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Cooperative regulation of C1-domain membrane recruitment polarizes atypical Protein Kinase C

Kimberly Jones, Michael Drummond, Rhiannon Penkert, and Kenneth Prehoda

Corresponding Author(s): Kenneth Prehoda, University of Oregon

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January 31, 2022

Re: JCB manuscript #202112143

Prof. Kenneth E Prehoda
University of Oregon
Institute of Molecular Biology
1229 University of Oregon
Eugene, OR 97403

Dear Prof. Prehoda,

Thank you for submitting your manuscript entitled "Cooperative regulation of C1-domain membrane recruitment polarizes atypical Protein Kinase C". Your manuscript has been assessed by expert reviewers, whose comments are appended below. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

As you will see, all three reviewers found the work of interest, particularly as it contrasts with earlier reports. However, all have suggestions for solidifying your major claims, either by adding new data or toning down some of the conclusions. We'd like to see a revision that addresses all of the comments in some way, but particularly suggest focus on: 1) Experimentally clarifying whether the polybasic vs C1 domain requirements for aPKC localization reflect differences in the tissue examined (Reviewer 1 point 1 and Reviewer 3 point 5)), 2) quantification of some key points (e.g. HA-aPKC fusions with better control over expression levels and polarity quantification 3) Firming up effectiveness of RNAi (Reviewer 2). Some of the more extensive future work suggested by Reviewer 2 could be addressed in the Discussion.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

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If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Mark Peifer
Monitoring Editor
Journal of Cell Biology

Tim Fessenden
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

How aPKC localizes at the cell cortex in a polar manner is a question of interest for the readership of JCB. A recent JCB paper from the Hong lab (Dong et al, 2020) proposes that Drosophila aPKC can directly interact with phospholipids via a polybasic/pseudo-substrate (PS) domain but that this interaction is auto-inhibited (presumably via an intramolecular interaction between the kinase domain and its pseudo-substrate) unless binding of Par6 to aPKC allosterically relieves this auto-inhibition.

Here, the authors provide experimental evidence for a slightly different scenario. The authors show that the C1 domain of Drosophila aPKC interacts with a broad array of phospholipids in an in vitro GUV pellet assay. Additionally, a protein fragment of aPKC containing this domain but lacking the catalytic domain localizes at the cell cortex of neuroblasts (NBs). Importantly, deletion of the C1 domain in the context of full-length aPKC strongly reduces cortical localization in NBs. This indicates that the C1 domain is required for the localization of aPKC at the cortex of NBs and that aPKC can directly interact with the membrane via its C1 domain. However, this interaction appears to be restricted to the apical domain of mitotic NBs. Since Par3 and Par6-Cdc42 localize at the apical cortex of dividing NBs, it is conceivable that membrane binding is somehow auto-inhibited and that the binding of Par3 and/or Par6-Cdc42 to aPKC relieves this inhibition.

As indicated above, Dong et al (2020) showed that aPKC can directly interact with phospholipids via a polybasic/pseudo-substrate (PS) domain. Here, the authors show that mutating the polybasic domain in aPKC AADAA did not disrupt the polar distribution of aPKC in mitotic NBs. This suggest that the polybasic region is largely dispensable for membrane binding in NBs. This piece of data appears to contradict the observation that mutating the polybasic region, in aPKC KR8Q, disrupts membrane binding in epithelia in both embryos and larvae (Dong et al. JCB, 2020). Thus, the role of the polybasic region in aPKC localization is controversial. It would be important to resolve this discrepancy by testing whether this difference in localization results from molecular differences in mutant aPKC or from differences in localization assay. Specifically, the authors could test whether aPKC KR8Q localize at the cortex in neuroblasts and whether aPKC AADAA is cytoplasmic in embryonic and larval epithelia. If aPKC AADAA localizes to the cortex in epithelia but not in NBs, could the authors test whether the epithelium-specific expression of Crumbs contribute to this difference in localization?

The authors further show that a kinase-dead version of aPKC, K293W, is cortical in NBs and that its localization at the cortex did not strictly depend on Cdc42 and Par6. This contrasts with wild-type aPKC. This observation is consistent with a direct Par3- and Cdc42-independent interaction that is otherwise inhibited by the catalytic domain. Whether and how the K293W mutation might disrupts auto-inhibition remains unclear. Nevertheless, this observation suggests a model whereby Par3 and Cdc42 might cooperate to relieve the auto-inhibitory interaction between the kinase domain and the PS-C1 domains. This is an interesting model. It is distinct from the one proposed by the Hong lab (JCB 2020). One aspect of the model remains, however, speculative,

i.e. the notion of cooperative regulation (title) was not addressed experimentally. Since addressing cooperativity may go well beyond the scope of the current study, I would suggest to tone-down the title/abstract of the paper (but discussion and Fig 5F are fine).

Minor points

1. The C1 domain of aPKC did not detectably localize to the cell cortex at interphase (Fig 4c). Rather, it seems to be largely nuclear. Is the C1 domain targeted by mitotic kinases? Is phosphorylation of the C1 domain important for aPKC localization at the cell cortex? Is the cortical localization of PB1-C1 also restricted to mitotic cells? And what about the localization of the C1 domain in epithelia: does it accumulate at the cortex in a non-polar manner?

2. HA- aPKC appeared to localize in both nucleus and cytoplasm at interphase (Fig 1d). Nuclear accumulation can also be seen for aPKC K293W and D388A at interphase (Fig 1d). Please comment.

3. line 95: please discuss how a difference in ATP binding between the D388A and K293W mutations might relate to Mira polarization (Fig 1c). Could it be that the binding of ATP in the catalytic domain of aPKC D388A modulates the intramolecular auto-inhibitory interaction between the kinase domain and the PS-C1 region?

4. The authors conclude that aPKC K293W does not rely on Cdc42 or Baz for cortical targeting (line 120). This seems to be a strong statement given that aPKC K293W is more cytoplasmic in Cdc42 and baz RNAi NBs (Fig 3a,c) than in control NBs (Fig 1c).

5. Could the authors discuss whether membrane binding is sufficient for the activation of aPKC-CAAX? or does aPKC-CAAX also require its auto-inhibition to be relieved by Par3 and/or Cdc42?

Reviewer #2 (Comments to the Authors (Required)):

A key step in the polarization of many cell types, including Drosophila neuroblasts, is the asymmetric localization and activation of the polarity kinase aPKC. While localized loading and activation of aPKC has previously been shown to depend on its association with other PAR proteins Baz/Par6/Cdc42, how membrane loading is gated is unclear. It is commonly assumed that aPKC is anchored via its association with these molecules, but this has never really been tested properly. This is where the manuscript by Jones et al comes in. Jones et al report on a novel role for the C1 domain in aPKC in membrane targeting, suggesting it plays a somewhat similar role to C1-type domains in other PKCs, which regulate membrane targeting in response to upstream signals. The key data here is that the C1 domain of aPKC constitutively binds the plasma membrane both in vivo and in vitro. They further show that a C1 mutant form of aPKC fails to bind the membrane, suggesting that the C1 is a key membrane determinant. Because inactivation of aPKC kinase activity also leads to constitute membrane targeting (shown here as well as by several others previously), the authors propose a model in which the kinase domain occludes the C1 domain until the enzyme associates with other members of the PAR complex, which would then displace the C1 domain and allow membrane association, arguing that aPKC inhibition may mimic this transition. While the observations regarding the C1 domain are nicely demonstrated, the paper does not explore how this regulation occurs and thus their cooperative model, while potentially attractive, remains largely speculation at this point.

Specific Comments

- Using two kinase inactivating mutations, the authors show in Figures 1-2 that inhibition of aPKC kinase activity is sufficient to dis-regulate membrane recruitment leading to uniform membrane association and loss of polarity. Moreover, loss of kinase activity causes reduction of PAR-6 polarity, but leaves Baz relatively intact. These experiments are nice, but they largely recapitulate prior work in neuroblasts and C. elegans embryos (Hannaford al, Rodriguez et al).

- The key difference here is the observation that aPKC(kd) mutants constitutively bind the plasma membrane independently of Baz/Cdc42 (Figure 3) which then supports the idea that the C1 domain is driving localization in this context. This is somewhat different from the C. elegans data in which aPKC(inhibited or ts) becomes independent of Par3 but dependent of Cdc42 (Rodriguez et al). How confident are the authors that they have sufficiently depleted Cdc42 to claim that localization is independent of Cdc42? While WT aPKC localization appears affected and this is rescued by the K293W mutation, Mira appears asymmetric in Figure 3A suggesting some level of Cdc42 may still be active in these cells. Also, without the control RNAi condition, it is difficult to determine the effect of Cdc42(RNAi) on K293W membrane binding. The Baz(RNAi) data suggest somewhat incomplete RNAi as aPKC(WT) is still clearly detectable (albeit reduced) at the apical membrane and the residual Mira I can see looks basal in the image provided.

- In Figure 1, HA and pan-aPKC antibodies are used. However, without a sense of relative expression levels, it is difficult to interpret these data. For example, Figure 1C, D388A, but not K293W, shows uniform "aPKC". This could either be a dominant effect on endogenous aPKC or that the D388A mutant is expressed at higher levels relative to endogenous and hence

dominates the observed signal.

- In Figure 4, how do the authors interpret the failure of the PB1 domain to bind Par6 at the apical membrane? The PB1 has been shown to enable formation of a stable aPKC-Par6 complex and so one might have expected a properly structured PB1 domain to interact with Par6 and hence be able to go apical. Are PB1 constructs not able to bind Par6? What is going on here?

- In Figure 5 is it possible that the C1 deletion is destabilizing full length aPKC? For example, the authors have shown previously that Par6/aPKC proteins can be destabilised if they cannot bind to their partner proteins. Can the authors be sure that the targeting defect of the C1 deletion is due to the lack of C1-mediated membrane binding as opposed to defective interactions with other proteins? For example, do C1 mutants still interact and bind to Par6?

- The authors propose an interesting model that postulates a C1-inhibited core configuration and speculate that mutations / inhibition of the active site somehow open this up, but these ideas are not tested. In fact I was somewhat surprised that mutation of the pseudosubstrate, which the authors have previously shown cooperates with the C1 to inhibit the kinase domain, does not release the C1 domain to trigger membrane association. Thus, their model would seem to suggest that only very specific states of the kinase domain are sufficient to expose the C1. If it is structural, one ought to be able to release the C1 via a variety of mutations that are not directly linked to activity. The new Alphafold structures could be a reasonable starting point for such an investigation. On a related point, can the authors rule out that it is not kinase activity that is required for regulating membrane association and/or C1 exposure?

Minor points:

- Figure 1E and Figure 3B seem not to match up. In Figure 1E, K293W apical:cytoplasm = 1.0, so I believe they must be plotting polarity not apical:cytoplasm. Figure 4E and 5E appears to use the same WT dataset and strongly suggests this is polarity not M:C (the re-use of this dataset should probably be noted in the legend). Would be nice to see the M:C ratios.

- I found the manuscript difficult to follow as a number of Figure notations in the text did not seem to match the corresponding figure and some language was a bit confusing.

Line 64 - Figure 1B cited to describe aPKC WT rescue experiments, but this is not shown (and I couldn't find it).

Line 70/71 - Figure 1A cited for experiment, but this is a schematic

Line 72 - Figures 1B-F cited to show cells exhibiting uniform Mira, but only B and only shows one example.

Line 155 - Figure 4B-E is probably Figure 5?

Line 94 - expression of aPKC K293W "restored Mira polarity" - this is strange language as this experiment is done in a WT background. So should be "did not impact" Mira polarity. Could this be a relative expression issue (see point above)?

- The authors show generic membrane binding via GUVs. Is there a reason they didn't address PIPs as I would have thought the lipids tested would be rather generic to non-PM membranes.

- While not essential, it would have been nice to see more discussion placing the work in the broader context, particularly around prior works that have addressed this question but come up with different answers. It may well be that the finding are context-specific, but some discussion of how to potentially reconcile the findings here with other works would be helpful to the reader.

Reviewer #3 (Comments to the Authors (Required)):

In this manuscript, Jones et al address the question of how aPKC is recruited to the plasma membrane and show that this does not depend in neuroblasts on the polybasic domain as previously reported, but on the adjacent C1 domain, which is both necessary and sufficient for plasma membrane recruitment. They also show that the availability of the C1 domain to bind membrane lipids depends on kinase activation. This work solves an important question about how this key polarity kinase is localised and regulated and is therefore suitable in principle for publication in JCB. However, there are a number of confusing issues in the manuscript that need to be addressed.

1) One of the more striking results is that the kinase dead form of aPKC (aPKC D388A) acts as a dominant negative and inhibits the activity of the endogenous wild-type aPKC, as shown by the uniform cortical localisation of Miranda (Fig 1C). This result is not discussed or explained, leaving the reader confused. One possibility is that aPKC D388A sequesters aPKC activators like Par-6, Baz and Cdc42 from endogenous aPKC and prevents its activation. This seems unlikely, however, because endogenous aPKC is still localised normally and its localisation depends on these factors. Do the authors have a possible explanation for this result?

2) The kinase dead aPKC mutants are described as "localizing to the entire cortex", but their distributions in Figures 1C and 2A look more like apical and basal crescents with an equatorial gap. A plot of the signal intensities around the circumference of several neuroblasts would help resolve this question.

3) What are the expression levels of the HA-tagged aPKC constructs compared to endogenous aPKC? It looks as if aPKC K293W is expressed at lower levels than D388A as the basal signal is barely detectable in the aPKC antibody staining for the former (Figure 2), whereas the HA-staining for D338A looks the same as the anti-aPKC staining. Could differences in the levels of expression account for the different effects on endogenous aPKC activity?

4) The membrane localisation of the kinase dead aPKCs in interphase is not very clear. Both K293W and D388A show significant nuclear signal, and the membrane-associated signal is mainly basal and could be in the adjacent GMCs.

5) This study reaches a different conclusion from Dong et al (2020) who claimed that the pseudosubstrate domain was responsible for aPKC's membrane association. It would be helpful to have some discussion about the reason for this discrepancy. Is this because aPKC localisation is different in epithelial cells and neuroblasts?

Minor points:
1) "As shown in Figure 4B, D-E, aPKC PB1-C1 (i.e. ΔKD) was uniformly localized in NSCs". aPKC PB1-C1 is uniformly localised to the plasma membrane, which is not the same as uniformly localised.

2) "Interestingly, Mira localization was also disrupted in NSCs expressing aPKC ΔC1 suggesting that the C1 also plays a role in regulating aPKC's protein kinase activity in NSCs." It would be clearer to state that Miranda is cytoplasmic, indicating that aPKC ΔC1 is active.

