAMD/2.10 package. The NAMD simulations were performed with a time step of 2 fs, and were coupled with a thermostat at 310 K, and a barostat at 1 bar with a semi-isotropic Langevin scheme. A 1.2 nm cut-off (with force-switching between 1.0 and 1.2 nm) was used for van der Waals and for local electrostatic interactions. The long distance electrostatic interaction was treated by the Particle-Mesh Ewald (PME) method. The equilibrated NAMD simulations were transferred to Anton2; a specialized supercomputer for molecular dynamics simulation, and run on this computer for the remaining simulation time. Production simulations were carried out for 1 µs for simulations #1 to #5 of the modeled C-Raf^{RBD-CRD}: K-Ras4B complex.

**Free Energy Calculations**

In order to calculate the potential of mean force (PMF) of K-Ras4B or C-Raf CRD binding to the membrane, umbrella sampling simulations were performed. The reaction coordinate is set as the distance in the Z direction between the center of mass of the model membrane and the center of the mass of K-Ras4B helix 4 (residues 127 to 137), or the center of mass of a previously identified lipid binding loop (residues 143 to 151) of the CRD. The membrane is composed of 176 POPC and 44 POPS lipid molecules. The relative orientation of K-Ras4B is
set with the helix 4 axis facing the membrane, and CRD is placed with the 143-RKTFLKLAF-151 segment also in parallel facing the membrane. Umbrella windows with a 0.1 nm (Δr) interval were extracted from the simulation trajectories of two steered molecular dynamics (SMD) simulations using established protocols. K-Ras4B was pulled from a position 5.6 nm distant from the membrane center to the membrane surface (Z = 2.2 nm), and then was pulled back in the opposite direction. CRD was pulled from a pre-inserted state (located at Z = 2.0 nm, as the final structure of membrane inserted CRD in simulation #1) to a non-membrane associated position (Z = 5.0 nm), and was pulled back in the opposite direction. A constant pulling velocity of 10⁻⁷ nm per time step (1fs) was used (total 30-40 ns of each SMD simulation). Biased simulations were performed with a harmonic potential applied between the Cα atoms of selected protein groups (K-Ras4B helix 4 or CRD lipid binding loop) relative to the sliding z-coordinate with a force constant K of 10 kcal/(Mol.Å²), typical for such simulations. For K-Ras4B, the sampling simulations were performed for each window for 20 ns at least. For CRD, sampling simulations were applied longer for each window for 60 ns at least, as the CRD is partially inserted into the membrane at the lowest energy state. The last 10 ns (K-Ras4B) and 20 ns (CRD) of the umbrella sampling trajectories were used in order to calculate the PMF, with the application of the weighted histogram analysis method (WHAM).8 Uncertainty estimates are determined using the equation 1 of Zhu and Hummer.9 The variance in free energy estimators is given by var[G(xₙ)] = (KΔr)^2Σᵢ₌₁ⁿ var[xᵢ], n is nᵗʰ simulation window. var[xᵢ] is squared error in the estimate of the mean position of x in window i. Variance is estimated using the block averaging method of Flyvbjerg and Petersen.10 The standard deviation σ(var[G(xₙ)]) is the cumulative statistical error as shown in Fig. 6b. In total, these simulations amounted to 3.6 µs of trajectories. Although the sampling within each window is longer than used in other recent publications of proteins in and at membranes,11 very substantial configurational reorganizations may not be sampled, a standard caveat using a 1D reaction coordinate.

