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Peer Review File

Positive cooperativity between RAS-binding and cysteine-rich domains regulates RAF membrane binding kinetics via lateral rebinding

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, Jimenez Salinas et al. investigate how the tandem RAS-binding domain (RBD) and cysteine-rich domain (CRD) of RAF cooperatively regulate membrane binding kinetics using a supported lipid bilayer-based reconstitution system. They show that RBD binding to RAS facilitates CRD engagement with phosphatidylserine (PS) lipids, thereby extending RAF membrane dwell time via a proposed lateral rebinding mechanism. The authors further suggest that this prolonged residence could enable kinetic proofreading to enhance signaling specificity in RAS nanoclusters.

While the study utilizes multiple biophysical approaches and presents kinetic measurements, it lacks critical experimental information that compromises both reproducibility and interpretation. Key parameters—such as buffer composition, salt concentration, protein concentration, temperature, and protein solubility/stability—are either absent or inadequately described. In addition, no methodological detail is provided for the generation, expression, or biochemical validation of mutant and linker-variant constructs, making it impossible to assess whether these proteins are comparably expressed, folded, or stable relative to wild-type controls.

Beyond these technical limitations, the conceptual contribution of the study is modest. The major conclusions—positive cooperativity between RBD and CRD, and the role of CRD–lipid interactions have been established previously through structural, computational, and biochemical studies. This work largely validates existing models in a minimal in vitro system and provides limited advancement in our mechanistic understanding of RAF activation.

Specific concerns:

The study does not use full-length BRAF constructs, including autoinhibited and active forms resolved by cryo-EM, which are necessary to explore physiologically relevant conformational transitions and regulation. The study uses BRAF cryoEM structures and utilizes isolated CRAF RBD, CRD, and RBD-CRD constructs.

The intrinsic instability of the isolated CRD domain relative to RBD or RBD-CRD is not acknowledged or controlled for, despite likely influencing the observed behaviors. Previous studies have suggested utilizing high salt concentration to keep it soluble/stable.

The authors use an HRAS(1–184) construct, even though mature HRAS is 185 residues long. They do not specify which cysteine is modified by maleimide or address the implications of this for membrane anchoring and orientation.

Maleimide conjugation is a non-physiological means of membrane tethering that does not replicate the native prenylation of KRAS, which has been extensively utilized in the recent past. This limitation is not discussed, despite its potential impact on membrane interaction kinetics.

The manuscript omits critical methodological details: the final composition of protein storage buffers, assay buffers, salt concentrations, assay temperatures, and precise protein concentrations used in kinetic and diffusion experiments are not reported.

There is no description or validation of the linker mutant or extended linker constructs. The authors provide no data on their expression levels, solubility, or folding.

The rationale for the L136A/T178A double mutant is unclear. T178 is not part of the linker, and its selection is not justified. Similarly, the purpose/rationale of introducing a 24-residue flexible linker is not explained.

The claim that the T178A mutation stabilizes the extended linker construct is not supported by any biochemical data.

The known ~2-fold higher affinity of RBD-CRD for RAS compared to RBD alone is not factored into the interpretation of results. Many observations attributed to RBD-CRD vs RBD or CRD could reflect this baseline affinity difference.

The proposed enhancement of RBD–RAS association by CRD–lipid interaction is not clearly demonstrated or mechanistically explained.

The claim that RAS·GTP mediates membrane recruitment of RBD-CRD species not bound to RAS is conceptually inconsistent, especially under conditions where all RAS binding sites are presumably occupied.

The term “RAS unbinding” is used ambiguously throughout and should be clarified to distinguish between RAS–RAF dissociation and membrane detachment.

The central conclusion regarding nanocluster-specific signaling is not experimentally supported. The lipid compositions used do not promote RAS nanocluster formation, and the system lacks the complexity to model this behavior. Therefore, extrapolating these findings to nanocluster biology is not justified.

Reviewer #2

(Remarks to the Author)
The manuscript by Salinas et al. presents a well-thought-out and comprehensive kinetic analysis of the RAF1 RBD-CRD domain interactions with HRAS and a simple supported lipid bilayer (SLB). The SLB is composed of DOPS, DOPC, and a functionalized DOPE that links HRAS to the bilayer via maleimide chemistry through an available cysteine in HRAS’s hypervariable region. I believe this study proposes a model that is both intriguing and valuable to the field, and that the supporting data and analysis are sound.

In brief, the authors propose that, following initial binding of RAF to RAS on the membrane, subsequent lipid interactions involving the RAF CRD domain are sufficient to extend RAF’s residence time. Nanoclustering of RAS and a CRD–lipid intermediate increases the likelihood of subsequent rebinding to other RAS monomers, enabling the full RAF activation sequence—including RAF dimerization—to proceed. This model has important implications for understanding the RAF activation cycle, particularly the possibility of a kinetic proofreading function.

Therefore, I recommend the manuscript for publication, provided the following points are addressed:

• The binding and dissociation rates of RAS/RAF complexes were calculated in a previous single-molecule pull-down study: Lee, H.W., Kyung, T., Yoo, J., et al. Nat Commun 4, 1505 (2013). In this study, RAS was immobilized, and RAF molecules were pulled down from a lysate onto coverslips and imaged using TIRF microscopy. Residence times and kinetic parameters were measured. The authors should explicitly compare their data to this study. Moreover, as in Lee et al., they should account for potential photobleaching effects to ensure that their measured rates are not significantly influenced by the photophysics of the fluorophores.

• Lee et al. describe the formation of “pre-equilibrating encounter complexes.” The authors should clarify whether these results are consistent with or differ from those in the current manuscript, especially with respect to the proposed lateral rebinding model. Although Lee et al. used immobilized RAS (i.e., non-diffusing and in a lipid-free system), it is possible that their findings contradict the current interpretation. Are there alternative explanations that support the proposed lateral rebinding and kinetic proofreading model?

• The authors cite a recent study using KRAS4b on SLBs, which analyzes KRAS4b/RBD-CRD complexes on simple and complex lipid bilayers: Shrestha R., Carpenter T.S., Van Q.N., et al. Commun Biol. 2024; 7(1):242. The authors should comment on the specific CRD-lipid contact points identified by the simulations and NMR in Shrestha et al. and evaluate experimentally whether mutations of these lipid-penetrating CRD residues affect lateral rebinding interactions in the RBD-CRD complex or the proposed RBD-CRD–PS intermediate.

• In the final sentence of paragraph 1 in the section titled “RAS density modulates RAF membrane residence through lateral rebinding,” the authors state: “we exclude the scenario where RBD-CRD first loses lipid contact yet remains bound to RAS, as a lipid-free RAS:RBD-CRD complex was not observed in single-particle tracking experiments (Fig. 2e).” To more robustly support this claim, the authors should measure the diffusion of fluorescently labeled RAS in the presence of RBD-CRD on the same SLB and compare those diffusion rates to the measurements where the fluorescent tag is on RBD-CRD. Comparing the distributions and fractional occupancies of the different diffusion states in both labeling conditions would

provide a stronger test of the assertion that lipid-free RAS:RBD-CRD complexes do not form or persist. I would also encourage a different term than “lipid-free.” We know that there are extensive and dynamic contacts between RAS and lipids. There is probably more precise language to talk about the conformational state of the complex and contact with the lipids in the bilayer.

Reviewer #3

(Remarks to the Author)
This is an interesting and potentially impactful article that would be suitable for publication in a major journal such as Nature Communications, following adequate attention to significant shortcomings. The article summarizes well-reasoned biophysical studies of the multi-pronged interactions between the important signaling proteins CRaf, HRas and anionic lipids (phosphatidylserine or PS) during CRaf membrane binding, 2D-diffusion and dissociation on a lipid bilayer possessing anchored HRas molecules. The quite promising results suggest that, as often observed for signaling proteins that target the plasma membrane, the CRaf molecule possesses cooperative, multi-component binding contacts involving both protein-lipid and protein protein interactions that together provide adequate specificity and affinity for the correct membrane surface. In CRaf this multi-component interaction is provided by its distinct cysteine-rich domain (CRD) and Ras binding domain (RBD) that provide protein-lipid (CRD-PS) and protein-protein (RBD-HRas), respectively. While these results are not surprising, this manuscript is the first to address the cooperative nature of these essential Ras:CRaf:PS interactions in a quantitative, biophysical manner. The following major and minor points should be addressed prior to publication.

1) The article places a major emphasis on Raf targeting, retention, and activation in Ras nanoclusters with language about relevance to nanoclusters on 10 pages of the text including the Abstract, Introduction, Results, Discussion, and Figure Legends. However, as the article itself notes, the system employed observes individual Ras:RBD-CRD complexes interacting with PS lipids on the target bilayer with no detectable clusters (pg 8, bottom half of paragraph). Moreover, it appears that Ras densities and clustering are not measured in most or all experiments. Thus the article should be more careful about its claims of relevance to clustered systems, and should limit those claims to the description of the plausible, but as yet unproven, model in the Discussion.

2) The experiments are well chosen and the data is promising, but there is inadequate attention to reproducibility which could lead to questions about the rigor of the work. In general, statistical tests of significance are absent and there is information on replicates for only two of the many experiments (in both cases N=3, presumably representing technical rather than biological replicates). Perhaps I missed it, but I could find no indication that experiments were carried out more than once, and were repeated on multiple days. Such lack of information about replicates and statistical tests makes it impossible to judge the reproducibility and statistical significance of the findings.

3) The slow diffusion observed for Ras:RBD-CRD:PS complexes on the DO-lipid bilayer raises the possibility that, in addition to the frictional drag of the Ras anchor lipid and the bound PS molecules against the bilayer, there may well be additional frictional drag provided by protein insertion into the bilayer. See PMID 23701821.

4) The article is missing key references to directly relevant single molecule studies of Ras-effector interactions, and of the effects of membrane environment on the affinities and kinetics of protein-protein and protein-membrane interactions. See, for example, PMID 29211993, 39217418, 22263647.

Reviewer #4

(Remarks to the Author)
This manuscript provides interesting results and confirms several observations from previous experimental and computational studies. I recommend that authors stress the novelty of their findings in the context of broader Ras biology during the revision of this manuscript.

The manuscript reports that RBD binds to RAS, allowing CRD to bind to negatively charged PS lipids (as determined by TIRF microscopy). They propose two kinetically distinct binding steps. Also, CRD binds to PS in a RAS-GTP-dependent manner. It ensures RAF membrane binding (by CRD) is strictly driven by RAS activation (by RBD). Using FRET, they demonstrate that CRD-lipid binding enhances the association between RBD and RAS (as measured by FRET). RBD and CRD binding to RAS and PS lipids exhibit cooperativity. RAF undergoes lateral rebinding with RAS (using kinetic simulations in SimBiology and TIRF microscopy). Weak CRD-lipid interactions play a role in lateral rebinding. This extends the RAF's membrane dwell time under high RAS density (e.g., nanoclusters). This prolonged membrane residence increases the likelihood of completing RAF's multistep activation sequence (i.e., facilitates kinetic proofreading of RAF activation).

Comments:

This work would have had a significant impact if the authors had investigated the kinetics in the presence of PIP2 lipid and distinguished the binding mechanisms.

Additionally, DOPC is not a particularly abundant component of the plasma membrane. Any reason for its selection? (CRD is more likely to bind to POPC:POPS membranes than DOPC:DOPS)

Also, is a 20% PS concentration sufficient to mimic biomembranes?