Response to reviewers JCB 202112143

We would like to thank the reviewers for their thoughtful comments on our manuscript. We have significantly revised the manuscript including the results of several new experiments. The changes are summarized directly below, followed by detailed responses to individual reviewer comments. We hope that you agree that the revised manuscript is suitable for publication in *JCB.*

With respect to the three key points that were noted in the review summary,

1) *Experimentally clarifying whether the polybasic vs C1 domain requirements for aPKC localization reflect differences in the tissue examined (Reviewer 1 point 1 and Reviewer 3 point 5))*

We added an analysis of the "AADAA" and C1 domain localization in an epithelial tissue

2) q*uantification of some key points (e.g. HA-aPKC fusions with better control over expression levels and polarity quantification*

We improved the polarity quantification and more carefully state our reasoning when expression level differences may be important. For example, we observed a difference in Miranda localization in NSCs expressing aPKC D388A or K293W (kinase inactivating mutations). This difference correlates with an effect on total aPKC localization (both endogenous and UAS-driven variant). In the revised manuscript we note:

> We do not know the origin of the differential effects of aPKC K293W and aPKC D388A on Mira localization, but it may arise from differences in the amounts of the two proteins and how endogenous aPKC is affected.

We used this approach because the difference in Miranda localization isn't an important observation for our conclusions and we've also found that measurements compare expression levels (e.g. westerns) in this class of experiments are unreliable.

3) *Firming up effectiveness of RNAi (Reviewer 2).*

We evaluated the effectiveness of *baz* and *cdc42* RNAi based on the reported phenotype (the extent to which WT aPKC localization is disrupted). Our conclusions are based on whether or not we can detect a difference between WT and K293W localization in NSCs expressing the relevant RNAi. Our results suggest that the *baz* RNAi is not complete as WT aPKC still has a detectable membrane enrichment. Nevertheless, we are able to detect a relatively increased enrichment in aPKC 293W. However, we added the following in the revision to more carefully and correctly state what can be inferred from the data:

> We also examined the localization of the aPKC K293W variant in NSCs expressing Baz RNAi. In this context, less aPKC is recruited to the apical membrane and Mira becomes depolarized as previously reported. However, aPKC K293W's remained highly enriched on the membrane (i.e., more than WT aPKC) when Baz was reduced (Figure 3C-D). While we cannot completely exclude a role for Cdc42 and Baz in recruiting aPKC K293W to the membrane, we conclude that aPKC K293W is targeted to the membrane significantly more than WT aPKC in metaphase NSCs with reduced Cdc42 or Baz function.

Overall, we have added significant new data to the manuscript:

- An analysis of the role of the aPKC PB1 domain in membrane recruitment using a full domain deletion (ΔPB1) and a point mutation (D77A) that disrupts aPKC's interaction with Par-6. These results lead us to conclude that the PB1 is required to regulate membrane targeting and the interaction with Par-6 is required to activate targeting. Together with our results on the role of the kinase domain in regulating membrane association and previous work on Par-3's role, we believe these results provide strong support for a cooperative activation model.
- An analysis of the aPKC pseudosubstrate variant "AADAA" and the isolated C1 domain's localization in an epithelial tissue. We found the localization largely recapitulates our results using neural stem cells.

Additionally, we more carefully explain how our results compare to Dong et al. (2019). While our results aren't consistent with an absolute requirement of the aPKC pseudosubstrate region for membrane recruitment, we note that we do detect a small reduction in membrane recruitment in our assays when the pseudosubstrate is inactivated.

Reviewer #1
How aPKC localizes at the cell cortex in a polar manner is a question of interest for the readership of JCB. A recent JCB paper from the Hong lab (Dong et al, 2020) proposes that Drosophila aPKC can directly interact with phospholipids via a polybasic/pseudo-substrate (PS) domain but that this interaction is auto-inhibited (presumably via an intramolecular interaction between the kinase domain and its pseudo-substrate) unless binding of Par6 to aPKC allosterically relieves this auto-inhibition.

Here, the authors provide experimental evidence for a slightly different scenario. The authors show that the C1 domain of Drosophila aPKC interacts with a broad array of phospholipids in an in vitro GUV pellet assay. Additionally, a protein fragment of aPKC containing this domain but lacking the catalytic domain localizes at the cell cortex of neuroblasts (NBs). Importantly, deletion of the C1 domain in the context of full-length aPKC strongly reduces cortical localization in NBs. This indicates that the C1 domain is required for the localization of aPKC at the cortex of NBs and that aPKC can directly interact with the membrane via its C1 domain. However, this interaction appears to be restricted to the apical domain of mitotic NBs. Since Par3 and Par6-Cdc42 localize at the apical cortex of dividing NBs, it is conceivable that membrane binding is somehow auto-inhibited and that the binding of Par3 and/or Par6-Cdc42 to aPKC relieves this inhibition.

As indicated above, Dong et al (2020) showed that aPKC can directly interact with phospholipids via a polybasic/pseudo-substrate (PS) domain. Here, the authors show that mutating the polybasic domain in aPKC AADAA did not disrupt the polar distribution of aPKC in mitotic NBs. This suggest that the polybasic region is largely dispensable for membrane binding in NBs. This piece of data appears to contradict the observation that mutating the polybasic region, in aPKC KR8Q, disrupts membrane binding in epithelia in both embryos and larvae (Dong et al. JCB, 2020). Thus, the role of the polybasic region in aPKC localization is controversial. It would be important to resolve this discrepancy by testing whether this difference in localization results from molecular differences in mutant aPKC or from differences in localization assay. Specifically, the authors could test whether aPKC KR8Q localize at the cortex in neuroblasts and whether aPKC AADAA is cytoplasmic in embryonic and larval epithelia. If

aPKC AADAA localizes to the cortex in epithelia but not in NBs, could the authors test whether the epithelium-specific expression of Crumbs contribute to this difference in localization?

As the reviewer notes, Dong et al reported that the aPKC PS domain is required for membrane localization in epithelial cells. We note that this conclusion was based on unquantified data and that they did not present any data showing that the PS is sufficient for localization, even in their predominant assay system, cultured HEK293 cells. Our data indicate that the PS is not required for membrane localization or polarity of aPKC in NSCs, but we more carefully note in the revision that we did detect a decrease in localization compared to WT. We expressed aPKC AADAA (and also aPKC C1) in the epithelium of the inner proliferation center and obtained similar results to what we found in NSCs– that AADAA is slightly less membrane enriched than WT (revised Figure 5F-H).

The authors further show that a kinase-dead version of aPKC, K293W, is cortical in NBs and that its localization at the cortex did not strictly depend on Cdc42 and Par6. This contrasts with wild-type aPKC. This observation is consistent with a direct Par3- and Cdc42-independent interaction that is otherwise inhibited by the catalytic domain. Whether and how the K293W mutation might disrupts auto-inhibition remains unclear. Nevertheless, this observation suggests a model whereby Par3 and Cdc42 might cooperate to relieve the auto-inhibitory interaction between the kinase domain and the PS-C1 domains. This is an interesting model. It is distinct from the one proposed by the Hong lab (JCB 2020). One aspect of the model remains, however, speculative, i.e. the notion of cooperative regulation (title) was not addressed experimentally. Since addressing cooperativity may go well beyond the scope of the current study, I would suggest to tone-down the title/abstract of the paper (but discussion and Fig 5F are fine).

To address the issue of cooperativity more directly, we created two new aPKC variants – aPKC ΔPB1 and aPKC D77A – the first completely removes the domain that interacts with Par-6 while the second introduces a point mutation in that domain to disrupt the interaction with Par-6. We examined the localization of these constructs in mitotic NSCs and found that aPKC ΔPB1 is membrane enriched but unpolarized whereas aPKC D77A is cytoplasmic. These results indicate that the PB1 is required to repress aPKC's membrane binding the PB1's binding to Par-6 is required for polarized membrane binding. Together with our previous results, we more clearly demonstrate that the interactions of multiple domains, including the kinase and PB1, are required to regulate aPKC's membrane association and polarity.

Minor points
1. The C1 domain of aPKC did not detectably localize to the cell cortex at interphase (Fig 4c). Rather, it seems to be largely nuclear. Is the C1 domain targeted by mitotic kinases? Is phosphorylation of the C1 domain important for aPKC localization at the cell cortex? Is the cortical localization of PB1-C1 also restricted to mitotic cells? And what about the localization of the C1 domain in epithelia: does it accumulate at the cortex in a non-polar manner?

The reviewer raises several important points that we did not clearly explain in the initial submission. First, the C1 domain is clearly mostly nuclear in interphase, consistent with previous literature reporting the presence of a nuclear localization signal in this region. In the revised manuscript, we cite these reports and and more clearly state that our conclusion derives from the relative amounts at the membrane and cytoplasm, not including the nuclear signal. We have also added an analysis of C1 expressed in the epithelium of the inner proliferation center

and found that it behaves similarly to in interphase NSCs – predominantly nuclear, but enriched at the membrane relative to the cytoplasm.

2. HA- aPKC appeared to localize in both nucleus and cytoplasm at interphase (Fig 1d). Nuclear accumulation can also be seen for aPKC K293W and D388A at interphase (Fig 1d). Please comment.

As described above, the nuclear localization of aPKC is an important point that we neglected to discuss in the submitted version. In the revised version we cite previous studies on a possible aPKC nuclear localization signal and clarify how the nuclear signal affected our analysis (we focused on the relative amounts at the membrane and cytoplasm)

3. line 95: please discuss how a difference in ATP binding between the D388A and K293W mutations might relate to Mira polarization (Fig 1c). Could it be that the binding of ATP in the catalytic domain of aPKC D388A modulates the intramolecular auto-inhibitory interaction between the kinase domain and the PS-C1 region?

As the reviewer notes, the Miranda localization in NSCs expressing D388A or K293W aPKC is surprisingly different, with depolarized Miranda in D388A but polarized Miranda in K293W. We interpret the differential effect of Miranda not in terms of differences in catalytic activity of D388A or K293W, but in terms of their effect on endogenous aPKC (see aPKC column of Fig 1C). For unknown reasons, D388A may disrupt the localization of endogenous aPKC whereas K293W has less of an effect. While we mention this difference in the revised manuscript, we do not pursue the difference further as it is unclear if the difference is related to the subject of the manuscript.

4. The authors conclude that aPKC K293W does not rely on Cdc42 or Baz for cortical targeting (line 120). This seems to be a strong statement given that aPKC K293W is more cytoplasmic in Cdc42 and baz RNAi NBs (Fig 3a,c) than in control NBs (Fig 1c).

We measured mean cortical/cytoplasmic ratios of:
- 2.7 ± 0.8 for K293W in otherwise wild type NSCs (Figure 1D)
- 1.6 ± 0.3 for K293W in *cdc42* RNAi NSCs (Figure 3B)
- 2.0 ± 0.7 for K293W in *baz* RNAi NSCs (Figure 3D)

Although the difference is likely not statistically distinguishable, we revised the text to note that we cannot rule out some role for Cdc42 and Baz in recruiting aPKC K293W to the membrane based on these data.

5. Could the authors discuss whether membrane binding is sufficient for the activation of aPKC-CAAX? or does aPKC-CAAX also require its auto-inhibition to be relieved by Par3 and/or Cdc42?

The reviewer raises an interesting question but not one that we believe we can comment on without additional evidence.

Reviewer #2 (Comments to the Authors (Required)):
- The key difference here is the observation that aPKC(kd) mutants constitutively bind the plasma membrane independently of Baz/Cdc42 (Figure 3) which then supports the idea that the

C1 domain is driving localization in this context. This is somewhat different from the C. elegans data in which aPKC(inhibited or ts) becomes independent of Par3 but dependent of Cdc42 (Rodriguez et al). How confident are the authors that they have sufficiently depleted Cdc42 to claim that localization is independent of Cdc42? While WT aPKC localization appears affected and this is rescued by the K293W mutation, Mira appears asymmetric in Figure 3A suggesting some level of Cdc42 may still be active in these cells. Also, without the control RNAi condition, it is difficult to determine the effect of Cdc42(RNAi) on K293W membrane binding. The Baz(RNAi) data suggest somewhat incomplete RNAi as aPKC(WT) is still clearly detectable (albeit reduced) at the apical membrane and the residual Mira I can see looks basal in the image provided.

We have tempered the conclusion that aPKC K293W membrane targeting is completely independent of Cdc42 (and Baz) in the revised manuscript, instead more clearly stating that our measurements indicate targeting is less dependent on these proteins compared to WT.

- In Figure 1, HA and pan-aPKC antibodies are used. However, without a sense of relative expression levels, it is difficult to interpret these data. For example, Figure 1C, D388A, but not K293W, shows uniform "aPKC". This could either be a dominant effect on endogenous aPKC or that the D388A mutant is expressed at higher levels relative to endogenous and hence dominates the observed signal.

The reviewer is correct that we do not know the underlying reason for D388A and K293W's differential effects on Miranda. We have added a short explanation in the revised text clarifying this point.

- In Figure 4, how do the authors interpret the failure of the PB1 domain to bind Par6 at the apical membrane? The PB1 has been shown to enable formation of a stable aPKC-Par6 complex and so one might have expected a properly structured PB1 domain to interact with Par6 and hence be able to go apical. Are PB1 constructs not able to bind Par6? What is going on here?

In the absence of aPKC, Par-6 is not targeted to the NSC membrane (c.f. Rolls et al. *JCB* 2003). We believe it is difficult to predict whether or not the aPKC PB1 alone should be sufficient to induce Par-6 membrane targeting without knowing why Par-6 can't target on its own. Our data suggest that the aPKC PB1 domain isn't sufficient to induce Par-6 targeting, potentially because the C1 domain's interaction with membrane phospholipids is also required.

- In Figure 5 is it possible that the C1 deletion is destabilizing full length aPKC? For example, the authors have shown previously that Par6/aPKC proteins can be destabilised if they cannot bind to their partner proteins. Can the authors be sure that the targeting defect of the C1 deletion is due to the lack of C1-mediated membrane binding as opposed to defective interactions with other proteins? For example, do C1 mutants still interact and bind to Par6?

We believe aPKC ΔC1 is not destabilized as it influences the localization of Miranda in NSCs (Figure 5C). We also know the aPKC PB1 domain does not depend on the C1 domain for its structure because of evidence from previous structural studies (Hirano et al. *JBC* 2004).

- The authors propose an interesting model that postulates a C1-inhibited core configuration and speculate that mutations / inhibition of the active site somehow open this up, but these ideas are

not tested. In fact I was somewhat surprised that mutation of the pseudosubstrate, which the authors have previously shown cooperates with the C1 to inhibit the kinase domain, does not release the C1 domain to trigger membrane association. Thus, their model would seem to suggest that only very specific states of the kinase domain are sufficient to expose the C1. If it is structural, one ought to be able to release the C1 via a variety of mutations that are not directly linked to activity. The new Alphafold structures could be a reasonable starting point for such an investigation. On a related point, can the authors rule out that it is not kinase activity that is required for regulating membrane association and/or C1 exposure?