**Analysis**

Unless stated otherwise, we regarded the initial 50 ns from each trajectory as equilibration, did not include it in our analyses. The trajectories of C-Raf<sup>BD-CRD</sup> were clustered using Wordom (Fig. 1).12 The clustering were based on the RMSD using a quality threshold-like algorithm and a cut-off distance of 5 Å. The Fusion Protein Modeller program 13 was used to explore the configurational space of C-Raf<sup>BD-CRD</sup> by rotating residues in the shorter linker (132 to 137) (Fig. S1b). The contact maps between one protein domain with another (RBD: CRD, RBD: RAS, CRD: RAS) were made by counting the contact events, i.e. when the distance between residues of one domain and residues of another domain is less than 5.0 Å. Occupancy is the % number of simulation frames where the interaction is present (Fig. S1c and Fig. S3). The criteria for a cation-π interaction is that the distance between all the aromatic ring atoms and the choline nitrogen are below 7 Å and that there is no more than a 1.5 Å difference between these distances
For presence of hydrogen bonds a distance cut off of 3.5 Å (Donor atom-acceptor atom) and angular cut-off of 30 degree (Donor atom-hydrogen atom-acceptor atom) was used.

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Table S1: List of simulations.

| Simulation   | Length (ns) | Number of Atoms | Orientation of K-Ras4B Catalytic Domain | Distance of CRD to membrane center (nm) | Remarks*          |
|--------------|-------------|-----------------|-----------------------------------------|-----------------------------------------|-------------------|
| Simulation U1| 1000        | 128,331         |                                         |                                         | Raf in solution   |
| Simulation U2| 500         | 128,331         |                                         |                                         | Raf in solution   |
| Simulation U3| 500         | 79590           |                                         |                                         | Raf in solution   |
| Simulation #1| 1000        | 233,181         | Away from membrane                      | 5.7                                     |                   |
| Simulation #2| 1000        | 233,121         | Away from membrane                      | 6                                       |                   |
| Simulation #3| 1000        | 237,509         | Away from membrane                      | 8.5                                     |                   |
| Simulation #4| 1000        | 229,875         | Away from membrane                      | 4.8                                     |                   |
| Simulation #5| 1000        | 229,953         | Binding to membrane                     | 6.8                                     |                   |
| Simulation #6| 240         | 196,893         | Binding to membrane                     | 5.6                                     | CRD close to switch II region of K-Ras4B |
Figure S1: (a) 10 major clusters of the simulation configurations of an isolated C-Raf$^{\text{RBD-CRD}}$ in solution, superimposed on RBD. Cluster #1 to #10 are denoted as red, pink, mauve, magenta, green, yellow, tan, silver, purple and cyan in order. (b) 10 configurations of C-Raf$^{\text{RBD-CRD}}$ predicted by FPModeller. Regions of the RBD that are unreachable for the CRD are evident by absence of clusters on the lower side of the RBD (near α1-β1) in the left diagram in row a). Left panel: top view, with RBD behind (as in Fig. 2c); Right panel: side view. (c) Contact maps between residues of the RBD and residues of the CRD, averaged over the three independent simulations. The heat map denotes the % number of simulation frames where residue (RBD)-residue (CRD) interactions are seen. Consistent with the clustering and FPModeller, a number of regions do not have contacts.
Figure S2: Configurations of C-Raf^{RBD-CRD} when bound to membrane anchored K-Ras4B. (a) Dihedral angle $\phi$ characterizes the orientation of the CRD relative to the RBD. This angle is measured as the angle between a surface made up of the C$\alpha$ atom of residues M83, E125 and F130 and a surface made of residues E125, F130 and a dummy atom representing the center of mass of the CRD. (b) The time evolution of the dihedral angle in each simulation. (c) The distribution of the dihedral angle, averaged over all K-Ras4B bound simulations. The configurational properties of the C-Raf^{RBD-CRD} were compared to the simulation of an isolated C-Raf^{RBD-CRD} in solution. The dihedral angle population plot presents four peaks similar to the dihedral angle distribution for the isolated C-Raf^{RBD-CRD}. The differences are seen as the peak shift reflecting a shift in the preferred orientations. (d) Distribution of radius of gyration of C-Raf^{CRD-RBD}. 
Figure S3: Contact maps (frequency in %) (a) between residues of K-Ras4B of switch I and II and the Raf RBD, and (b) between residues of K-Ras4B and the C-Raf CRD, averaged over the 5 simulations. In simulations #1 to #5, contacts between CRD and K-Ras4B are seen occasionally, mainly between residues K42/Q43 of K-Ras4B and residues V185/D186 at the flexible C-terminus of the CRD, with largest occupancy about 28% (averaged over all simulations). (c) Snapshots for simulation #6 (left: initial, right: final structure). Several studies suggested C-Raf CRD interactions with the K-Ras switch region. Therefore, we performed one additional simulation by placing the CRD near the switch-2 (since switch-1 is occupied by RBD) and since GDP and GTP differ the most in switch conformations. The CRD gradually moved away from the membrane over the course of the simulation. Thus, within the current time-frame of our simulation, our studies do not show the C-Raf CRD to be in contact with either switch regions or with the extreme end of the HVR region.
Figure S4: Time evolution of the distance of the center of K-Ras4B CD2 (residues 87 to 166) or of C-Raf CRD to the membrane center. Snapshots are taken at various time points from (a) simulation #2, (b) simulation #4 and (c) simulation #5.

Figure S5: Contour maps of the relative orientation of K-Ras4B with respect to the membrane. $D_{CD1}$ is the distance (in nm) between the vertical center of the membrane and that of the K-Ras4B CD1 subdomain/lobe. $\alpha$ is the cross angle (in degree) between the normal direction of the membrane and a directional vector of the K-Ras4B catalytic domain as explained in Li et al (Ref. 30 in main text). The color scale from gray to red denotes the relative population of K-Ras4B in different orientation states ($D_{CD1}$, $\alpha$). The population is normalized (maximum occupancy point as 1).
Figure S6: Cation-π and hydrogen bonding interactions between CRD and membrane. The analysis is for simulation #1. (a) Residue vs. simulation time plot showing instances of cation-π interactions between aromatic rings of phenylalanine or tryptophan and choline nitrogen. (b) Frequency of hydrogen bonds formed per residue of C-Raf to lipid headgroups.

Figure S7: Distributions of variables D1, distance of K-Ras4B CD2, and D2, distance of CRD to membrane center. There is substantial overlap between the configurational space that is being sampled by simulations #2–#5 (see a similar extent of sampling of K-Ras4A at a POPS containing bilayer in our previous study (Ref. 30 in main text)); however, as noted, simulation #1 samples a more restricted space, mostly with D2 near 2.8 nm, i.e. a partial insertion of the CRD into the membrane bilayer, while the Ras catalytic domain is only loosely interacting with the bilayer. Even in the instances where the Ras attaches to the membrane, the interaction surfaces of neither K-Ras nor of the CRD are optimal with the membrane, as noted in the main text.
Figure S8: CRD binding to the membrane. When the CRD is bound to the membrane, K-Ras4B can be away from or close to the membrane. When it is close to the membrane, typically it only uses few residues (mainly containing the loop 3 of K-Ras4B and less Helix 5, H5) to contact the membrane. However, H3 and H4 are away from the membrane.
Figure S9: (a) Sequence alignments of C-RAF (RAF1) RBD-linker-CRD region (5 mammalian and 1 bird species). (b) Sequence alignment of C-RAF RBD-linker-CRD comparing human C-, B- and A-RAF. The CRD-RBD linker is indicated by line in both. B-Raf is mutated in ~8% of all cancers. A-RAF and C-RAF mutations are very rare in cancer. The majority of mutations are observed in the activation loop (A-loop) near V600, or in the GSGSFG phosphate binding loop (P-loop) at residues 464–469. The mutation frequency in the linker and RBD-CRD regions is moderate in C- as well as B-Raf.; there are several mutations in the Ras binding interface of the C-Raf RBD and in the regions we find that are interacting with the membrane. However, with exception of L86P and Q156 stop, these are all single count according to the COSMIC database. In the linker region, there are two mutations, L136P and R143L with a count of two.