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Engineered Synaptic Tools Reveal Localized cAMP Signaling in Synapse Assembly

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Corresponding Author(s): Richard Sando, Vanderbilt University and Thomas Südhof, Stanford University School of Medicine

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October 18, 2021

Re: JCB manuscript #202109111

Dr. Richard Sando
Vanderbilt University
Pharmacology
465 21st Avenue, MRBIII room 7160A
Nashville, Tennessee 37240-7933

Dear Dr. Sando,

Thank you for submitting your manuscript entitled "Engineered Synaptic Tools Reveal Localized cAMP Signaling in Synapse Assembly". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

I am glad to report that the assessments of the reviewers are generally positive, but both reviewers have some comments that require your attention. Especially, concerning to me, I would like to call your attention to the comment of the second reviewer who has some reservations about the specificity of your phosphodiesterase-anti-homer nanobody and phosphodiesterase-homer fusion. As I considered this issue, I decided that I should also ask you to ensure that the specificity controls that you used in Figure 3 especially, for use of a very efficient enzyme did not have more widespread impact. My concern does not require more experimentation, but I think some modest modification of your summary to ensure that this is appreciated would be helpful to readers. I encourage you to resubmit and we expect to consider a revision at the editorial level. I would ask that you include a letter describing your responses to the various comments and in addition a PDF of the manuscript with changes highlighted.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Editorially, your study seems more suitable as a Tool as opposed to an Article, while the formatting is the same please prepare and submit your revised study as a Tool.

Please direct any editorial questions to the journal office.

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IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

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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.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

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 in this letter.

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

Sincerely,

Louis Reichardt, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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

The paper by Sando et al. is reports a new tool to reduce the concentration of cAMP locally at the synapse. By using this tool, the authors demonstrated the local cAMP signaling is required for the formation and maintenance of excitatory but not inhibitory synapses. Overall, this study is carefully and rigorously carried out and I do not see any major issues. It opens up new questions such as what is the upstream of cAMP and how it acts. I believe it has sufficient novelty for publication in JCB.

Minor issue.
Entorhinal-CA1 pathway should be correctly called temporoammonic pathway, not perforant pathway.

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

Synapse formation is a continuous process throughout life of animals. Multiple synaptic cell adhesion molecules (CAMs) take part in synapse establishment, organization, and plasticity. These CAMs are increasingly known to be critical for trans-synaptic organizations and synapse formation. But whether, and if yes, how CAMs transduce intracellular signals are much less understood. Recent studies from Sudhof lab reported that CAM-initiated cAMP-PKA signalling pathway directly regulates synapse formation (Jiang et al., 2021; Sando and Sudhof, 2021). cAMP signalling is involved in multifarious cellular processes, and its compartmentalized signalling likely determines the specificity of various cAMP signalling processes. Recent studies have documented compartmentalization of cAMP in cells (Bock et al., 2020; Zhang et al., 2020), but how to perturb cAMP signalling at specific subcellular compartments remains a challenge. In this manuscript, the authors developed a synaptic targeting strategy called "SynTAMs" by fusing phosphodiesterase or PKA inhibitor to Homer1, a well-known excitatory postsynaptic density (ePSD) marker. Importantly, overexpression of Homer1 alone does not cause obvious changes to synaptic development, thus these Homer1-fused "cargoes" can be specifically targeted to ePSD for specific modulation of cAMP signalling in PSD compartments. The authors have also used an alternative synaptic targeting approach by fusing phosphodiesterase or PKA inhibitor to nanobodies specifically targeting Homer1 (Dong et al., 2019). Together, the authors demonstrated that postsynaptic cAMP-PKA signalling is critical for the formation of excitatory synapses of hippocampal neurons in cultures and in vivo. The authors also showed that postsynaptic cAMP-PKA signalling is required for synaptic connectivity in hippocampal circuits.
In addition to modulating postsynaptic cAMP signalling, the method developed in this work can be readily adapted to modulate many other postsynaptic signalling processes. Although numerous attempts have been made in the past to specifically target molecular cargoes to PSD compartments, essentially all these methods suffer from perturbation of certain synaptic functions. The method reported in the current study appears to have largely avoided this problem (although this will need more experimental validations in future). As such, the study will be valuable to many colleagues in the field of synaptic biology.
This reviewer has some minor concerns/comments on the manuscript:
1. PDE7b with no targeting group: theoretically, PDE7b will convert all accessible cAMP to AMP, and thus is expected to cause broader/more serious impacts than the ePSD targeted group. (1) Although the authors showed that expression of untagged PDE7b does not change the dendritic development of neurons, but the cell morphology shown in the most right panel in Fig. 1b looks sufficiently different from the rest of the panels. (2) Fig 2a-c, PDE group behaves more or less the same as the empty vector control, is this expected? The authors stated in the manuscript (lines 207-208), "perinuclear cAMP signaling is essential for neuronal survival and axonal development". It is strange that perturbing the whole cell cAMP level does not have any impact on the synaptic activity of neurons. (3) The authors mentioned that PDE7b alone is less enriched in the PSD (extended Fig 1i-j), would this suggest cAMP outside the PSD does not participate in synapse formation? (4) Fig 2d-f, if the authors recorded cells with PDE7b alone, would mIPSC be affected?
2. Fig 2j-l, to distinguish cAMP's impact on initial synapse formation or on destabilization of already formed synapses, the authors infected neurons at DIV0, which resulted in lower mEPSC frequency along the entire recording period (DIV6-DIV16). It would be informative to test whether cAMP is important for maintenance of synapses by expressing PDE-Hr1 in neurons that have already formed synapses (e.g., infect neurons at DIV12 or later). Similarly, for data in Figs 5&6, what might happen if