What would be the effect of having the kinase domain? Past experiments were based on the same RBD-CRD construct. Engaging the kinase domain and seeing the effect of homodimerization can be quite novel.

I find it challenging to convince myself that RAF unbinding occurs primarily by detaching from RAS first, then from the membrane, rather than the other way around (end of first paragraph on p. 12). Strong arguments to support their claim can be useful.

Several xray structures clearly show that CRD creates contacts with RAS. I agree that these contacts may not be formed in the absence of RBD. Therefore, lipids are not the only driving force for membrane interaction. Claiming that CRD is driven only by lipids is not completely correct.

It is interesting that RBD alone has a higher k_on to RAS-associated membranes than RBD-CRD; however, the presence of CRD lowers the off rate, resulting in RBD-CRD having a stronger binding affinity than RBD alone. Additionally, the rate constants for RBD alone and RBD-CRD are not affected by PS concentration, so only k_off is influenced by PS levels. I don't quite understand that easily. Some clarity would be helpful.

Additionally, I find it puzzling that the RBD-CRD linker does not affect the initial RBD-RAS association but is relevant for the membrane dissociation rate, and thus for the cooperative binding of RBD-CRD to RAS and PS lipids.

Reviewer #5

(Remarks to the Author)
In this study, the authors use quantitative TIRF microscopy to analyze the binding kinetics and equilibrium behavior of two RAF domains (RBD and CRD). Particularly, they looked at RBD/CRD interactions with phosphatidylserine lipids, RAS, and cooperative binding interactions with each other. The purpose of this study was to develop a model for membrane-dependent RAF activation, which is dependent on recruitment to the membrane by GTP-RAS, and retention on the membrane through weak PS interactions which cooperatively strengthen binding to RAS. The carefully designed experiments elucidate KD values, koff rates, and kon rates to elucidate the impact of each component in the system. The RAF-MEK-ERK signaling cascade is a classical biochemistry interaction that is fundamentally important to numerous cell signaling events. Understanding activation of this pathway is critical to being able to regulate cell signaling in cancer cells. This work will be of high interest to a number of fields because of the extreme importance of RAF activation in cancer.

Overall the conclusions of the study are supported by the results. The experiments are well-designed, and the interpretations are logical. There are some minor points where the language and reporting in the manuscript could be clarified, as noted below. The manuscript is well written and the figures are generally of good production quality. The biggest concern to this reviewer is there may be an over-extrapolation of these results to connections concerning RAS nanoclusters, RAF auto-inhibition, and involvement of the SHOC2-MRAS-PP1C complex. I feel that these results provide a convincing argument about RBD/CRD cooperativity, interactions with PS lipids, and importance of RAS concentration. However, the attempt to explain a full mechanism of RAF activation seems to be speculation and extrapolation based on other published works. I recommend the manuscript undergo minor revisions to address the points listed below, where the issue of over-extrapolation is elaborated on in the last point.

Points to address:
• Some additional clarifications could be added to the introduction to aid readers who are not in this field:
o It was not immediately obvious to me that "14-3-3" is the name of a protein. Please add a (very) brief explanation. Even just add the word "protein" after it the first time it appears.
o Please briefly explain what are BRAF, KRAS, and CRAF, compared to just RAF and RAS.
• Evaluating how the RBD-CRD had measured KD values of 14 nM vs 5 nM for binding 2% and 20% PS membranes as a "evident dependence on PS" seemed strange in contrast to the previous comparison of 222 nM vs 163 nM as "depended little on upon PS content". Please adjust the language or clarify.
• Was there a 0% PS control performed for any of these samples? Why were 2% and 20% PS concentrations chosen for the experiments? Please explain the lipid choice briefly.
• The number of reported significant figures should be reduced for reported KD values in caption for Figure 1. Example: 222 +/- 88 should be changed to 220 +/- 90
• Figure 2a, middle panel: I think I'm seeing some low-intensity spots indicating protein binding in the -RAS condition. However, I can't rationalize how you can have less than one molecule showing up, unless this is a product of partial occupancy over the duration of a given exposure time? Please explain.
• It is odd that the experiment wasn't successful for the 4XGGGGGS linker construct, but it was successful for the 4XGGGGGS/T178A construct. Can the authors please provide their thoughts on this? What part of the experiment was unsuccessful? Could the protein not be expressed? Perhaps this doesn't need to be explained in the manuscript, but I think it is appropriate for discussion during the review.
• The statement in the discussion "Consistent with this, we observed stronger binding of RBD-CRD on RAS-free membranes" is unclear. After a few minutes of consideration, I am now thinking that perhaps you mean that RBD-CRD has

stronger binding on RAS-free membranes compared to RBD alone or CRD alone? When I originally read this sentence, I was interpreting it as RBD-CRD has stronger binding on RAS-free membranes, compared to membranes containing RAS. Please revise for clarity.
• To improve clarity, it would be helpful to reference figure numbers throughout the discussion.
• This point is in regards to the statement in the discussion: "… whereby molecules that remain on the membrane for extended periods – such as those within RAS nanoclusters – are disproportionately more likely to become activated." Based on the evidence presented here, I don't see how a RAS nanocluster would retain RAF any more than a high concentration of monomeric RAS. I'm seeing how this is related to concentration, but not necessarily how it's related to nanoclusters. This needs to be made clearer. Perhaps making it explicit that clustering is creating a high local concentration, and RAF will be retained due to this high local concentration. This is distinct, for example, from requiring a patch of RAS that is a certain diameter or particular shape.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I acknowledge that the authors have resolved the earlier technical issues and that the strengthened controls and lipid-free assays make the experimental framework sound. However, as I noted in my first-round review, the conceptual advance remains limited: the reported RBD–CRD cooperativity aligns with established structural expectations, and the proposed lateral rebinding mechanism appears to be an expected consequence of multivalent interactions on crowded membrane surfaces rather than a genuinely new insight. Although the manuscript offers careful quantitative confirmation of established ideas, it still falls short of delivering a substantive mechanistic advance.

Reviewer #2

(Remarks to the Author)
Thank you again for addressing my comments. I see no need for further revisions, and this manuscript should be accepted for publication.

Reviewer #3

(Remarks to the Author)
My previous concerns have all been adequately addressed, and I recommend publication.

Reviewer #4

(Remarks to the Author)
I have no further comments. Most of previous comments have been addressed in the revision

Reviewer #5

(Remarks to the Author)
The authors have satisfactorily addressed all of my comments.

Response to the reviewers' comments

We are grateful to reviewers for their constructive comments, all of which have been addressed in the revised manuscript. Below, we respond to each comment point-by point. The inclusion of additional replicates and standardizing significant figures led to small changes in some reported values; these revisions do not alter any interpretation and conclusions. We also made minor edits to maintain the logical flow and the manuscript length after incorporating the reviewers' suggestions and the new data.

The original comments from the reviewers are in ***bold italic***; our responses are in regular font. Changes in the manuscript are colored in blue.

Table of Contents

Reviewer #1 (Remarks to the Author):

In this manuscript, Jimenez Salinas et al. investigate how the tandem RAS-binding domain (RBD) and cysteine-rich domain (CRD) of RAF cooperatively regulate membrane binding kinetics using a supported lipid bilayer-based reconstitution system. They show that RBD binding to RAS facilitates CRD engagement with phosphatidylserine (PS) lipids, thereby extending RAF membrane dwell time via a proposed lateral rebinding mechanism. The authors further suggest that this prolonged residence could enable kinetic proofreading to enhance signaling specificity in RAS nanoclusters.

While the study utilizes multiple biophysical approaches and presents kinetic measurements, it lacks critical experimental information that compromises both reproducibility and interpretation. Key parameters—such as buffer composition, salt concentration, protein concentration, temperature, and protein solubility/stability—are either absent or inadequately described. In addition, no methodological detail is provided for the generation, expression, or biochemical validation of mutant and linker-variant constructs, making it impossible to assess whether these proteins are comparably expressed, folded, or stable relative to wild-type controls.

We have addressed all issues in our point-by-point responses below.

Beyond these technical limitations, the conceptual contribution of the study is modest. The major conclusions—positive cooperativity between RBD and CRD, and the role of CRD–lipid interactions have been established previously through structural, computational, and biochemical studies. This work largely validates existing models in a minimal in vitro system and provides limited advancement in our mechanistic understanding of RAF activation.

We respectfully propose that our study is the first quantitative biophysical analysis providing a kinetic model for RAF–membrane interaction, with novel mechanistic insights at the molecular level. Reviewer #3 also acknowledged that our manuscript is "*the first to address the cooperative nature of these essential Ras:CRaf:PS interactions in a quantitative, biophysical manner.*"

We agree that positive cooperativity between RBD and CRD has been extensively studied; however, many questions remain regarding their functional and structural communication, particularly within the membrane context. This was one of the central questions in this work: how do RBD and CRD cooperate to integrate protein and lipid inputs in membrane environments? We sought to address these questions through quantitative measurements in simplified model membrane systems, moving beyond qualitative description.

Specifically, previous structural and NMR studies have reported positive cooperativity between the RBD and CRD, in which nucleotide-dependent KRAS–RBD binding promotes transient interactions between KRAS and the CRD. In contrast, our study provides a comprehensive kinetic analysis of protein–protein and protein–lipid interactions within membrane environments. Our findings reveal membrane-specific positive cooperativity and resulting kinetics in which CRD–lipid engagement enhances the protein–protein interaction within the RAS:RAF complex, and vice versa. We also identified the distinct mechanistic roles of RAS–RBD, RAS–CRD, and

CRD–lipid interactions in modulating RAF membrane binding kinetics. Importantly, we discovered that RAF membrane residence is modulated by RAS density through a lateral rebinding mechanism, in which a weak CRD–lipid interaction plays a key role. This lateral rebinding mechanism in RAF has not been previously observed or proposed. Because RAF activation involves a series of multistep processes on the membrane, these findings provide critical mechanistic insight into how coordinated RAS and lipid interactions collectively drive full RAF activation.

To better reflect this point, we modified the introduction as follows (page 3, line 64):

"Prior studies reported cooperativity between RBD and CRD in which RAS–RBD binding induces a transient interaction between RAS and CRD, and mapped CRD–lipid interfaces[16]. However, how these interactions control membrane kinetics has remained unclear. A quantitative understanding of membrane-binding kinetics and the underlying mechanisms is crucial because RAF is exclusively activated on cellular membranes[14]."

Specific concerns:

1. The study does not use full-length BRAF constructs, including autoinhibited and active forms resolved by cryo-EM, which are necessary to explore physiologically relevant conformational transitions and regulation. The study uses BRAF cryoEM structures and utilizes isolated CRAF RBD, CRD, and RBD-CRD constructs.

The reviewer raises two concerns: (1) our use of truncated CRAF constructs rather than full-length BRAF, and (2) our use of BRAF cryo-EM structure while utilizing CRAF constructs. We will address each point below. :

(1) Why use truncated CRAF constructs rather than full-length BRAF constructs?