The reviewer raises some very interesting points that get to the heart of the mechanism we propose. In the revised manuscript we include additional data showing that deletion of the PB1 domain also induces membrane targeting, consistent with the reviewer's conclusion that "one ought to be able to release the C1 via a variety of mutations that are not directly linked to activity". We also note that we do see a reduction in polarity for aPKC AADAA compared to WT suggesting that the C1 is somewhat activated in this protein. This point is more carefully explained in the revised manuscript.

We agree with the reviewer that alpha fold structures could be a useful starting point for a more detailed analysis of the structural mechanism of C1 regulation. In the revised manuscript we include an alpha fold structure (Figure 7A) that suggests that the PB1 and C1 interact with one another and a discussion of how this interaction may influence the regulatory model.

As to whether aPKC's kinase activity is required for membrane targeting, we note that introduction of the activity of endogenous aPKC (Figure 1C) did not rescue the depolarized localization of aPKC D388A (Figure 1B).

Minor points:

- Figure 1E and Figure 3B seem not to match up. In Figure 1E, K293W apical:cytoplasm = 1.0, so I believe they must be plotting polarity not apical:cytoplasm. Figure 4E and 5E appears to use the same WT dataset and strongly suggests this is polarity not M:C (the re-use of this dataset should probably be noted in the legend). Would be nice to see the M:C ratios.
- I found the manuscript difficult to follow as a number of Figure notations in the text did not seem to match the corresponding figure and some language was a bit confusing.
Line 64 - Figure 1B cited to describe aPKC WT rescue experiments, but this is not shown (and I couldn't find it).
Line 70/71 - Figure 1A cited for experiment, but this is a schematic
Line 72 - Figures 1B-F cited to show cells exhibiting uniform Mira, but only B and only shows one example.
Line 155 - Figure 4B-E is probably Figure 5?

We apologize for the numerous inconsistencies present in the submitted version. We have corrected these errors in the revised manuscript.

Line 94 - expression of aPKC K293W "restored Mira polarity" - this is strange language as this experiment is done in a WT background. So should be "did not impact" Mira polarity. Could this be a relative expression issue (see point above)?

We agree with the reviewer that the wording was confusing in this section of the submitted manuscript and have clarified it in the revised version.

- The authors show generic membrane binding via GUVs. Is there a reason they didn't address PIPs as I would have thought the lipids tested would be rather generic to non-PM membranes.

We did not test PIPs because they are typically introduced at a small percentage into PC:PS vesicles and C1 already binds nearly completely to PC and PS especially PS.

- While not essential, it would have been nice to see more discussion placing the work in the broader context, particularly around prior works that have addressed this question but come up with different answers. It may well be that the finding are context-specific, but some discussion of how to potentially reconcile the findings here with other works would be helpful to the reader.

Unfortunately without more specific information we are unsure which works the reviewer is referring to. A key finding is that the aPKC kinase domain is directly involved in regulating aPKC membrane localization, which provides a potential explanation for previous results of experiments using perturbations of the catalytic domain. Our work also provides a potential explanation for why several gene products (i.e. Baz and Par-6) are required for aPKC membrane targeting. While our results are somewhat in conflict with those of Dong et al., specifically with respect to the role of the PS region in membrane targeting, we note that the only difference is that Dong et al. claim that the PS is absolutely required for targeting whereas we detect a small decrease in membrane association when the PS is inactivated. We discuss this difference more carefully in the revised manuscript.

Reviewer #3 (Comments to the Authors (Required)):
1) One of the more striking results is that the kinase dead form of aPKC (aPKC D388A) acts as a dominant negative and inhibits the activity of the endogenous wild-type aPKC, as shown by the uniform cortical localisation of Miranda (Fig 1C). This result is not discussed or explained, leaving the reader confused. One possibility is that aPKC D388A sequesters aPKC activators like Par-6, Baz and Cdc42 from endogenous aPKC and prevents its activation. This seems unlikely, however, because endogenous aPKC is still localised normally and its localisation depends on these factors. Do the authors have a possible explanation for this result?

The reviewer raises and important point about the dominant negative effect of aPKC D388A. We believe the reviewer's interpretation that aPKC D388A is likely correct because the anti-aPKC antibody shows depolarized aPKC for this variant but not for aPKC K293W. We are unsure what causes this difference but it could be simply a small difference in expression level. We added a discussion of this point to the revised manuscript.

2) The kinase dead aPKC mutants are described as "localizing to the entire cortex", but their distributions in Figures 1C and 2A look more like apical and basal crescents with an equatorial gap. A plot of the signal intensities around the circumference of several neuroblasts would help resolve this question.

We refined the language in the revised manuscript to make it more clear that our measurements are based on the apical and basal membrane near the poles and that the signal can often remain variable across the membrane for depolarized proteins (however, this is not the case for metaphase polarized proteins which are typically continuous membrane domains).

3) What are the expression levels of the HA-tagged aPKC constructs compared to endogenous aPKC? It looks as if aPKC K293W is expressed at lower levels than D388A as the basal signal is barely detectable in the aPKC antibody staining for the former (Figure 2), whereas the HA-staining for D338A looks the same as the anti-aPKC staining. Could differences in the levels of expression account for the different effects on endogenous aPKC activity?

We agree with the reviewers proposal that a likely explanation for the difference in “dominant negative” effects between the two proteins is a difference in expression level. We have noted this in the revised manuscript.

4) The membrane localisation of the kinase dead aPKCs in interphase is not very clear. Both K293W and D388A show significant nuclear signal, and the membrane-associated signal is mainly basal and could be in the adjacent GMCs.

We have revised the discussion of interphase aPKC localization to clarify that the K293W and D388A variants are clearly predominantly nuclear, probably because of a previously characterized nuclear localization sequence. We have cited the relevant literature and more clearly state that our conclusions regarding membrane enrichments in these cells are based on a comparison of membrane and cytoplasmic signals at sites distinct from progeny cell contacts (c.f. Figure 1F,G).

5) This study reaches a different conclusion from Dong et al (2020) who claimed that the pseudosubstrate domain was responsible for aPKC's membrane association. It would be helpful to have some discussion about the reason for this discrepancy. Is this because aPKC localisation is different in epithelial cells and neuroblasts?

We have addressed this important issue in several ways. First, we have more clearly explained the differences between our observations and Dong et al.’s. Second, we have examined the localization of the pseudosubstrate “AADAA” variant and the isolated C1 domain in an epithelial tissue.

Minor points:
1) "As shown in Figure 4B, D-E, aPKC PB1-C1 (i.e. ΔKD) was uniformly localized in NSCs". aPKC PB1-C1 is uniformly localised to the plasma membrane, which is not the same as uniformly localised.

We thank the reviewer for bringing the confusing language in this point and point 2 below to our attention and have corrected it in the revised manuscript.

2) "Interestingly, Mira localization was also disrupted in NSCs expressing aPKC ΔC1 suggesting that the C1 also plays a role in regulating aPKC's protein kinase activity in NSCs." It would be clearer to state that Miranda is cytoplasmic, indicating that aPKC ΔC1 is active.

See above.

April 21, 2023

Re: JCB manuscript #202112143R

Prof. Kenneth E Prehoda
University of Oregon
Institute of Molecular Biology
1229 University of Oregon
Eugene, OR 97403

Dear Prof. Prehoda,

Thank you for submitting your revised manuscript entitled "Cooperative regulation of C1-domain membrane recruitment polarizes atypical Protein Kinase C". The manuscript has been seen by the original reviewers whose full comments are appended below. While the reviewers continue to be overall positive about the work in terms of its suitability for JCB, some important issues remain.