expression of PDE-Hr1 is performed at P10 or later.
3. To overcome the overexpression effect in PDE-Hr1, the authors fused PDE to Homer1 nanobodies. Both nanobodies target Homer1. However, the two nanobodies were not thoroughly characterized. In the paper by Dong et al, the nanobodies were only shown not to affect the distribution or size of Homer1 puncta (Dong et al., 2019). Homer1 is a small scaffold protein capable of interacting with multiple proteins including Shank, mGluR, Dynamin, Drebrin, etc. One might need to consider whether the two nanobodies may affect Homer1 in binding to any of its target proteins. For example, the effects of Nb7-PDE and Nb25-PDE are not exactly the same (e.g., Fig 4g and 4h with Nb25-PDE more effective in lowering mEPSC amplitude), does this suggest that Nb7 and Nb25 target different regions of Homer1? An earlier study (Gross et al., 2013) generated an intrabody targeting PSD-95 (FingR). FingR comes with a self-expression level control system. It is curious why the authors did not consider FingR for targeting PDE7b or PKI to ePSD.
4. Extended Fig 1e, western blot of Nb7-PED and Nb25-PDE, why there are two bands?

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Response to the Reviewers' Comments for Sando et al., "Engineered Synaptic Tools Reveal Localized cAMP Signaling in Synapse Assembly"

(Journal of Cell Biology manuscript #202109111)

Introduction

We would like to thank the reviewers for their careful and constructive comments about our study. For the revision, we have revised the text and figures to reflect the important changes requested by the reviewers. We agree that these revisions help strengthen our study, and again thank the reviewers for their comments.

In the following, we cite the reviewers' comments in full in *italic* typeface, and provide our responses in **bold** typeface.

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

The paper by Sando et al. is reports a new tool to reduce the concentration of cAMP locally at the synapse. By using this tool, the authors demonstrated the local cAMP signaling is required for the formation and maintenance of excitatory but not inhibitory synapses. Overall, this study is carefully and rigorously carried out and I do not see any major issues. It opens up new questions such as what is the upstream of cAMP and how it acts. I believe it has sufficient novelty for publication in JCB.

We thank the Reviewer for the positive comments – we try to be solid and rigorous, and are very happy about the 'rigorously performed' remark. We agree that our study raises new questions, including the question of what mechanisms upstream/downstream of cAMP in postsynaptic signaling nanodomains mediate excitatory synapse formation. The biological principles of synapse formation are a relatively new area of neuroscience. We believe our study will provide new insights into and approaches towards understanding this question.

Minor issue.
Entorhinal-CA1 pathway should be correctly called temporoammonic pathway, not perforant pathway.

We thank the Reviewer for identifying this oversight, and have edited the text and figures accordingly. However, we have kept the term 'Entorhinal-CA1 pathway' in parallel because that term, even if not completely correct, is widely used by the community.

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

Synapse formation is a continuous process throughout life of animals. Multiple synaptic cell adhesion molecules (CAMs) take part in synapse establishment, organization, and plasticity. These CAMs are increasingly known to be critical for trans-synaptic