RAF activation is a complex process that involves multiple protein–protein and protein–lipid interactions within membrane environments. We strategically planned to dissect this complex process with the conserved regulatory module (RBD-CRD) in a minimal, well-controlled setting. This approach enabled high-resolution kinetic analysis and revealed a previously unrecognized membrane-interaction mechanism—lateral rebinding—by which RAF integrates protein and lipid inputs to achieve prolonged membrane residence. CRAF was chosen because its regulatory domains are extensively characterized biochemically and structurally, enabling structure-driven hypotheses and direct comparison between solution and membrane contexts. Given the modular domain architecture and conservation across RAF, we expect that the positive cooperativity and lateral rebinding observed for the RBD-CRD module will also be present in the full-length proteins. We agree that full-length protein is essential to understand the complete activation process, but full-length RAF study is beyond the scope of this work. Our current work will provide a mechanistic foundation for future work with full-length RAF.

We now make this limitation explicit in our planned extensions to full-length RAF reconstitutions (page 17, line 418):

"To investigate how membrane-binding kinetics influence activation outcomes under more physiologically relevant conditions, it will be necessary to use the autoinhibited full-length RAF:14-3-3:MEK complex."

(2) *Why show BRAF cryo-EM structures while using CRAF constructs experimentally?* The cryo-EM structure of autoinhibited BRAF was used as the current structural archetype for RAF because autoinhibited CRAF structures were not yet officially available at the time of submission; autoinhibitory mechanisms observed in BRAF are widely accepted to be conserved in CRAF (Ref. 27; Part et al., *Nature* 2019, 575, 545). Recently, autoinhibited CRAF structures—including a conserved autoinhibited structure similar to BRAF—have been released (Ref. 30; Jang *et al. Nat Commun* 2025, 16, 8150). We have replaced the BRAF cryo-EM structure in Figure 1b with the fully autoinhibited CRAF structure.

To reflect this change, we have modified the main text as follows (page 2, line 47):

"The inactive monomeric CRAF:MEK1:14-3-3 complex can adopt multiple conformations, including a fully autoinhibited confirmation (Fig. 1b) that is closely resembles that of BRAF, as well as more open conformations in which the CRD is structurally liberated[30]."

Please note that all mutational designs and linker variants in our experiments were guided by the CRAF RBD-CRD structure (Ref. 35; Tran et al., *Nat. Commun*. 2021, 12:1176), not BRAF.

Regarding the BRAF cryo-EM structure, we originally stated that RAS binding to the RBD alone is insufficient to release RAF from autoinhibition, based on the reported structure of the RAS:BRAF recruitment complex (Ref. 29; Park *et al*. *Nat Commun* 2023, 14, 4580). However, more recent structural and biochemical studies by Ritt *et al.* (Ref. 31; *PNAS* 2025, 122, 38.) have shown that RAS binding to the RBD initiates the disassembly of the autoinhibited complex. We have updated the manuscript to reflect this most up-to-date understanding by deleting the previous statement and adding the following text (page2, line 54):

"For BRAF, RBD–RAS binding initiates the disassembly of the autoinhibited state, and CRD–lipid interaction is required for full activity[31]."

2. The intrinsic instability of the isolated CRD domain relative to RBD or RBD-CRD is not acknowledged or controlled for, despite likely influencing the observed behaviors. Previous studies have suggested utilizing high salt concentration to keep it soluble/stable.

We appreciate the reviewer's thoughtful comment regarding this important control. During the final size-exclusion chromatography (SEC) purification step of purification (buffer: 20 mM HEPES [pH 7.3], 500 mM NaCl, 1 mM TCEP, and 10% glycerol), the CRD eluted at the

expected volume based on protein standards, and SDS–PAGE analysis confirmed high purity. These results indicate that the CRD was robustly expressed and properly purified.
In our membrane assays, we used a buffer containing 40 mM HEPES (pH 7.4), 150 mM NaCl, and 5 mM $MgCl_2$. We did not observe any detectable aggregation on membranes in either ensemble or single-molecule binding assays. To examine this more thoroughly, we nonspecifically immobilized CRD-mNG on a glass surface in our assay buffer and analyzed its fluorescence intensity distribution at the single-molecule level. The CRD exhibited a slightly broadened distribution with a mean intensity approximately 25-30% higher than other constructs, and the distribution tail extended only modestly (Supplementary Fig. 5). These results suggest that the CRD may contain a small fraction of dimers but no evidence of larger oligomeric species.

To reflect this discussion in Results, the following text has been added to the Results (page 5, line 112):

"Data demonstrating protein quality—SEC and SDS–PAGE, and single-molecule intensity analyses—are provided in Supplementary Figure 4–6. The single-molecule intensity distribution suggests the presence of a minor dimeric fraction of the CRD-mNG construct, with no evidence of larger oligomers, at 150 mM NaCl, while all other proteins were purely monomeric"
Additionally, a previous study has shown that the CRD is sufficiently stable at a physiological salt concentration to allow characterization of its membrane binding. Spencer-Smith et al. (Ref. 2; Spencer-Smith *et al. Mol Cell* 2022, 82, 4262-4272) quantified CRD binding to nanodiscs containing 20% PS at 150 mM NaCl using SPR. They observed minimal binding at 0.3 μM, and micromolar concentrations were required to detect substantial SPR signals. This weak binding of the CRD at nanomolar concentrations is consistent with our observations (Fig. 1g). Similarly, Travers et al. (Ref. 60; Travers *et al. Biophys J* 2020, 119, 1-14) also reported comparably low levels of CRD binding at 1 μM on 30% PS liposomes at 250 mM NaCl.

3. The authors use an HRAS(1–184) construct, even though mature HRAS is 185 residues long. They do not specify which cysteine is modified by maleimide or address the implications of this for membrane anchoring and orientation.

Details of the specific residues used for maleimide-conjugation of HRAS and the validity of this method are described in the Result (page 4, line 100) with relevant references. We used its native cysteine palmitoylation sites (C181 and C184) to covalently link to lipids via maleimide chemistry. Maleimide conjugation has been extensively used in membrane reconstitution studies and has been validated to preserve the biological function and membrane orientation of RAS at levels comparable to those of the fully processed protein. The relevant references are provided (Ref. 40-47, including three newly added references).

Although HRAS orientation is less studied than other RAS isoforms, prior work shows that the NRAS G-domain remains largely free of lipid interaction, exhibiting rapid free rotational mobility (Ref. 48; A. Werküller et al. *ChemPhyChem* 2013, 14, 3698-3705). Furthermore, a recent NMR study revealed that ~90% of KRAS adopts the membrane-distal state in which the G-domain does not directly contact the membrane, but it is tethered via its C-terminal HVR (Ref.

49; Van *et al. PNAS* 2020, 117, 24258-24268), facilitating the capture of effector proteins. Consistent with these observations, we expect the HRAS G-domain to undergo rapid free rotational diffusion on membranes, remaining largely distal from the membrane surface and thereby facilitating efficient RBD binding.

Our experimental results also indicate that G-domain of maleimide-conjugated HRAS is freely available for RBD binding. The measured K_d for RBD–RAS binding on the membrane surface (Fig. 1e and Supplementary Fig. 9a,b) agrees well with K_d determined in solution (Ref. 52; Zheng *et al. PNAS* 2022, 119, e2119990119) (page 6, line 141).

To reflect this discussion, we have added the following sentence (page 5, line 104):

"Maleimide-conjugated HRAS is expected to undergo rapid free rotational diffusion on membranes, allowing ready access for effector protein binding, as previously observed in fully processed NRAS and KRAS[48,49]."

4. Maleimide conjugation is a non-physiological means of membrane tethering that does not replicate the native prenylation of KRAS, which has been extensively utilized in the recent past. This limitation is not discussed, despite its potential impact on membrane interaction kinetics.

We chose maleimide conjugation here because the study aims to determine membrane kinetic mechanisms in a minimal system with precise control of HRAS surface density. Covalent attachment to maleimide lipids provides advantages that are difficult to achieve with prenylated KRAS under our flow-based assays: (i) stable tethering that withstands multiple wash/flow steps; and (ii) avoidance of variability from uncontrolled reversible farnesyl insertion/extraction, which would otherwise couple RAS association/dissociation to the measured RAF kinetics and confound rate estimates. This robustness enables unambiguous interpretation of relative kinetic effects, because all measurements were performed at a stable, well-defined RAS density.

Moreover, recent SPR studies using KRAS robustly demonstrated that farnesylation (F) and methylation (Me) do not measurably alter its affinity for CRAF RBD-CRD, and that the CRD interacts with neither fully processed KRAS (KRAS-FMe) nor the fully processed hypervariable region (HVR-FMe) (Ref. 35; Tran *et al.*, *Nat. Commun*. 2021, 12:1176). Therefore, the absence of prenylation in our tethering strategy is unlikely to meaningfully affect RBD-CRD or CRD binding to RAS, or to alter our conclusions.

5. The manuscript omits critical methodological details: the final composition of protein storage buffers, assay buffers, salt concentrations, assay temperatures, and precise protein concentrations used in kinetic and diffusion experiments are not reported.

We appreciate the reviewer's attention to these details and apologize for the omission of the final protein storage buffer composition. Buffer A (20 mM HEPES, pH 7.3, 500 mM NaCl, 1 mM TCEP, and 10% glycerol) was used throughout the protein purification process, including the

final SEC step. The composition is described under the section *Protein Purification* in the Methods.

We identified that the assay buffer composition for the competitive FRET assay was missing and apologize for this omission. The complete buffer composition has now been added. The compositions of all other assay buffers were originally provided as abbreviations in the Methods, with full definition given at their first mention. To improve clarity, we have now revised the manuscript to present full buffer compositions—including salt concentrations—throughout, without using abbreviations.

In addition, the assay temperature is now specified alongside the full buffer compositions in the Methods.

We also found the omission of some protein concentrations. All protein concentrations have been specified in figure captions. The availability of this information has been indicated in the Methods.

6. There is no description or validation of the linker mutant or extended linker constructs. The authors provide no data on their expression levels, solubility, or folding.

Glycine-rich flexible linkers are widely used to connect two protein domains without interfering with their individual structures and functions. (Chen et al., *Adv Drug Deliv Rev* 2013, 65, 1357-1369). The L136A/T178A mutant and short linker (GGGGGGS) and extended linker (4xGGGGGGS) variants were expressed at levels comparable to wild-type RBD-CRD and were stable under our assay conditions. Each protein eluted at the expected volume on SEC (calibrated with protein standards, Supplementary Fig. 3) and was monomeric in the assay buffer (40 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$), as indicated by single-molecule fluorescence intensity distributions (Supplementary Fig. 5). Additionally, we acquired CD spectra (Supplementary Fig. 24) for these constructs and found no evidence of folding variation among them.

To include protein quality analysis and to reflect this discussion, the manuscript has been modified as follows (page 12, line 289):

"Protein quality analyses (Supplementary Fig. 3–5) and circular dichroism (Supplementary Fig. 24) confirmed that all RBD-CRD linker variants were properly purified and folded. The reduced membrane binding observed for these constructs is therefore attributed to perturbations in the relative positioning between the RBD and CRD caused by the increased linker flexibility and length."

7. The rationale for the L136A/T178A double mutant is unclear. T178 is not part of the linker, and its selection is not justified. Similarly, the purpose/rationale of introducing a 24-residue flexible linker is not explained.

(1) Rationale for the L136A/T178A double mutant: This set of measurements (Figure 4) was performed to identify structural elements that mediate positive cooperativity between the RBD and CRD. The mutations and linker variants were selected based on the X-ray structure of KRAS:RBD-CRD(CRAF) complex reported by Tran et al. (Ref. 35; Tran *et al.*, *Nat. Commun*. 2021, 12:1176). In this structure, the RBD and CRD bind RAS through distinct interfaces, forming a trimer (Figure 4a). The L136A/T178A double mutant was selected to disrupt this assembly. To clarify the rationale for the selection of the L136A/T178A double mutant, we have revised the corresponding text as follows (page 11, line 273):

"L136, located within the linker region, is buried within a hydrophobic pocket formed by F130 in the RBD and M183 in the CRD, bringing the two domains into close proximity[35]. The CRD residue T178 mediates contact with RAS through hydrogen bonding[35]. The L136A and T178A mutations are expected to disrupt the linker–RBD and CRD–RAS interactions, respectively, which would destabilize the RAS:RBD-CRD trimer complex. A previous study reported that L136A reduced KRAS binding affinity fourfold and T178A decreased RAF activity in cells[35]."

(2) Rationale for the linker variants: The flexible linker variants were designed to induce greater perturbations in the RBD-CRD arrangement, particularly in how the CRD can be positioned (or oriented) relative to the RBD. To clarify the rationale for introducing the short GGGGGS linker, we have revised the corresponding text as follows (page 12, line 283):

"This flexible linker [GGGGGS] is expected to eliminate residue-specific interactions while increasing overall flexibility, which disrupts the proper orientation of the CRD."

To clarify the rationale for introducing the extended 4×GGGGGS linker, we have revised the corresponding text as follows (page 12, line 286):

"To introduce a greater perturbation by increasing the physical separation and flexibility between the RBD and CRD, we characterized a construct containing an extended linker of four GGGGGS repeats (4×GGGGGS; 24 amino acids total)."

8. The claim that the T178A mutation stabilizes the extended linker construct is not supported by any biochemical data.

In the original manuscript, we were unable to express the RBD-CRD(4×GGGGGS) construct despite multiple attempts. The open reading frame of the plasmid had been verified by Sanger sequencing, so the lack of expression was puzzling. During the revision, we performed whole-plasmid sequencing and found an unexpected insertion that had not been detected by earlier Sanger sequencing. After re-cloning the plasmid, we were able to purify RBD-CRD(4×GGGGGS). The RBD-CRD(4×GGGGGS/T178A) construct was used out of necessity because we were initially unable to express the RBD-CRD(4×GGGGGS) construct. Now that the RBD-CRD(4×GGGGGS) construct has been successfully expressed, we have replaced the RBD-CRD(4×GGGGGS/T178A) data with RBD-CRD(4×GGGGGS) in Figure 4. The manuscript has

been revised to reflect this change and provide adequate description as follows (page 12, line 288):

"The extended linker constructs showed a greater reduction in membrane binding (cyan, Fig. 4b). Protein quality analyses (Supplementary Fig. 3–5) and circular dichroism (Supplementary Fig. 24) confirmed that all RBD-CRD linker variants were properly purified and folded. The reduced membrane binding observed for these constructs is therefore attributed to perturbations in the relative positioning between the RBD and CRD caused by the increased linker flexibility and length."

The 4×GGGGGS construct occasionally exhibited a minor residual signal, about 5% higher than others, in the normalized desorption curve (Fig. 4d). The absolute residual intensities were comparable across constructs, suggesting that its lower equilibrium binding intensity may have caused the apparent residual fraction after normalization. The dissociation rate of the 4×GGGGGS construct was nonetheless faster than that of the other constructs.

Additionally, we found that RBD-CRD(4×GGGGGS) exhibits relatively fast equilibrium kinetics, making it difficult to reliably compare relative association rates using ensemble association assays. Therefore, we quantified k_{on} using single-molecule recruitment assays for the WT RBD-CRD, the L136A/T178A mutant, and the two linker variants. All k_{on} values remained unchanged, consistent with our previous conclusions. The manuscript has been revised to include this new data in Figure 4c.

9. The known ~2-fold higher affinity of RBD-CRD for RAS compared to RBD alone is not factored into the interpretation of results. Many observations attributed to RBD-CRD vs RBD or CRD could reflect this baseline affinity difference.

We appreciate the Reviewer's critical feedback. We agree that it is important to disentangle the contributions of protein–protein and protein–lipid interactions to the high affinity of RBD-CRD on RAS-functionalized membranes. To account for the intrinsically higher protein–protein affinity between RBD-CRD and RAS, we performed additional experiments measuring the K_d values of RBD and RBD-CRD for RAS in the absence of membranes (PEGylated surfaces) and compared them with the corresponding K_d values for membrane-tethered RAS.

Briefly, we observed that an additional protein contact between RAS and the CRD increases the overall affinity of RBD-CRD for RAS by approximately fivefold. The CRD–PS interaction further enhances the overall affinity of RBD-CRD for membrane-tethered RAS by approximately sevenfold. To reflect this new result, we have revised the main text describing the K_d measurements and have added K_d values determined on lipid-free PEG surfaces as Supplementary Fig 8 as follows (page 6, line 131):

"To better understand how RAF integrates protein and lipid inputs for membrane binding, we sought to disentangle the contributions of protein interaction and lipid interaction to the enhanced affinity of RBD-CRD on RAS-functionalized membranes. To quantify the affinity arising from solely from protein–protein interactions, the apparent dissociation constants (K_d) of

RBD and RBD-CRD for RAS were determined on PEGylated (non-membrane) surfaces. An additional protein contact between RAS and the CRD increases the overall affinity of RBD-CRD by approximately fivefold—220 ± 80 nM for RBD and 40 ± 10 nM for RBD-CRD (Supplementary Fig. 8)—a trend similar to the twofold enhancement previously observed with KRAS[35]. For membrane-tethered RAS, the RBD exhibited K_d that depended little upon PS content, measuring 270 ± 90 nM and 240 ± 20 nM at 2% and 20% PS, respectively (Fig. 1e and Supplementary Fig. 9a,b). These values are consistent with our membrane-free measurements and previously reported affinities of the CRAF RBD for HRAS[35,52]. In contrast, RBD-CRD exhibited an affinity for membrane-tethered RAS more than an order of magnitude higher than that of the RBD alone (K_d = 25 ± 10 nM and 6 ± 3 nM at 2% and 20% PS, respectively; Fig. 1f and Supplementary Fig. 9c,d). Removing RAS essentially abolished this high-affinity binding (Fig. 1f). The progressive increase in affinity to RAS—from K_d = 220 nM for RBD (PEG surface), to 40 nM for RBD-CRD (PEG surface), to 6 nM for RBD-CRD (20% PS membrane)—demonstrates that low-nanomolar membrane affinity arises from the cooperative contributions of RAS–RBD binding, the additional RAS–CRD contact, and CRD–PS engagement."

Additionally, we quantified the dissociation rate of RBD-CRD from RAS on lipid-free PEGylated surfaces and compared it with that measured for RAS tethered to 20% PS membranes. We found that the lipid interaction is essential to achieve the prolonged membrane residence observed in Figure 2c. To reflect this new result, we have revised the main text describing the dissociation kinetics (Fig. 2c) as follows (page 8, line 182):

"Such a long dwell time cannot be achieved solely from protein–protein interactions. The RBD-CRD dissociates from RAS on a lipid-free PEGylated surface with a half-life of a few seconds (Supplementary Fig. 15a). This kinetic parameter analysis reveals that a crucial role for the CRD–PS interaction in selectively regulating membrane dissociation kinetics without affecting the association rate."

10. The proposed enhancement of RBD–RAS association by CRD–lipid interaction is not clearly demonstrated or mechanistically explained.

The conclusion is derived from the competitive FRET assay shown in Figure 3. This assay measures energy transfer between fluorescently labeled RAS and RAF (RBD or RBD-CRD), thereby selectively reporting the dissociation kinetics of RAF from membrane-tethered RAS. As such, the FRET decay curve reflects only the kinetics of the protein–protein dissociation. Using this setup, we examined how this protein–protein (RAS–RAF) interaction is influenced by CRD–lipid engagement. A key conclusion arises from comparing the FRET dissociation curves at 2% and 20% PS membranes. The dissociation at 20% PS was slower than that at 2% PS, indicating that CRD–lipid interactions strengthen the protein–protein interaction. If the protein-protein interaction were unaffected by CRD–lipid interaction, the FRET dissociation kinetics would be identical between 2% and 20% PS.

Thanks to the additional experiments measuring the dissociation kinetics of RBD-CRD from RAS on lipid-free PEGylated surfaces (related to Reviewer 1, Comment #9), we were able to identify the contribution of the RAS-CRD interaction to the observed slow FRET dissociation

kinetics. The manuscript has been revised to clarify the role of the RAS-CRD interaction in the slower dissociation kinetics, as follows (page 10, line 250):

"The slower dissociation for RBD-CRD at 2% PS is primarily due to an additional interaction between the CRD and RAS, as its FRET dissociation curve closely follows the ensemble dissociation curve of RBD-CRD measured on lipid-free PEG surfaces (Supplementary Fig. 15b). Importantly, 20% PS lipids, which promote a greater degree of CRD–lipid interaction, further accentuates this slowed dissociation for RBD-CRD (Fig. 3b). These findings indicate that CRD–lipid interactions enhance the binding strength between protein domains (RAS:RBD and RAS:CRD), thereby stabilizing the complex and slowing its dissociation."

The potential mechanism underlying this behavior may involve reduced fluctuations of the RAS:RBD complex due to additional anchoring by CRD–lipid interactions, as suggested by MD simulations (ref. 57; *Structure* 2018, 26, 513–525). This mechanism is described in the Result (page 10, line 237–239) and the Discussion (page 15, line 373–375).

11. The claim that RAS·GTP mediates membrane recruitment of RBD-CRD species not bound to RAS is conceptually inconsistent, especially under conditions where all RAS binding sites are presumably occupied.

We found that the major population of lipid-engaged RBD-CRD species not bound to RAS (the RBD-CRD:PS complex) is primarily generated through the dissociation of RAS from the RAS:RBD-CRD:PS complex. Therefore, RAS·GTP indirectly mediates the membrane recruitment of the RBD-CRD:PS species. This process is described in detail in the Results (page 13, line 313–315; Fig. 5b).

We believe the confusion may have arisen because this observation was introduced too early—before the full explanation of the lateral rebinding mechanism that accounts for the formation of the RAS-free RBD-CRD:PS species. To improve clarity, we have removed this earlier description on the formation of the RBD-CRD:PS species, this process is described in detail in the later section *RAS density modulates RAF membrane residence through lateral rebinding*.

12. The term "RAS unbinding" is used ambiguously throughout and should be clarified to distinguish between RAS–RAF dissociation and membrane detachment.

We appreciate for the suggestion. The term *"RAS unbinding"* has been revised to *indicate "RAS dissociation from the RAS:RBD:PS complex."* (page 13, line 313; page 14, line 336; page 15, line 376)

13. The central conclusion regarding nanocluster-specific signaling is not experimentally supported. The lipid compositions used do not promote RAS nanocluster formation, and the system lacks the complexity to model this behavior. Therefore, extrapolating these findings to nanocluster biology is not justified.

We appreciate the reviewer's critical feedback. The description of RAS nanoclusters has been removed from the Abstract and Results. Additionally, we have substantially revised the Discussion section to present this concept briefly as an unproven hypothesis, as follows (page 16, line 409):

"It is worth mentioning that the average density of RAS on the plasma membrane is in the range of 30–150 RAS molecules per μm^2 [68]. RAS is not uniformly distributed; instead, it forms dynamically exchanging nanoclusters that serve as the primary sites for recruitment and activation of RAF[32]. The radius of a nanocluster is estimated to be 6–12 nm, with a local surface density of ~4000 to ~16,000 RAS per μm^2 [32,33,68]. Such high local RAS densities within nanoclusters may facilitate RAF activation by extending its membrane residence time through lateral rebinding, although this hypothesis remains to be experimentally validated."

Reviewer #2 (Remarks to the Author):

The manuscript by Salinas et al. presents a well-thought-out and comprehensive kinetic analysis of the RAF1 RBD-CRD domain interactions with HRAS and a simple supported lipid bilayer (SLB). The SLB is composed of DOPS, DOPC, and a functionalized DOPE that links HRAS to the bilayer via maleimide chemistry through an available cysteine in HRAS's hypervariable region. I believe this study proposes a model that is both intriguing and valuable to the field, and that the supporting data and analysis are sound.

In brief, the authors propose that, following initial binding of RAF to RAS on the membrane, subsequent lipid interactions involving the RAF CRD domain are sufficient to extend RAF's residence time. Nanoclustering of RAS and a CRD–lipid intermediate increases the likelihood of subsequent rebinding to other RAS monomers, enabling the full RAF activation sequence—including RAF dimerization—to proceed. This model has important implications for understanding the RAF activation cycle, particularly the possibility of a kinetic proofreading function.

Therefore, I recommend the manuscript for publication, provided the following points are addressed:

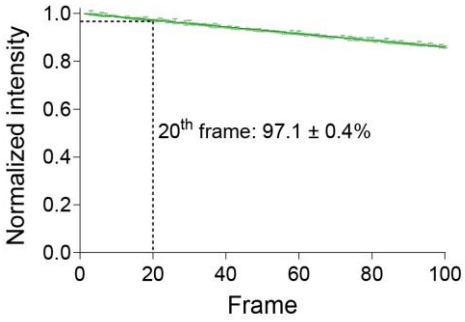
1. The binding and dissociation rates of RAS/RAF complexes were calculated in a previous single-molecule pull-down study: Lee, H.W., Kyung, T., Yoo, J., et al. Nat Commun 4, 1505 (2013). In this study, RAS was immobilized, and RAF molecules were pulled down from a lysate onto coverslips and imaged using TIRF microscopy. Residence times and kinetic parameters were measured. The authors should explicitly compare their data to this study. Moreover, as in Lee et al., they should account for potential photobleaching effects to ensure that their measured rates are not significantly influenced by the photophysics of the fluorophores.

We appreciate the reviewer's thoughtful suggestions. First, we would like to clarify that, unlike the previous study by Lee *et al.* (*Nat Commun* 2013), pre-equilibrating encounter complexes were not observed in our system (see also our response to Reviewer 2, Comment #2). Therefore, the reported k_{on} values in the *Nat Commun* 2013 study and in the present work describe different processes and cannot be directly compared. In our measurements, the k_{on} for the RBD binding to RAS was estimated to be ~0.006 $nM^{-1}s^{-1}$ (Fig. 2b) and the k_{off} was ~1.4 s^{-1} (converted from a half-life of ~1 s, Fig. 2c). From these results, the corresponding $K_d = k_{off}/k_{on}$ was estimated to be ~230 nM, which agrees well with independently determined K_d values (270 nM on 2% PS and 240 nM on 20% PS membranes) obtained from titration experiments (Fig. 1e and Supplementary Fig. 9a,b). This close agreement supports the validity of our kinetic rate estimates. The transient nature of RBD–RAS interaction observed in our study is also consistent with that reported by Lee *et al.* (~300 ms), indicating that both systems exhibit dynamic, fast exchange kinetics.

Because the k_{on} values in the previous study and in our present study represent different processes, we chose not to compare them directly. To highlight the dynamic nature of RBD–RAS interaction observed in both studies, we have revised the manuscript and provide the reference as follows (Page 7, line 178, related to Fig. 2c):

"The RBD exhibited highly dynamic and transient interactions with a half-life of ~1 s, consistent with a previous study using proteins pulled down from cell lysates[53]."

Regarding the potential effect of photobleaching on k_{off} we observed minimal impact. In our study, k_{off} was determined using an ensemble desorption assay, in which fluorescence intensity decay was measured at specified time intervals rather than by continuous streaming acquisition. Each measurement involved the acquisition of fewer than 20 frames. To quantify the contribution of photobleaching to the observed intensity decay, we measured the photobleaching kinetics of mNG stably tethered to supported lipid bilayers. The laser was illuminated every 0.5 seconds using the same laser power and exposure time as in the desorption assays. Only a minor photobleaching effect was observed over 100 frames, with a 3% decrease in fluorescence at the 20th frame, clearly demonstrating that photobleaching had little to no effect on our measurements.



Supplementary Fig. 14: Photobleaching kinetics has a minimal impact on determining k_{off}.

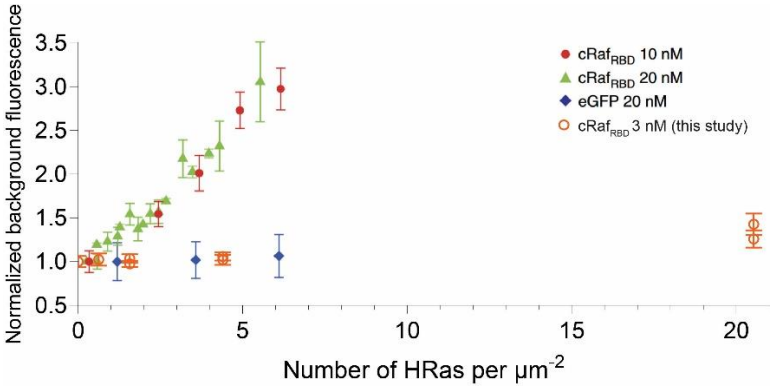
To reflect this discussion, the manuscript has been revised to include the photobleaching data as Supplementary Figure 14 and the following statement for describing Figure 2c (page 8, line 181):

"Photobleaching had a minimal impact on the estimation of k_{off} (Supplementary Fig. 14)."

2. Lee et al. describe the formation of "pre-equilibrating encounter complexes." The authors should clarify whether these results are consistent with or differ from those in the current manuscript, especially with respect to the proposed lateral rebinding model. Although Lee et al. used immobilized RAS (i.e., non-diffusing and in a lipid-free system), it is possible that their findings contradict the current interpretation. Are there alternative explanations that support the proposed lateral rebinding and kinetic proofreading model?

We examined the possibility of pre-equilibrating encounter complex formation in our system by conducting an additional experiment based on a similar approach in Figure 2f of Lee *et al*. (*Nat Commun* 2013). In this experiment, we measured background fluorescence intensity as a function of membrane-bound RAS surface density, in the presence of 3 nM RBD-mNG. According to Lee *et al.*, an increase in background signal at higher RAS densities would indicate formation of pre-equilibrating encounter complexes. We calculated background intensity by

averaging fluorescence signals from membrane regions excluding detectable single-molecule RBD spots. The 3 nM concentration was chosen to maintain low enough particle density for robust particle exclusion during background analysis. Across RAS densities titrated up to ~20 molecules/μm^2, we observed little to no change in background intensity (Response Fig. 1). The trend closely followed control measurements using eGFP reported by Lee *et al.*, which does not interact with RAS. This result suggests the absence—or minimal presence—of pre-equilibrating encounter complexes in our system. Thus, we conclude that such complexes, if they exist, do not significantly contribute to RAF membrane binding dynamics under our experimental conditions. Thus, the observed RAS density-dependent modulation of RAF dwell time is best explained by the lateral rebinding mechanism in which RAF forms a transient RBD-CRD:lipid intermediate on membrane surface (Fig. 5b). This is supported by additional data (see also our response to Reviewer 2, Comment #3), showing that disruption of CRD–lipid interactions by mutations reduces the RAS density dependence of RAF dissociation kinetics (Supplementary Fig. 25c).



Response Figure 1: Normalized background fluorescence in the presence of fluorescent RBD. Data from RAS-functionalized lipid membranes (open red circles, this study) are compared with those from RAS-functionalized PEGylated surfaces (solid symbols, Lee *et al. Nat. Commun.* 2013). No change in background fluorescence was detected on RAS-functionalized lipid membranes, indicating the absence of pre-equilibrating encounter complex formation under our experimental conditions.

3. The authors cite a recent study using KRAS4b on SLBs, which analyzes KRAS4b/RBD-CRD complexes on simple and complex lipid bilayers: Shrestha R., Carpenter T.S., Van Q.N., et al. Commun Biol. 2024; 7(1):242. The authors should comment on the specific CRD-lipid contact points identified by the simulations and NMR in Shrestha et al. and evaluate experimentally whether mutations of these lipid-penetrating CRD residues affect lateral rebinding interactions in the RBD-CRD complex or the proposed RBD-CRD–PS intermediate.

We thank the reviewer for the thoughtful suggestion. Based on the study by Shrestha et al. (*Commun Biol* 2024), we generated an RBD-CRD construct containing tetra-alanine mutations (K148A, L149A, K157A, and F158A; hereafter referred to as 4A). We characterized membrane binding, diffusion, and desorption kinetics for the 4A construct. To reflect this new result, the manuscript has now been revised to include the data as Supplementary Fig. 25 and the following text (Page 14, line 339):

"Positively charged and hydrophobic residues in loops 1 and 2 of the CRD penetrate the bilayer and mediate membrane binding[55]. To test how these specific CRD–lipid interactions contribute to lateral rebinding, we characterized an RBD-CRD^{4A} construct containing four alanine mutations (K148A, L149A, K157A, and F158A; hereafter referred to as 4A). The 4A construct exhibited a diffusion behavior similar to that of the isolated RBD, and its membrane binding was substantially reduced compared to WT RBD-CRD (Supplementary Fig. 25a,b). Notably, the extension of membrane dwell time at higher RAS densities was significantly attenuated, confirming the critical role of CRD–lipid interactions in lateral rebinding and in the formation of the RBD-CRD:PS intermediate (Supplementary Fig. 25c)."

4. In the final sentence of paragraph 1 in the section titled "RAS density modulates RAF membrane residence through lateral rebinding," the authors state: "we exclude the scenario where RBD-CRD first loses lipid contact yet remains bound to RAS, as a lipid-free RAS:RBD-CRD complex was not observed in single-particle tracking experiments (Fig. 2e)." To more robustly support this claim, the authors should measure the diffusion of fluorescently labeled RAS in the presence of RBD-CRD on the same SLB and compare those diffusion rates to the measurements where the fluorescent tag is on RBD-CRD. Comparing the distributions and fractional occupancies of the different diffusion states in both labeling conditions would provide a stronger test of the assertion that lipid-free RAS:RBD-CRD complexes do not form or persist. I would also encourage a different term than "lipid-free." We know that there are extensive and dynamic contacts between RAS and lipids. There is probably more precise language to talk about the conformational state of the complex and contact with the lipids in the bilayer.