Your revised version addresses many of the issues raised by the three reviewers, and two of the reviewers are now satisfied. However, we agree with Reviewer 2 that you have not effectively addressed some of their points, including some which we emphasized in our original decision letter. We would be open to one additional revision, addressing all of the major issues raised by Reviewer 2--many of these can be addressed by text changes, tempering conclusions, and discussing alternatives. However, the data presentation issues noted in point 5 by Reviewer 2 must also be addressed.

Our general policy is that papers are considered through only one revision cycle; however, given that the suggested changes are relatively minor we are open to one additional short round of revision. Please submit the final revision within one month, along with a cover letter that includes a point by point response to the remaining reviewer comments.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact me or the scientific editor listed below at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Mark Peifer
Monitoring Editor
Journal of Cell Biology

Tim Fessenden
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The authors have satisfactorily addressed my comments.

Reviewer #2 (Comments to the Authors (Required)):

The manuscript by Jones et al presents new and interesting evidence for a role for the C1 domain in membrane binding and they have now added some additional data to support the idea of a cooperative mechanism for regulating PM binding through inhibition of C1 membrane targeting. The PB1 data is a nice addition, though I would note that it is consistent with Dong et al (2020) who show that expression of the Par6 PB1 domain is sufficient to trigger relocalization of aPKCzeta to the PM - though I may have missed it, this data curiously isn't mentioned in this manuscript. Overall, the demonstration of a role for the C1 domain is interesting, but feel that the relatively strong claims made around dependence on Baz/Cdc42 and the contributions of the C1 vs PS domains, which the authors frame as drawing a clear distinction from prior results, are not well supported.

In terms of my prior concerns, several caveats remain:

(1) Interpretation of the Baz/cdc42 RNAi depletion data. The manuscript strongly argues for the C1 as a constitutive membrane targeting molecule and that inhibition of aPKC triggers this C1 targeting by preventing the normal restriction provided by cooperative interactions. The language remains too strong in my opinion. For example, the manuscript states: "Our results indicate that the uniform membrane localisation of aPKC with inactive kinase domains (K293W) is independent of both Baz and Cdc42." I don't believe the data support this statement. To make this claim one would expect no change for K293W {plus minus}cdc42 or {plus minus}Baz. But this is not what the authors show. They simply show that K293W is less affected by depletion than WT. To make the proper comparison, the reader is forced to eyeball the behaviour of K293W between Figures 1 and 3. Indeed in the rebuttal, the authors state that both cdc42 and baz reduce the efficiency of membrane targeting. Given that RNAi is unlikely to completely deplete these proteins, the data do not support the language used. One explanation is that these mutants retain some of the negative regulatory behaviour and thus membrane binding remains dependent, albeit to a lesser degree, on Baz and Cdc42? I don't think that this would dramatically undercut the main conclusion. As presented, I feel the current statements and misleading and will add to confusion in the literature, especially when these differences in dependencies found by different groups are not really discussed.

(2) I also still have issues with how the authors score uniform membrane binding. What I still do not understand is why they often observe bipolar HA staining. First there is often basal HA signal for WT HA::aPKC, though lower than apical. Moreover, for the conditions that lose specificity, localizations generally appear bipolar rather than uniform. This is inconsistent with a model of constitutive membrane targeting. All three referees noted this issue and I don't feel that the authors have provided a satisfactory explanation for this.

(3) Similarly, my concerns with interpreting the total aPKC stainings have not been addressed. The "total aPKC" is used to assess the effects on endogenous aPKC, but the staining will show the combined signal of ectopic and endogenous aPKC. Without knowing the relative contributions of the two, it is impossible to interpret these data. For example, if over expressed, the mutant forms of aPKC could dominate the "total" signal preventing any conclusions about the effects of the mutants on the endogenous protein. That said, while I think they need to strongly caveat the interpretations around this data, I don't think these caveats necessarily undermine the main finding that, consistent with several prior reports, blocking aPKC kinase activity leads to de-regulation of membrane localization. But a much stronger disclaimer is warranted - i.e. because total aPKC recognises both HA and endogenous aPKC and we do not know the relative concentrations of the fusions relative to each other or wild-type, we cannot draw firm conclusions with respect to differences in behaviour of the two mutations.

(4) The authors contrast the behaviour of the C1 with the pseudosubstrate (PS) domain with new experiments, but it is not really fair to compare a C1 deletion with an AADAA mutation. I note in Dong et al, the AADAA only compromises 4 of 8 polybasic residues and shows a less severe phenotype compared to the KR8Q or PS deletion constructs. As the authors have not tested a PS deletion or stronger charge mutants, it is entirely possible that they would see a similar result to Dong. While the effect of AADAA here does seem less severe than in Dong, I am not convinced that the PS is not playing a larger role than currently articulated in the manuscript. I would also note that while Dong et al did not show membrane localisation of PS alone in cells, the isolated PS domain was capable of binding PIP/PIP2 containing liposomes and I am not sure how the authors can claim that the data for membrane binding in Dong "was based on unquantified data" as I clearly see numerous quantifications in Figures 2, 3, 5, 6. The key data in this work vis a vis Dong et al is the failure of the PB1-PS construct to target the membrane, whereas C1 or PB1-PS-C1 both did. But the authors do not look at PS alone. Is it possible that the PB1 binds to the PS and inhibits lipid binding (there is precedent for interactions between PS and PB1 domains from other proteins in regulating aPKC - 10.1074/jbc.M115.676221)? Such a result would reconcile the two works - i.e. both domains contribute to some degree to membrane targeting. I concede that there may be differences between the relevant model systems leading to distinct results, but I don't think the experiments are clean enough to judge. At minimum a more thorough discussion of the similarities, differences, and caveats of the two works is necessary to avoid further muddying the field.

(5) I continue to be somewhat concerned about what appears to be sloppy data handling. There is unmentioned data reuse and what appear to be missing/inconsistent data points. I'm fine with showing data multiple times for reference/comparison purposes, but the authors should indicate where this is and is not the case. If it is not indicated it suggests the samples were retested in parallel with mutants, which is unlikely to be the case here. Where sample numbers don't match for measurements that would presumably be performed on the same datasets, it should be clear why. Either they analysed distinct datasets for the two measures for some reason or some NSCs were included in one measurement, but not in the other. To stress, I am not insinuating any malintent here, but I would suggest the authors carefully re-examine their datasets and be more clear about the origin of data (e.g. separate vs parallel experiments, data exclusion, etc.) Examples:

Figure 1D vs 1E: Are D and E taken from different datasets? I would have imagined these would be measured from the same set of samples, but the number of datapoints don't match up between D and E.

Figure 4C vs 4D: Again sample numbers don't always match (e.g. C1). It is also odd that the WT data for 4D is the same WT data for 1E, but the WT data for 4C doesn't match 1D. Looks like one data point may have been omitted for the WT in 4C?

Figure 5D and 5E: WT data are reproduced from Figures 1D and 1E, but not noted.

Figure 6C and 6D: WT data are reproduced again for comparison (again not noted), but 6C appears to truncate the topmost

data point (as occurs in 4C).

Reviewer #3 (Comments to the Authors (Required)):

This revised version of the manuscript contains considerable additional data, and has adequately addressed all of the concerns raised by the referees. I can therefore recommend that it be accepted.

We would like to sincerely thank reviewer 2 for their thorough comments on our revised manuscript. We appreciate the opportunity to incorporate their feedback to properly frame our arguments and importantly to correct any errors in the presentation.