organizations and synapse formation. But whether, and if yes, how CAMs transduce intracellular signals are much less understood. Recent studies from Sudhof lab reported that CAM-initiated cAMP-PKA signalling pathway directly regulates synapse formation (Jiang et al., 2021; Sando and Sudhof, 2021). cAMP signalling is involved in multifarious cellular processes, and its compartmentalized signalling likely determines the specificity of various cAMP signalling processes. Recent studies have documented compartmentalization of cAMP in cells (Bock et al., 2020; Zhang et al., 2020), but how to perturb cAMP signalling at specific subcellular compartments remains a challenge. In this manuscript, the authors developed a synaptic targeting strategy called "SynTAMs" by fusing phosphodiesterase or PKA inhibitor to Homer1, a well-known excitatory postsynaptic density (ePSD) marker. Importantly, overexpression of Homer1 alone does not cause obvious changes to synaptic development, thus these Homer1-fused "cargoes" can be specifically targeted to ePSD for specific modulation of cAMP signalling in PSD compartments. The authors have also used an alternative synaptic targeting approach by fusing phosphodiesterase or PKA inhibitor to nanobodies specifically targeting Homer1 (Dong et al., 2019). Together, the authors demonstrated that postsynaptic cAMP-PKA signalling is critical for the formation of excitatory synapses of hippocampal neurons in cultures and in vivo. The authors also showed that postsynaptic cAMP-PKA signalling is required for synaptic connectivity in hippocampal circuits.
In addition to modulating postsynaptic cAMP signalling, the method developed in this work can be readily adapted to modulate many other postsynaptic signalling processes. Although numerous attempts have been made in the past to specifically target molecular cargoes to PSD compartments, essentially all these methods suffer from perturbation of certain synaptic functions. The method reported in the current study appears to have largely avoided this problem (although this will need more experimental validations in future). As such, the study will be valuable to many colleagues in the field of synaptic biology.

We also thank this Reviewer for her/his very constructive comments.

This reviewer has some minor concerns/comments on the manuscript:
1. PDE7b with no targeting group: theoretically, PDE7b will convert all accessible cAMP to AMP, and thus is expected to cause broader/more serious impacts than the ePSD targeted group. (1) Although the authors showed that expression of untagged PDE7b does not change the dendritic development of neurons, but the cell morphology shown in the most right panel in Fig. 1b looks sufficiently different from the rest of the panels.

Indeed, neuronal morphology is highly variable in culture, and we attempted to display this variability by selecting representative images in an unbiased manner. This can be appreciated by comparing representative images of PDE-overexpressing cells to controls in Extended Figure 1i, which appear more extensively branched than those in Fig. 1b. We agree that non-specific cAMP manipulations likely cause phenotypic consequences that our assays do not detect. Given our focus on compartmentalized postsynaptic signaling, we implemented assays to rigorously examine excitatory synapse formation and function, both of which do not appear significantly perturbed by untargeted PDE expression. We completely concur that

examining other cell biological parameters would likely reveal perturbations following untargeted PDE expression. Moreover, our immunocytochemical analyses suggest that untargeted PDE does not significantly diffuse past the neuronal soma and primary dendrites (extended data Figure 1i and j), suggesting that local PDE concentrations in more distal areas of the neuron and in synaptic compartments may not be substantial enough to produce synaptic effects in our assays.

(2) Fig 2a-c, PDE group behaves more or less the same as the empty vector control, is this expected? The authors stated in the manuscript (lines 207-208), "perinuclear cAMP signaling is essential for neuronal survival and axonal development". It is strange that perturbing the whole cell cAMP level does not have any impact on the synaptic activity of neurons.

Similarly, while untargeted PDE overexpression, which results in PDE being distributed throughout the neuronal cytosol, failed to produce significant alterations in mEPSCs in primary culture (Fig 2a-c), we agree that it is likely we would detect phenotypes in this condition if we expanded our array of analyses in this condition and studied other neuronal features besides synapse formation. However, we feel that this was outside the scope and focus of our current study, which was centered in compartmentalized postsynaptic signaling in synapse formation. Thus, we concentrated on developing and employing molecular tools to target and enrich PDE in excitatory postsynaptic compartments combined with assays to probe excitatory synapse formation and function.

(3) The authors mentioned that PDE7b alone is less enriched in the PSD (extended Fig 1i-j), would this suggest cAMP outside the PSD does not participate in synapse formation?

Indeed, PDE7b overexpressed alone is virtually undetectable in postsynaptic excitatory compartments (Extended data Fig 1i and j). This raises two potential possibilities: (1) cAMP outside the PSD does not participate in synapse formation; or (2) untargeted PDE7b overexpression does not sequester PDE7b in a high enough local concentration in other subcellular compartments involved in synapse formation. Given the emerging evidence of highly spatially-organized cAMP signaling nanodomains, we feel that the latter scenario is likely the case. cAMP likely plays roles in other subcellular compartments that are not substantially perturbed in our untargeted PDE7b conditions (i.e. presynaptic terminals, etc.). Future studies employing targeting approaches similar to ours to determine this will be of great interest. We thank the Reviewer for raising this important and interesting question.

(4) Fig 2d-f, if the authors recorded cells with PDE7b alone, would mIPSC be affected?