We appreciate the reviewer's suggestion. However, we believe the proposed experiment would not directly address the presence or absence of RAS:RBD-CRD complexes that lack CRD–lipid engagement (hereafter referred to as CRD-disengaged RAS:RBD-CRD complexes). The main complication originates from interpretating the diffusion behavior of fluorescently labeled RAS* (the asterisk denotes a fluorescent label). Based on our findings, CRD-disengaged RAS*:RBD-CRD complexes are expected to diffuse similarly to free RAS*, as the diffusion of free RAS* and RAS:RBD* are nearly identical (Figure 2e). Therefore, it would be difficult to distinguish whether fast-diffusing RAS* corresponds to free RAS or CRD-disengaged RAS:RBD-CRD complexes. Diffusion analysis of labeled RBD-CRD* on membranes provides a more direct mean for detecting CRD-lipid engagement. In our experiment, we did not observe a fast-diffusing population of RBD-CRD* (Figure 2e), suggesting the majority of membrane-bound RBD-CRD remains engaged with lipids.

To revise a term *"lipid-free"*, the corresponding sentence has been modified as follow (page 13, line 317):

"We exclude the scenario where RBD-CRD first loses lipid contact yet remains bound to RAS, as a RAS:RBD-CRD complex with the CRD disengaged from lipids was not observed in single-particle tracking experiments (Fig. 2e)."

Reviewer #3 (Remarks to the Author):

This is an interesting and potentially impactful article that would be suitable for publication in a major journal such as Nature Communications, following adequate attention to significant shortcomings. The article summarizes well-reasoned biophysical studies of the multi-pronged interactions between the important signaling proteins CRaf, HRas and anionic lipids (phosphatidylserine or PS) during CRaf membrane binding, 2D-diffusion and dissociation on a lipid bilayer possessing anchored HRas molecules. The quite promising results suggest that, as often observed for signaling proteins that target the plasma membrane, the CRaf molecule possesses cooperative, multi-component binding contacts involving both protein-lipid and protein protein interactions that together provide adequate specificity and affinity for the correct membrane surface. In CRaf this multi-component interaction is provided by its distinct cysteine-rich domain (CRD) and Ras binding domain (RBD) that provide protein-lipid (CRD-PS) and protein-protein (RBD-HRas), respectively. While these results are not surprising, this manuscript is the first to address the cooperative nature of these essential Ras:CRaf:PS interactions in a quantitative, biophysical manner. The following major and minor points should be addressed prior to publication.

1. The article places a major emphasis on Raf targeting, retention, and activation in Ras nanoclusters with language about relevance to nanoclusters on 10 pages of the text including the Abstract, Introduction, Results, Discussion, and Figure Legends. However, as the article itself notes, the system employed observes individual Ras:RBD-CRD complexes interacting with PS lipids on the target bilayer with no detectable clusters (pg 8, bottom half of paragraph). Moreover, it appears that Ras densities and clustering are not measured in most or all experiments. Thus the article should be more careful about its claims of relevance to clustered systems, and should limit those claims to the description of the plausible, but as yet unproven, model in the Discussion.

We appreciate the reviewer's critical feedback. The description of RAS nanoclusters has been removed from the Abstract and Results. Additionally, we have substantially revised the Discussion section to present this concept briefly as an unproven hypothesis, as follows (page 16, line 409):

"It is worth mentioning that the average density of RAS on the plasma membrane is in the range of 30–150 RAS molecules per μm^2 [68]. RAS is not uniformly distributed; instead, it forms dynamically exchanging nanoclusters that serve as the primary sites for recruitment and activation of RAF[32]. The radius of a nanocluster is estimated to be 6–12 nm, with a local surface density of ~4000 to ~16,000 RAS per μm^2 [32,33,68]. Such high local RAS densities within nanoclusters may facilitate RAF activation by extending its membrane residence time through lateral rebinding, although this hypothesis remains to be experimentally validated."

RAS densities were quantified for all experiments. They are presented in either figures or figure captions.

2. The experiments are well chosen and the data is promising, but there is inadequate attention to reproducibility which could lead to questions about the rigor of the work. In general, statistical tests of significance are absent and there is information on replicates for only two of the many experiments (in both cases N=3, presumably representing technical rather than biological replicates). Perhaps I missed it, but I could find no indication that experiments were carried out more than once, and were repeated on multiple days. Such lack of information about replicates and statistical tests makes it impossible to judge the reproducibility and statistical significance of the findings.

We thank the reviewer for directing our attention to the significance of statistical rigor and reproducibility. We apologies the omission of this information. We have now revised manuscript to report the number of total technical replicates (n = SLBs in total) and independent experiments (N) in figure captions, with the addition of replicate experiments. In some cases—particularly those dependent on RAS surface density—performing identical replications on different days was challenging because the resulting RAS densities often varied. However, all RAS densities were quantified, and in such cases we clearly indicated in the figure captions that similar results were obtained in independent experiments, with references to the corresponding data in the Supplementary Information.

The experiments are highly reproducible, largely due to our ability to directly measure the absolute surface density of proteins—including RAS—using a TIRF-FCS calibration standard. This allows quantitative reconstitution of well-defined protein-functionalized membranes.

Additionally, statistical tests have been performed for k_{on} measurements in Figure 2b (p values in Supplementary Table 1) and, as well as ensemble binding measurements in Supplementary Fig. 10.

3. The slow diffusion observed for Ras:RBD-CRD:PS complexes on the DO-lipid bilayer raises the possibility that, in addition to the frictional drag of the Ras anchor lipid and the bound PS molecules against the bilayer, there may well be additional frictional drag provided by protein insertion into the bilayer. See PMID 23701821.

We fully agree with this interpretation. The possibility of membrane insertion by CRD loop 1 and 2 is described in the Results (page 9, line 217–220).

To further highlight this point, we have now included the suggested reference (added to Ref. 59; Ziemba et al. *Chem Phys Lipids* 2013) and the following text (page13, line 319):

"The slow diffusion coefficient of RBD-CRD is comparable to that of PKC regulatory domains known to penetrate into the hydrocarbon core of the bilayer[59], indicating persistent lipid engagement of the CRD through its basic and hydrophobic residues in loop1 and loop 2[16,55]."

4. The article is missing key references to directly relevant single molecule studies of Ras-

effector interactions, and of the effects of membrane environment on the affinities and kinetics of protein-protein and protein-membrane interactions. See, for example, PMID 29211993, 39217418, 22263647.

We appreciate for providing important references relevant to our work. We have now added the following three references in the main text (page 5, line 102):

Ref. 45: Buckles et al., *Biophys* J 2017, 113, 2396-2405
Ref. 46: McCombs et al., *Biophys J* 2024, 123, 3277-3280
Ref. 47: Buckles et al., *Biophys J* 2020, 229, 2305-2218

"This widely used lipidation method preserves the biological function and structure of RAS and has been applied to all three RAS isoforms (H-, N-, and KRAS) and other membrane-anchored small GTPases[40–47]."

Reviewer #4 (Remarks to the Author):

This manuscript provides interesting results and confirms several observations from previous experimental and computational studies. I recommend that authors stress the novelty of their findings in the context of broader Ras biology during the revision of this manuscript.
We appreciate the reviewer's constructive feedback. To emphasize the novelty and significance of our study in the context of broader RAS biology, we have revised our manuscript to include the following text in the Introduction section (page 3, line 64):

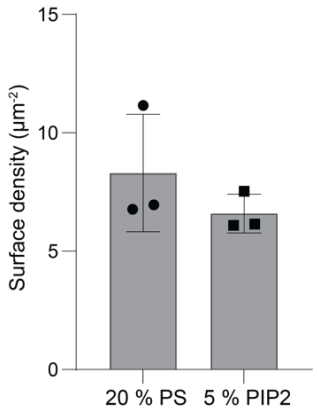
"Prior studies reported cooperativity between RBD and CRD in which RAS–RBD binding induces a transient interaction between RAS and CRD, and mapped CRD–lipid interfaces[16]. However, how these interactions control membrane kinetics has remained unclear. A quantitative understanding of membrane-binding kinetics and the underlying mechanisms is crucial because RAF is exclusively activated on cellular membranes[14]. RAF is also among the least abundant signaling proteins in many cancer cell types[36]. Upon receptor activation, only a small number of RAF molecules—estimated to be as few as 100–200 per cell—are recruited to the membrane, where they serve as a stoichiometric bottleneck that determines the overall signaling outcome[37]. Therefore, understanding RAF membrane dynamics is fundamental for elucidating how normal and oncogenic RAS activities are transduced into ERK signaling[38]."

The manuscript reports that RBD binds to RAS, allowing CRD to bind to negatively charged PS lipids (as determined by TIRF microscopy). They propose two kinetically distinct binding steps. Also, CRD binds to PS in a RAS-GTP-dependent manner. It ensures RAF membrane binding (by CRD) is strictly driven by RAS activation (by RBD). Using FRET, they demonstrate that CRD-lipid binding enhances the association between RBD and RAS (as measured by FRET). RBD and CRD binding to RAS and PS lipids exhibit cooperativity. RAF undergoes lateral rebinding with RAS (using kinetic simulations in SimBiology and TIRF microscopy). Weak CRD-lipid interactions play a role in lateral rebinding. This extends the RAF's membrane dwell time under high RAS density (e.g., nanoclusters). This prolonged membrane residence increases the likelihood of completing RAF's multistep activation sequence (i.e., facilitates kinetic proofreading of RAF activation).

Comments:

1. This work would have had a significant impact if the authors had investigated the kinetics in the presence of PIP2 lipid and distinguished the binding mechanisms.

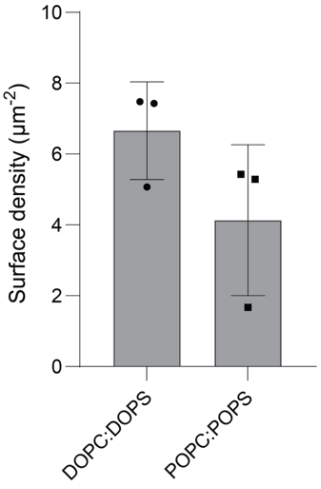
Lipid specificity of RAF kinases is an important question. PIP2 is involved in upstream signaling events such as RAS activation by SOS. Several molecular dynamics simulations and experimental studies suggest that the CRD engages with PIP2 (Johnson et al., *Biochemistry* 2005, 44(9), 3432–3440; Carpenter et al., *Biophys. J.* 2026, PMID: 40849684). Our lab is actively investigating this topic. Preliminary experiments comparing RBD-CRD binding to supported lipid bilayers containing 5% PIP2 versus 20% PS (both carry the same –20 net charge) in the absence of RAS show largely similar association behavior (Revision Fig. 2). While we agree that this is a significant question, we believe it requires a more detailed investigation in the presence of RAS, which we plan to focus on in a separate study.



Revision Figure 2. Equilibrium surface densities of 100 nM RBD-CRD on RAS-free DOPC membranes containing either 20% DOPS or 5% PIP2. Both conditions show similar levels of membrane binding. Data represent means ± standard deviations from three technical replicates (n = 3 SLBs).

2. Additionally, DOPC is not a particularly abundant component of the plasma membrane. Any reason for its selection? (CRD is more likely to bind to POPC:POPS membranes than DOPC:DOPS)

We thank the reviewer for this comment. We selected DO-lipids based on prior success in generating high-quality supported lipid bilayers. We have recently begun using PO-lipids in our experiments. Our latest measurements indicate that RBD-CRD binds to POPC:POPS membranes with comparable (or slightly lower) affinity with respect to DOPC:DOPS membranes (Revision Fig. 3). However, this small to modest difference does not affect the main conclusions of this study.



Revision Figure 3. Equilibrium surface densities of 100 nM RBD-CRD on RAS-free membranes composed of either DOPC:DOPS or POPC:POPS. Both membranes contain 20% PS. Data represent means ± standard deviations from three technical replicates (n = 3 SLBs).

3. Also, is a 20% PS concentration sufficient to mimic biomembranes?

We believe that 20% PS is sufficiently close to physiological levels for studying RAF–PS interactions. According to recent lipidomics data from Lorent et al. (*Nat. Chem. Biol.* 2020, 16, 644–652), PS constitutes approximately 26% of the inner leaflet lipids in erythrocyte plasma membranes. Currently, 20% PS is a technical upper limit we can reliably incorporate while maintaining high-quality supported lipid bilayers and efficient protein coupling, although further optimization might be possible.

To provide this information, we have revised the manuscript to include the following sentence and cite the study by Lorent et al. (page 4, line 98):

"A 20% PS composition was used to closely approximate the physiological fraction (~26%) of PS in the inner leaflet of plasma membranes while maintaining high-quality bilayers[39]."

4. What would be the effect of having the kinase domain? Past experiments were based on the same RBD-CRD construct. Engaging the kinase domain and seeing the effect of homodimerization can be quite novel.

We strategically designed this study to dissect complex interactions between RAF and membranes with the conserved regulatory module (RBD-CRD) in a minimal, well-controlled setting. We plan to extend this work to investigate the membrane dynamics of full-length RAF. Based on our observations, we expect that kinase domain dimerization would further extend membrane dwell time, as increased multivalency (two RBD-CRD modules) could enhance the lateral rebinding process, which in turn increases sensitivity to RAS density. This hypothesis requires experimental validation with full-length proteins.

We provided our planned future studies in the final paragraph of the Discussion section as follows (page 17, line 418):

"To investigate how membrane-binding kinetics influence activation outcomes under more physiologically relevant conditions, it will be necessary to use the autoinhibited full-length RAF:14-3-3:MEK complex. These advancements will enable us to rigorously recapitulate the sequential steps of RAF activation and elucidate the underlying molecular mechanisms and kinetics with high resolution."

5. I find it challenging to convince myself that RAF unbinding occurs primarily by detaching from RAS first, then from the membrane, rather than the other way around (end of first paragraph on p. 12). Strong arguments to support their claim can be useful.

We identified the primary membrane detachment pathway of RAF as sequential dissociation from RAS followed by unbinding from lipids (hereafter referred to as Scenario A). This conclusion is supported by RAF diffusion analysis. The RBD, which is lacking the lipid-interacting CRD, diffuses at ~1 $\mu m^2/s$ when bound to RAS. However, this fast-diffusing species

was not observed for RBD-CRD:RAS complexes. Instead, these complexes exhibit much slower diffusion (~0.3 $\mu m^2/s$), indicating persistent lipid engagement. This slow diffusion coefficient is comparable to that of PKC regulatory domains known to penetrate into the hydrocarbon core of the bilayer (Ref. 59). Indeed, NMR and computational studies have shown that loop 1 and 2 of the CRD insert into the membrane (Ref. 16,55). The absence of the fast-diffusing population suggests that RBD-CRD persistently engages lipids via the CRD as long as it remains membrane-bound, making Scenario A favorable.

For detachment from RAS to be the final step in RAF membrane dissociation (hereafter referred to as Scenario B), all RBD-CRD:PS intermediates would need to laterally rebind to RAS, which is unlikely to occur. Scenario B also requires the CRD to disengage from lipids before RAS unbinding. However, our single-molecule tracking data (Supplementary Fig. 20) show that when RBD binds to RAS, the CRD engages lipids rapidly, within the 20 ms temporal resolution of our assay; which is much shorter than the dwell time of RBD:RAS (0.3 to 1 second, according to the present study and previous study by Lee *et al.* reported in Ref. 55 *Nat Commun* 2013, 4, 1505). This result suggests that if the CRD disengages from lipids, it is likely to reengage immediately as long as RBD-CRD is bound to RAS, making Scenario B unfavorable.

To provide a clearer rationale for selecting the primary RAF membrane dissociation pathway, we have now included the above discussion in the main text as follows (page 13, line 319):

"The slow diffusion coefficient of RBD-CRD is comparable to that of PKC regulatory domains known to penetrate into the hydrocarbon core of the bilayer[59], indicating persistent lipid engagement of the CRD through its basic and hydrophobic residues in loop1 and loop 2[16,55]. Even if the CRD momentarily dissociates from lipids, it is likely to re-engage rapidly while still bound to RAS, given the fast lipid engagement timescale observed in our single-molecule tracking analysis (Supplementary Fig. 20)."

6. Several xray structures clearly show that CRD creates contacts with RAS. I agree that these contacts may not be formed in the absence of RBD. Therefore, lipids are not the only driving force for membrane interaction. Claiming that CRD is driven only by lipids is not completely correct.

We appreciate the reviewer's constructive feedback. We performed additional experiments to measure the K_d values for RBD and RBD-CRD for RAS immobilized on lipid-free PEGylated surfaces. These experiments allowed us to quantitatively assess how the affinity of RBD-CRD increases progressively with the addition of each binding contact—RAS–RBD, RAS–CRD, and CRD–lipid interactions.

The main text describing Figure 2 has been revised to incorporate these new results. The contribution of the CRD–RAS interaction to the overall membrane binding affinity is now explicitly stated as follows (page 6, line 146):

"The progressive increase in affinity to RAS—from K_d = 220 nM for RBD (PEG surface), to 40 nM for RBD-CRD (PEG surface), to 6 nM for RBD-CRD (20% PS membrane)—demonstrates

that low-nanomolar membrane affinity arises from the cooperative contributions of RAS–RBD binding, the additional RAS–CRD contact, and CRD–PS engagement"

7. It is interesting that RBD alone has a higher k_{on} to RAS-associated membranes than RBD-CRD; however, the presence of CRD lowers the off rate, resulting in RBD-CRD having a stronger binding affinity than RBD alone. Additionally, the rate constants for RBD alone and RBD-CRD are not affected by PS concentration, so only k_{off} is influenced by PS levels. I don't quite understand that easily. Some clarity would be helpful.

We also found these results are interesting and unexpected. The observed trend is reproducible across multiple independent experiments (Fig. 2). First, we would like to clarify that the k_{on} values were measured by a single-molecule recruitment assay. In this assay, the time resolution is inherently set by the camera frame rate (20 ms) as described in the Methods section; this information has now been added to the Figure 2 caption as well. CRD–lipid interactions alone are highly transient and rarely result in detectable binding events at our current time resolution. We occasionally observed faint, short-lived diffusive traces of RBD-CRD on 20% PS membrane in the absence of RAS (see figure 2a for RBD-CRD, (-) RAS). However, these events are too transient to be reliably detected by the particle localization algorithm. Based on rough estimates, the dwell time of these interactions is likely on the order of a few to several milliseconds; even shorter-lived species may exist but are not visible under our experimental conditions (Supplementary Fig. 12).

The key observation is that these transient interactions between RBD-CRD and lipids do not lead to productive RAS engagement (clearly spatially resolved binding events). If they did, we would expect k_{on} to increase at a higher PS concentration, which was not observed. The highly transient nature of PS-recruited RBD-CRD and its inability to engage RAS prevent it from accumulating on the membrane. Therefore, PS does not contribute to the initial membrane recruitment of RAF.

To reflect this discussion, the manuscript has been modified to the following text in the Results (page 7, line 164):

"In line with our ensemble binding measurements, clearly spatially resolved membrane recruitment events required the interaction with RAS (Fig. 2a). Interestingly, we observed highly transient interactions of RBD-CRD (appearing as faint, diffusive traces) on RAS-free 20% PS membranes. Although those particles could not be localized, manual intensity analysis estimated their dwell times on the order of several milliseconds (Supplementary Fig. 12). Importantly, these short-lived interactions between RBD-CRD and lipids do not result in productive RAS engagement, as k_{on} values for both RBD and RBD-CRD were unaffected by PS concentration (Fig. 2b)."

We were also surprised that such a weak CRD–lipid interaction has a large impact on the dissociation rate (Fig. 2b). This arises from the combined effect of multiple factors: (1) promotion of CRD–PS engagement upon RAS–RBD binding (Fig. 1d,f), (2) stabilization of the RAS:RBD-CRD complex by PS engagement (Fig. 3), and (3) lateral rebinding (Fig. 5). A more detailed discussion of this point is provided in the Discussion (page 15, line 365-380).

Regarding the higher k_{on} for RBD compared to RBD-CRD, we speculate that the CRD may impose partial steric hindrance on RBD access to RAS. Although it is a plausible explanation, we do not have direct evidence.

8. Additionally, I find it puzzling that the RBD-CRD linker does not affect the initial RBD-RAS association but is relevant for the membrane dissociation rate, and thus for the cooperative binding of RBD-CRD to RAS and PS lipids.

This point is closely related to our response to Reviewer 4, Comment #7. RAF membrane interactions involve three different molecular contacts: (1) RBD–RAS interaction, (2) CRD–RAS interaction, and (3) CRD–lipid (PS) interaction. The CRD-mediated interactions (2) and (3) are dependent of RBD–RAS interaction (1) (Fig. 1g). Therefore, interactions (2) and (3) play no role in the initial membrane recruitment event. Instead, k_{on} is primarily governed by the RBD–RAS interaction (1) (Fig 2a,b).

Increasing the flexibility or length of the RBD-CRD linker likely alters the orientation and position of the CRD relative to RAS and the membrane surface (Ref. 35 and 58), thereby reducing the efficiency of both CRD–RAS and CRD–lipid interactions. Because these CRD-mediated interactions (2) and (3) primarily contribute to membrane stabilization and lateral rebinding rather than initial recruitment, their disruption selectively affects the membrane dissociation rate (k_{off}), without altering the initial RBD–RAS association.

To clarify the role of the linker, the manuscript has been revised to include the following text in the Result section (page 12, line 296):

"Thus, the CRD-mediated interactions (CRD–RAS and CRD–lipid) primarily contribute to membrane stabilization rather than to the initial association between RBD and RAS, consistent with the K_{on} and K_{off} analyses shown in Figure 2."

Reviewer #5 (Remarks to the Author):

In this study, the authors use quantitative TIRF microscopy to analyze the binding kinetics and equilibrium behavior of two RAF domains (RBD and CRD). Particularly, they looked at RBD/CRD interactions with phosphatidylserine lipids, RAS, and cooperative binding interactions with each other. The purpose of this study was to develop a model for membrane-dependent RAF activation, which is dependent on recruitment to the membrane by GTP-RAS, and retention on the membrane through weak PS interactions which cooperatively strengthen binding to RAS. The carefully designed experiments elucidate KD values, koff rates, and kon rates to elucidate the impact of each component in the system. The RAF-MEK-ERK signaling cascade is a classical biochemistry interaction that is fundamentally important to numerous cell signaling events. Understanding activation of this pathway is critical to being able to regulate cell signaling in cancer cells. This work will be of high interest to a number of fields because of the extreme importance of RAF activation in cancer.

Overall the conclusions of the study are supported by the results. The experiments are well-designed, and the interpretations are logical. There are some minor points where the language and reporting in the manuscript could be clarified, as noted below. The manuscript is well written and the figures are generally of good production quality. The biggest concern to this reviewer is there may be an over-extrapolation of these results to connections concerning RAS nanoclusters, RAF auto-inhibition, and involvement of the SHOC2-MRAS-PP1C complex. I feel that these results provide a convincing argument about RBD/CRD cooperativity, interactions with PS lipids, and importance of RAS concentration. However, the attempt to explain a full mechanism of RAF activation seems to be speculation and extrapolation based on other published works. I recommend the manuscript undergo minor revisions to address the points listed below, where the issue of over-extrapolation is elaborated on in the last point.

Points to address:
1. Some additional clarifications could be added to the introduction to aid readers who are not in this field:

(1) It was not immediately obvious to me that "14-3-3" is the name of a protein. Please add a (very) brief explanation. Even just add the word "protein" after it the first time it appears.

We have changed "14-3-3 dimer" to "dimeric 14-3-3 protein" when it first appears in the Introduction (page 2, line 44).

(2) Please briefly explain what are BRAF, KRAS, and CRAF, compared to just RAF and RAS.

We have now listed all three RAF isoforms in the Introduction as follows (page 2, line 26):

"RAF isoforms (A-, B-, and CRAF) share a conserved domain organization: an N-terminal regulatory region composed of the RAS-binding domain (RBD) and the cysteine-rich domain (CRD), and a C-terminal kinase domain (Fig. 1a)."

The three RAS isoforms are listed in the Result as follows (page 4, line 102):

"This widely used lipidation method preserves the biological function and structure of RAS and has been applied to all three RAS isoforms (H-, N-, and KRAS) and other membrane-anchored small GTPases[40–47]."

2. Evaluating how the RBD-CRD had measured KD values of 14 nM vs 5 nM for binding 2% and 20% PS membranes as a "evident dependence on PS" seemed strange in contrast to the previous comparison of 222 nM vs 163 nM as "depended little on upon PS content". Please adjust the language or clarify.

We appreciate the reviewer's detailed attention to our work. In the original submission, we evaluated the differences based on the mean values and 95% confidence intervals. The K_d values of RBD binding to RAS on 2% and 20% PS membranes largely overlapped due to wide confidence intervals, whereas those for RBD-CRD did not. During the revision, we performed additional experiments and now report mean values and standard deviations from three independent replicates. Incorporating these new data, the K_d of RBD was determined to be 270 ± 90 nM and 240 ± 20 nM on 2% and 20% PS membranes, respectively, showing little dependence on PS. The K_d for RBD-CRD was 25 ± 10 nM and 6 ± 3 nM on RAS-functionalized membranes containing 2% and 20% PS, respectively. The revised manuscript no longer directly compares the 2% and 20% PS data; instead, it focuses on comparing K_d values between lipid-free surfaces and 20% PS membranes. Therefore, we believe this issue has been fully addressed.

3. Was there a 0% PS control performed for any of these samples? Why were 2% and 20% PS concentrations chosen for the experiments? Please explain the lipid choice briefly.

We selected 20% PS as a physiologically relevant level, based on lipidomics showing ~26% PS in the erythrocyte inner leaflet plasma membrane (Lorent et al. *Nat. Chem. Biol.* 2020, 16, 644–652). 20% is the current technical upper limit for incorporating PS while maintaining high bilayer quality and efficient protein coupling; further optimization may increase this threshold. For a lower PS membrane, we selected 2% because complete removal of PS (0% PS) resulted in a higher frequency of membrane defects.

To provide this information, we have revised the manuscript to include the following sentence and cite the study by Lorent et al. (page 4, line 98):

"A 20% PS composition was used to closely approximate the physiological fraction (~26%) of PS in the inner leaflet of plasma membranes while maintaining high-quality bilayers[39]. "

4. The number of reported significant figures should be reduced for reported KD values in caption for Figure 1. Example: 222 +/- 88 should be changed to 220 +/- 90

We appreciate the reviewer's detailed attention to our work. We have revised our manuscript to reduce significant figures for all reported K_d values.

5. Figure 2a, middle panel: I think I'm seeing some low-intensity spots indicating protein binding in the -RAS condition. However, I can't rationalize how you can have less than one molecule showing up, unless this is a product of partial occupancy over the duration of a given exposure time? Please explain.

We agree with reviewer's interpretation. In the (–) RAS condition for RBD-CRD, the faint, low-intensity spots correspond to RBD-CRD-AZ647 recruited by PS interaction alone. These spots dwell much shorter than a single exposure (20 ms). The integrated signal is therefore proportionally reduced, which explains the appearance of *"less than one molecule."*

To explain this observation with an appropriate context, the manuscript has been revised to include the following text in the Results (page 7, line 164):

"In line with our ensemble binding measurements, clearly spatially resolved membrane recruitment events required the interaction with RAS (Fig. 2a). Interestingly, we observed highly transient interactions of RBD-CRD (appearing as faint, diffusive traces) on RAS-free 20% PS membranes. Although those particles could not be localized, manual intensity analysis estimated their dwell times on the order of several milliseconds (Supplementary Fig. 12). Importantly, these short-lived interactions between RBD-CRD and lipids do not result in productive RAS engagement, as k_{on} values for both RBD and RBD-CRD were unaffected by PS concentration (Fig. 2b)."

6. It is odd that the experiment wasn't successful for the 4XGGGGGS linker construct, but it was successful for the 4XGGGGGS/T178A construct. Can the authors please provide their thoughts on this? What part of the experiment was unsuccessful? Could the protein not be expressed? Perhaps this doesn't need to be explained in the manuscript, but I think it is appropriate for discussion during the review.

In the original manuscript, we were unable to express the RBD-CRD(4×GGGGGS) construct despite multiple attempts. The open reading frame of the plasmid had been verified by Sanger sequencing, so the lack of expression was puzzling. During the revision, we performed whole-plasmid sequencing and found an unexpected insertion that had not been detected by earlier Sanger sequencing. After re-cloning the plasmid, we were able to purify RBD-CRD(4×GGGGGS). The RBD-CRD(4×GGGGGS/T178A) construct was used out of necessity because we were initially unable to express the RBD-CRD(4×GGGGGS) construct. Now that the RBD-CRD(4×GGGGGS) construct has been successfully expressed, we have replaced the RBD-CRD(4×GGGGGS/T178A) data with RBD-CRD(4×GGGGGS) in Figure 4. The manuscript has been revised to reflect this change and provide adequate description as follows (page 12, line 288):

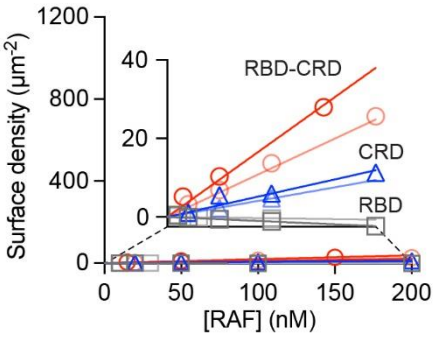
"The extended linker constructs showed a greater reduction in membrane binding (cyan, Fig. 4b). Protein quality analyses (Supplementary Fig. 3–5) and circular dichroism (Supplementary Fig. 24) confirmed that all RBD-CRD linker variants were properly purified and folded. The reduced membrane binding observed for these constructs is therefore attributed to perturbations in the relative positioning between the RBD and CRD caused by the increased linker flexibility and length."

The 4×GGGGGS construct occasionally exhibited a minor residual signal, about 5% higher than others, in the normalized desorption curve (Fig. 4d). The absolute residual intensities were comparable across constructs, suggesting that its lower equilibrium binding intensity may have caused the apparent residual fraction after normalization. The dissociation rate of the 4×GGGGGS construct was nonetheless faster than that of the other constructs.

Additionally, we found that RBD-CRD(4×GGGGGS) exhibits relatively fast equilibrium kinetics, making it difficult to reliably compare relative association rates using ensemble association assays. Therefore, we quantified k_{on} using single-molecule recruitment assays for the WT RBD-CRD, the L136A/T178A mutant, and the two linker variants. All k_{on} values remained unchanged, consistent with our previous conclusions. The manuscript has been revised to include this new data in Figure 4c.

7. The statement in the discussion "Consistent with this, we observed stronger binding of RBD-CRD on RAS-free membranes" is unclear. After a few minutes of consideration, I am now thinking that perhaps you mean that RBD-CRD has stronger binding on RAS-free membranes compared to RBD alone or CRD alone? When I originally read this sentence, I was interpreting it as RBD-CRD has stronger binding on RAS-free membranes, compared to membranes containing RAS. Please revise for clarity.

We now recognize that the original sentence could be confusing. Our intended meaning was that on RAS-free membranes, RBD-CRD exhibits stronger binding than CRD alone. We have demonstrated this behavior in a separate study (BioRxiv: https://www.biorxiv.org/content/10.1101/2025.08.03.668294v1).



Revision Figure 4. CRAF RBD-CRD exhibits higher binding to RAS-free membranes containing 20% PS compared to RBD.

However, we now recognize that the present study does not include sufficient data to support this conclusion without referencing our unpublished work. Therefore, we have removed the corresponding sentence. The revised text with the deletion now reads as follows (page 15, line 380):

"Previous computational and experimental studies have shown that RBD induces local anionic lipid enrichment independently of RAS, thereby enhancing the overall membrane affinity of RBD-CRD[60]. The RBD-induced local enrichment of anionic lipids may support lateral rebinding by slowing the dissociation of the transient RAS-free RBD-CRD:PS intermediate."

8. To improve clarity, it would be helpful to reference figure numbers throughout the discussion.

We have included figure numbers throughout the discussion.

9. This point is in regards to the statement in the discussion: "… whereby molecules that remain on the membrane for extended periods – such as those within RAS nanoclusters – are disproportionately more likely to become activated." Based on the evidence presented here, I don't see how a RAS nanocluster would retain RAF any more than a high concentration of monomeric RAS. I'm seeing how this is related to concentration, but not necessarily how it's related to nanoclusters. This needs to be made clearer. Perhaps making it explicit that clustering is creating a high local concentration, and RAF will be retained due to this high local concentration. This is distinct, for example, from requiring a patch of RAS that is a certain diameter or particular shape.

The original intent of the discussion on RAS nanoclusters was to propose a hypothesis that a high local concentration of RAS within nanoclusters may extend RAF membrane residence. To reflect the reviewer's feedback, the description of RAS nanoclusters has been removed from the Abstract and Results. Additionally, we have substantially revised the Discussion section to present this concept briefly as an unproven hypothesis, as follows (page 16, line 409):

"It is worth mentioning that the average density of RAS on the plasma membrane is in the range of 30–150 RAS molecules per μm^2 [67]. RAS is not uniformly distributed; instead, it forms dynamically exchanging nanoclusters that serve as the primary sites for recruitment and activation of RAF[32]. The radius of a nanocluster is estimated to be 6–12 nm, with a local surface density of ~4000 to ~16,000 RAS per μm^2 [32,33,67]. Such high local RAS densities within nanoclusters may facilitate RAF activation by extending its membrane residence time through lateral rebinding, although this hypothesis remains to be experimentally validated."

Additionally, we have removed detailed descriptions of the RAF activation mechanism involving RAS nanoclusters. We also revised the manuscript to explicitly state the need to use the autoinhibited full-length RAF protein in future studies to enable a more comprehensive mechanistic investigation, as follows (page 17, line 418):

"To investigate how membrane-binding kinetics influence activation outcomes under more physiologically relevant conditions, it will be necessary to use the autoinhibited full-length RAF:14-3-3:MEK complex. These advancements will enable us to rigorously recapitulate the sequential steps of RAF activation and elucidate the underlying molecular mechanisms and kinetics with high resolution."