Reviewer #2 (Comments to the Authors (Required)):

The manuscript by Jones et al presents new and interesting evidence for a role for the C1 domain in membrane binding and they have now added some additional data to support the idea of a cooperative mechanism for regulating PM binding through inhibition of C1 membrane targeting. The PB1 data is a nice addition, though I would note that it is consistent with Dong et al (2020) who show that expression of the Par6 PB1 domain is sufficient to trigger relocalization of aPKCzeta to the PM - though I may have missed it, this data curiously isn't mentioned in this manuscript.

We thank the reviewer for bringing these data to our attention and have added a reference to the Dong paper in the PB1 section of the paper.

(1) Interpretation of the Baz/cdc42 RNAi depletion data. The manuscript strongly argues for the C1 as a constitutive membrane targeting molecule and that inhibition of aPKC triggers this C1 targeting by preventing the normal restriction provided by cooperative interactions. The language remains too strong in my opinion. For example, the manuscript states: "Our results indicate that the uniform membrane localisation of aPKC with inactive kinase domains (K293W) is independent of both Baz and Cdc42." I don't believe the data support this statement. To make this claim one would expect no change for K293W {plus minus}cdc42 or {plus minus}Baz. But this is not what the authors show. They simply show that K293W is less affected by depletion than WT. To make the proper comparison, the reader is forced to eyeball the behaviour of K293W between Figures 1 and 3. Indeed in the rebuttal, the authors state that both cdc42 and baz reduce the efficiency of membrane targeting. Given that RNAi is unlikely to completely deplete these proteins, the data do not support the language used. One explanation is that these mutants retain some of the negative regulatory behaviour and thus membrane binding remains dependent, albeit to a lesser degree, on Baz and Cdc42? I don't think that this would dramatically undercut the main conclusion. As presented, I feel the current statements and misleading and will add to confusion in the literature, especially when these differences in dependencies found by different groups are not really discussed.

In the previous version of the manuscript we modified the language used to describe the *cdc42* and *baz* RNAi phenotypes in the revised but the reviewer is correct that we missed the sentence, "Our results indicate that the uniform membrane localisation of aPKC with inactive kinase domains (K293W) is independent of both Baz and Cdc42.” We apologize for the omission and have revised this sentence to state, “Our results indicate that the depolarized membrane localization of aPKC with inactive kinase domains (e.g., K293W) is at least partially independent of both Baz and Cdc42.”

(2) I also still have issues with how the authors score uniform membrane binding. What I still do not understand is why they often observe bipolar HA staining. First there is often basal HA signal for WT HA::aPKC, though lower than apical. Moreover, for the conditions that lose specificity, localizations generally appear bipolar rather than uniform. This is inconsistent with a model of constitutive membrane targeting. All three referees noted this issue and I don't feel that the authors have provided a satisfactory explanation for this.

We don't know why aPKC is somewhat unevenly distributed across the membrane but we note that this is a common effect (e.g. many images in Dong et al. show variable aPKC staining across the membrane). A possible source of the variability is the presence of structure in the membrane, and we mention this possibility in the current revision. Furthermore, since the key point is not whether aPKC is evenly distributed but whether or not it is apically polarized, we have clarified our language by changing "constitutive" (and "uniform") to "unpolarized but membrane bound".

(3) Similarly, my concerns with interpreting the total aPKC stainings have not been addressed. The "total aPKC" is used to assess the effects on endogenous aPKC, but the staining will show the combined signal of ectopic and endogenous aPKC. Without knowing the relative contributions of the two, it is impossible to interpret these data. For example, if over expressed, the mutant forms of aPKC could dominate the "total" signal preventing any conclusions about the effects of the mutants on the endogenous protein. That said, while I think they need to strongly caveat the interpretations around this data, I don't think these caveats necessarily undermine the main finding that, consistent with several prior reports, blocking aPKC kinase activity leads to de-regulation of membrane localization. But a much stronger disclaimer is warranted - i.e. because total aPKC recognises both HA and endogenous aPKC and we do not know the relative concentrations of the fusions relative to each other or wild-type, we cannot draw firm conclusions with respect to differences in behaviour of the two mutations.

The reviewer raises concerns about the signal arising from the anti-aPKC antibody and its use in assessing the effect on endogenous aPKC but we don't use this signal in the revised manuscript. Here is the relevant portion of the text (note that it relies on Mira localization and not the anti-aPKC signal):

"Interestingly, in cells expressing aPKC K293W Mira was basally polarized but in cells expressing aPKC D388A it was depolarized suggesting that aPKC D388A influences the localization or activity of endogenous aPKC (Figure 1C). We do not know the origin of the differential effects of aPKC K293W and aPKC D388A on Mira localization, but it may arise from differences in the amounts of the two proteins and how endogenous aPKC is affected."

We are unsure what the reviewer is referring to when they state, "a much stronger disclaimer is warranted".

(4) The authors contrast the behaviour of the C1 with the pseudosubstrate (PS) domain with new experiments, but it is not really fair to compare a C1 deletion with an AADAA mutation. I note in Dong et al, the AADAA only compromises 4 of 8 polybasic residues and shows a less severe phenotype compared to the KR8Q or PS deletion constructs. As the authors have not tested a PS deletion or stronger charge mutants, it is entirely possible that they would see a similar result to Dong. While the effect of AADAA here does seem less severe than in Dong, I am not convinced that the PS is not playing a larger role than currently articulated in the manuscript. I would also note that while Dong et al did not show membrane localisation of PS alone in cells, the isolated PS domain was capable of binding PIP/PIP2 containing liposomes and I am not sure how the authors can claim that the data for membrane binding in Dong "was based on unquantified data" as I clearly see numerous quantifications in Figures 2, 3, 5, 6. The key data in this work vis a vis Dong et al is the failure of the PB1-PS construct to target the membrane, whereas C1 or PB1-PS-C1 both did. But the authors do not look at PS alone. Is it

possible that the PB1 binds to the PS and inhibits lipid binding (there is precedent for interactions between PS and PB1 domains from other proteins in regulating aPKC - 10.1074/jbc.M115.676221)? Such a result would reconcile the two works - i.e. both domains contribute to some degree to membrane targeting. I concede that there may be differences between the relevant model systems leading to distinct results, but I don't think the experiments are clean enough to judge. At minimum a more thorough discussion of the similarities, differences, and caveats of the two works is necessary to avoid further muddying the field.

We agree with the reviewer that we observed an effect of AADAA that is “less severe than in Dong”. We also observed that the C1 domain is sufficient for membrane targeting. We agree with the reviewer that it remains possible that the PS is autoinhibited by a domain besides the kinase domain and we appreciate the reference noting such an example. We have revised the relevant text in the discussion to state:

“Our results suggest that the PS is not sufficient for membrane recruitment, but it remains possible that the PS is autoinhibited by other domains within aPKC besides the kinase domain. Consistent with this possibility, an interaction between the PS and a PB1 has been reported (Tsai et al., 2015).”

To clarify our comment in the previous reviewer response about the data in Dong et al. being unquantified, this was specifically in reference to their *epithelial* data.

(5) I continue to be somewhat concerned about what appears to be sloppy data handling. There is unmentioned data reuse and what appear to be missing/inconsistent data points. I'm fine with showing data multiple times for reference/comparison purposes, but the authors should indicate where this is and is not the case. If it is not indicated it suggests the samples were retested in parallel with mutants, which is unlikely to be the case here. Where sample numbers don't match for measurements that would presumably be performed on the same datasets, it should be clear why. Either they analysed distinct datasets for the two measures for some reason or some NSCs were included in one measurement, but not in the other. To stress, I am not insinuating any malintent here, but I would suggest the authors carefully re-examine their datasets and be more clear about the origin of data (e.g. separate vs parallel experiments, data exclusion, etc.)

Examples:

Figure 1D vs 1E: Are D and E taken from different datasets? I would have imagined these would be measured from the same set of samples, but the number of datapoints don't match up between D and E.

Figure 4C vs 4D: Again sample numbers don't always match (e.g. C1). It is also odd that the WT data for 4D is the same WT data for 1E, but the WT data for 4C doesn't match 1D. Looks like one data point may have been omitted for the WT in 4C?

Figure 5D and 5E: WT data are reproduced from Figures 1D and 1E, but not noted.

Figure 6C and 6D: WT data are reproduced again for comparison (again not noted), but 6C appears to truncate the topmost data point (as occurs in 4C).

We very much appreciate the reviewer finding and pointing out these errors. There is no excuse - we should have found them before submission. I replotted all of the data in the paper and confirmed that each plot correctly renders the data as recorded in the original analysis files. This led to differences in four panels - Figure 1D is the most different whereas 1E, 4D, and 6D contain one additional data point for WT (see below for explanations). While the corrected plots continue to support our conclusions, the errors were nevertheless serious and we are very thankful to the reviewer for bringing them to our attention and apologize for any inconvenience they caused.

- The data in figure 1D and 1E were plotted incorrectly. Our workflow includes a step where the data is copied from the original table where the measurements were recorded to a file that is specific for the plotting software we use and the data for these figures wasn't correct, likely due to a copy/paste error.
- In Figure 4D and 6D a point was missing from the WT dataset, likely due to a copy paste error.
- The figure legends have been revised to make it clear when the WT dataset is being reused.
- We took the opportunity to make the plots consistent so that apical/basal signal plots are for proteins with detectable membrane signal (i.e. apical to cytoplasmic ratio greater than one).

June 20, 2023

RE: JCB Manuscript #202112143RR

Prof. Kenneth E Prehoda
University of Oregon
Institute of Molecular Biology
1229 University of Oregon
Eugene, OR 97403

Dear Prof. Prehoda:

Thank you for submitting your revised manuscript entitled "Cooperative regulation of C1-domain membrane recruitment polarizes atypical Protein Kinase C".

We have sent your further revised manuscript to one of the Reviewers, who finds that their concerns, many of which had been raised during the initial review, have now been at least partially resolved. Based on the enthusiasm of the other two Reviewers we are ready to move forward. A final version must address the unresolved issue raised by Reviewer 2, concerning how localization measurements are normalized, by noting this choice in the discussion and noting how it may impact the conclusions reached. A final version must also discuss the new paper that has come out during revision mentioned by this reviewer.

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, http://jcb.rupress.org/submission-guidelines#revised. Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.

1) Text limits: Character count for Articles is < 40,000, not including spaces. Count includes abstract, introduction, results, discussion, and acknowledgments. Count does not include title page, figure legends, materials and methods, references, tables, or supplemental legends.

2) Figures limits: Articles may have up to 10 main figures and 5 supplemental figures/tables.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.
** Please include scale bars in Fig 4E and Fig 6E.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."
** please indicate n (biological replicates/technical replicates) in the figure legends for all plots.

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts. We also provide a report from SciScore and an associate score, which we encourage you to use as a means of evaluating and improving the methods section.
** Please describe purification of MBP-C1 purification.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible. Source Data files will be directly linked to specific figures in the published article.

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Sincerely,

Mark Peifer
Monitoring Editor
Journal of Cell Biology

Tim Fessenden
Scientific Editor
Journal of Cell Biology

Reviewer #2 (Comments to the Authors (Required)):

The primary finding of this paper is that the C1 domain of aPKC is sufficient for membrane localization and thus constitutes a membrane targeting mechanism. This is well documented. The authors have addressed most of my concerns regarding other claims and importantly resolved the data issues. However, they have not really attempted to assuage concerns around the strong statements regarding dependence on Baz/Cdc42 and the relative contributions of the C1 vs PS domains. I still do not see how the data in the paper support the title.

The authors have very modestly tempered their claims around dependence on CDC-42 to say "partially independent" which to me implies there is a pool that doesn't require CDC-42 as opposed to maybe simply being less dependent or able to bind at somewhat lower concentrations. I still feel that it is disingenuous to only show comparisons relative to wild-type RNAi, rather than comparisons to control RNAi, as the latter would clearly show that membrane localization remains dependent (at least to a substantial degree) on CDC-42/Baz. The authors did not respond to this point. Moreover, the title of the section still reads: "Kinase inactive aPKC may bind the NSC membrane independently of Cdc42 and Bazooka". I could easily using the same data to argue that kinase inactive aPKC remaiins sensitive to Cdc42 (albeit to a somewhat lesser degree than WT). The language "Cdc42 was absent" is rather strong given there is no sense of the magnitude of depletion. Perhaps I am being too pedantic here, but the data simply don't support the claims made.

They have added substantial caveats around the discussion of the psuedosubstrate vs C1 domain, which is a step in the right direction. I still feel the comparisons between the PS and C1 are not equivalent which makes it difficult to draw strong conclusions (point mutants vs deletions, expressed alone vs in a larger context). Seems to me the most likely outcome will be that the pseudosubstrate and C1 domain cooperate to bind the membrane. Indeed, in the past year since first submission there

is a paper that provides precisely such data (10.1016/j.jbc.2023.104847) suggesting that the Ps and C1 domains form an integrated membrane docking structure, which the authors may wish to address in their discussion.

Response to reviewers JCB 202112143

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We rewrote this section, including the section title, to address the issues raised by the reviewer. The revised section is copied below for convenience.

Kinase inactive aPKC membrane recruitment is less sensitive to the loss of Cdc42 and Bazooka

Membrane targeting of aPKC normally requires the activities of Baz and the small GTPase Cdc42 (Wodarz et al., 2000; Rolls et al., 2003; Atwood et al., 2007). We tested whether these upstream regulators are required for membrane localization of kinase inactive aPKC by examining the localization of aPKC K293W in NSCs expressing Baz or Cdc42 RNAi. We found that wild-type aPKC membrane enrichment was reduced in these contexts, as previously reported (Figure 3A-D) (Atwood et al., 2007; Rolls et al., 2003). We also observed a reduction of aPKC K293W on the membrane in NSCs expressing Cdc42 or Baz RNAi, but less so than for WT aPKC (Figure 3A-D), suggesting that aPKC K293W membrane recruitment is less sensitive to the loss of Cdc42 or Baz.

They have added substantial caveats around the discussion of the psuedosubstrate vs C1 domain, which is a step in the right direction. I still feel the comparisons between the PS and C1 are not equivalent which makes it difficult to draw strong conclusions (point mutants vs deletions, expressed alone vs in a larger context). Seems to me the most likely outcome will be that the pseudosubstrate and C1 domain cooperate to bind the membrane. Indeed, in the past year since first submission there is a paper that provides precisely such data (10.1016/j.jbc.2023.104847) suggesting that the Ps and C1 domains form an integrated membrane docking structure, which the authors may wish to address in their discussion.

We have added a citation to the paper referenced by the reviewer (line 250).

The PS could also cooperate with the C1 to mediate membrane binding and aPKC regulatory module localization in cultured cells supports this model (Cobbaut et al., 2023).

If it is ultimately found that the PS and C1 cooperate to mediate membrane binding, we feel our paper will have been an important contribution to that understanding (which did not include the contribution from the C1 before our work).