This is an interesting question that falls outside the scope of our current study, which focused on mechanisms of excitatory synapse formation and tools to probe these mechanisms. Future studies using molecular targeting approaches to unravel how inhibitory synapses form will be important.

2. Fig 2j-l, to distinguish cAMP's impact on initial synapse formation or on destabilization of already formed synapses, the authors infected neurons at DIV0, which resulted in lower mEPSC frequency along the entire recording period (DIV6-DIV16). It would be informative to test whether cAMP is important for maintenance of synapses by expressing PDE-Hr1 in neurons that have already formed synapses (e.g., infect neurons at DIV12 or later). Similarly, for data in Figs 5&6, what might happen if expression of PDE-Hr1 is performed at P10 or later.

Elucidating the mechanisms of synapse formation vs. maintenance is of great interest and importance towards understanding synaptic circuit assembly, maintenance and refinement. As the Reviewer points out in her/his introductory statements above, synapse formation is a continuous process that occurs through-out life and is intertwined with synapse maintenance/elimination. Thus, distinguishing between these processes is challenging and will require approaches such as live imaging combined with mechanistic perturbations to monitor synapses over time. Our rabies tracing experiments in Figure 8 begin to address the Reviewer's question, given that the viral injections were conducted at P21. While we still observed robust deficits in synaptic connectivity using this paradigm, it cannot distinguish synapse formation vs. elimination because synapse formation is a continual process even in established circuits of the hippocampus. The electrophysiological time-course experiments shown in Figures 2 and 6 are time-consuming and labor-intensive, and repeating them using later infections might be helpful but would require 4-6 months of work, placing them outside the scope of a revision experiment. Moreover, they would be only partly informative in view of the same limitations outlined above in our rabies tracing experiments. We agree with the Reviewer that dissecting the mechanisms of synapse formation vs. elimination are critically important and we will pursue them in future studies.

3. To overcome the overexpression effect in PDE-Hr1, the authors fused PDE to Homer1 nanobodies. Both nanobodies target Homer1. However, the two nanobodies were not thoroughly characterized. In the paper by Dong et al, the nanobodies were only shown not to affect the distribution or size of Homer1 puncta (Dong et al., 2019). Homer1 is a small scaffold protein capable of interacting with multiple proteins including Shank, mGluR, Dynamin, Drebrin, etc. One might need to consider whether the two nanobodies may affect Homer1 in binding to any of its target proteins. For example, the effects of Nb7-PDE and Nb25-PDE are not exactly the same (e.g., Fig 4g and 4h with Nb25-PDE more effective in lowering mEPSC amplitude), does this suggest that Nb7 and Nb25 target different regions of Homer1? An earlier study (Gross et al., 2013) generated an intrabody targeting PSD-95 (FingR). FingR comes with a self-expression level control system. It is curious why the authors did not consider FingR for targeting PDE7b or PKI to ePSD.

We chose to focus on Homer1-based targeting considering Homer1 is rapidly enriched in nascent excitatory synapses prior to PSD-95 (Meyer et al., *Neuron*, 2014).

Thus, this Homer1-based approach allowed us to perturb cAMP signaling early during synapse formation. Given our recordings detected no alterations in mEPSCs following Homer1 or Homer1-Nanobody fusion overexpression, we feel these tools serve our purposes. We agree that further in-depth characterizations would be helpful in future studies to determine whether Nb7 and Nb25 bind to distinct areas of Homer1, and whether they impede Homer1 interactions. However, we feel given our current sets of control experiments, that these approaches fulfilled our need for a protein cargo targeting system to nascent excitatory postsynaptic compartments.

4. Extended Fig 1e, western blot of Nb7-PED and Nb25-PDE, why there are two bands?

The double bands in the Nb7-PDE and Nb25-PDE immunoblots could be due to phosphorylation or other modifications, or be caused by a proteolytic cleavage of the fusion proteins under our experimental conditions when they are overexpressed. As our extensive imaging characterizations support, these do not interfere with targeting specificity or protein function.

We thank the reviewers for their valuable comments, and hope to have addressed all of their concerns.

November 18, 2021

RE: JCB Manuscript #202109111R

Dr. Richard Sando
Vanderbilt University
Pharmacology
465 21st Avenue, MRBIII room 7160A
Nashville, Tennessee 37240-7933

Dear Dr. Sando:

Thank you for submitting your revised manuscript entitled "Engineered Synaptic Tools Reveal Localized cAMP Signaling in Synapse Assembly". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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, https://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 Tools is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

2) Figures limits: Tools may have up to 10 main text figures.

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.

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."

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.

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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
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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. Tools may have up to 5 supplemental figures. At the moment, you currently have 8 such items, therefore we need you to reduce the count. Please be sure to correct the callouts in the text to reflect this change. 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.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

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The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Louis Reichardt, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology
