JCB

Mechanical stress impairs pheromone signaling via Pkc1-mediated regulation of the MAPK scaffold Ste5

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Re: JCB manuscript #201808161

Prof. Matthias Peter
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Dear Matthias -

We have now received three reports on your manuscript "Mechano-stress inhibits pheromone signaling via Pkc1-depndent regulation of the MAPK scaffold Ste5", from external reviewers with expertise in this area. As you will see from the attached reports, they were all positive, with comments that some of the data are compelling and valuable, and that the study is generally solid. However, each of the reviewers raises several important issues that would need to be addressed by additional experimental data before the work could be considered suitable for publication by the Journal. Therefore, we are unfortunately unable to accept the manuscript as written. Given the substantial revisions required, we propose that you submit a plan of their scope prior to resubmission, outlining the point-by-point response to each of the reviewer comments.

Rev #1 has two major issues: showing that the S185 phosphorylation state depends on Pkc1, and the role of phosphorylation during mating/relationship to mechanical stress, and a better demonstration that the action of Pkc1 is independent of the MAPK of the pathway, Mpk1. Their argument is that although phosphorylation of Ste5 is convincingly shown by MS, no evidence for a change in phosphorylation attributable to Pkc1 is provided. Perhaps a Phos-tag gel would help here? This reviewer also questions your model of an on-off switch and notes numerous issues with errors, omissions, etc., in the text.

Although Rev #2 finds the data for direct phosphorylation of Ste5 by Pkc1 convincing, they feel that the data do not support regulation of Far1 by Pkc1. The reviewer points out that Ste5 and Far1 are not redundant and the Ser residue in Far1 is not a Pkc1 consensus motif, and there are no direct data that Pkc1 phosphorylates Far1. The reviewer suggests removing or softening this conclusion.

Finally, Rev #3 notes there are numerous serious textual errors, as well as missing information regarding reagents and methods that were in a previous version they reviewed and have not been corrected (extensively listed). As experimental issues, this reviewer brings up a lack of evidence for Ste5 phosphorylation in vivo and general issues with controls to support that the results depend on mechanical stress. The reviewer has specific experimental suggestions regarding mechanical stress, specificity/effect of Pkc1, in vivo phosphorylation of Ste5. The reviewer also notes the Ser185 site of Ste5 does not conform to a PKC site.

Clearly there are a number of experimental issues that need to be addressed, most notably the link between Pkc1 and Ste5 and Far1 phosphorylation, plus the numerous textual changes that need to be made. 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. Our typical timeframe for revisions is three to four months; if submitted within this timeframe, novelty will not be reassessed. We would be open to resubmission at a later date; however, please note that priority and novelty would be reassessed.

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.

GENERAL GUIDELINES:
Text limits: Character count 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.

Figures: Your manuscript may have up to 10 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

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.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Your manuscript may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

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,

Ian Macara, Ph.D.
Editor, Journal of Cell Biology

Andrea L. Marat, Ph.D.
Scientific Editor, Journal of Cell Biology

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

Review of "Mechano-stress inhibits pheromone signaling via Pkc1-dependent regulation of the MAPK scaffold Ste5" by Frank van Drogen et al.

To some extent this is a follow up on a recent paper by the same group (Mishra et al PNAS 2017). In that paper they showed, relevant to this manuscript, that compression stress blocks the mating response, both the polarization and signaling aspects of it. In addition, they showed that Pkc1 causes dispersal of actin patches away from the mating projection in a Mpk1-independent fashion. Finally, they suggest that Pkc1 somehow might inhibit Fus3 to cause actin patches dispersal.
In this new manuscript, they claim that the action of Pkc1 on the pheromone pathway is by phosphorylating Ste5 in serine 185. Because S185 is at the core of the RING domain, with which Ste5 binds to Ste4, they claim that said modification blocks Ste5 function, causing inactivation of Fus3. Similar sites (in this case three) were found on Far1.
They show that Pkc1 action, by inhibiting pheromone signaling and polarization prevents lysis during pheromone response while being compressed (which they had already showed in 2015) and that it reduces lysis after successful cell-cell fusion.
The manuscript is solid is many respects, but there are some important aspects that need to be addressed.

Major comments:

1- The manuscript describes, convincingly, that Pkc1 binds to Ste5, that it can phosphorylate S185 (in vitro), and that mimicking that phosphorylation by replacement with aspartate blocks the ability of the RING domain (and presumably of the whole Ste5) to Ste4. Consistently, strains with Ste5-S185D cannot signal. The authors even show that a constitutively active Pkc1 can reduce pheromone dependent signaling only if S185 is available for modification.
What the manuscript doesn't show is that S185 changes its phosphorylation state in a given condition (compression or during mating). In fact, the only direct evidence that the site is phosphorylated in vivo at all comes from mass-spec. Thus, some other assay, such as a mobility shift of Ste5, showing a change in mobility after Pkc1 activation, a change that should be absent in S185A expressing yeast, is required.
2- The specificity of cercosporamide is not well established. A quick look in the web shows that it is marketed as an inhibitor of multiple kinases (see for example https://www.tocris.com/products/cercosporamide_4500, where they claim that it is "potent" inhibitor of Mnk2 and Jak3). Thus, we really don't know if the relevant target is Pkc1, another kinase or a combination of Pkc1 with another kinase. A way to get around this problem is to use a Pkc1 mutant that is resistant to cercosporimide. Given that Sussman et al in 2004 showed that this drug acts as an ATP competitor, it might be possible to mutate the ATP binding pocket of Pkc1 in such a way that it would not bind cercosporamide anymore. Without that kind of experiment, it is hard to know if the effects reported here are due (or just due) to inhibition of Pkc1.
The claim in the text that there are no off targets relevant because without stress cercosporamide does not block Fus3 doesn't prove that with stress there is no other relevant target.
3- That the phenotypes are independent of Mpk1 needs more evidence. As the authors are aware, there is plenty of previous evidence (beginning in the late 90's by work from the Errede lab) that deletion of mpk1 leads to extensive cell lysis after pheromone treatment. Thus, it is important demonstrating that there is another, Mpl1-independent way that lysis is prevented by the CWI pathway. The only evidence provided here is that cercosporamide increases cell lysis and deletion of Mpk1 does not. First, it is odd that the authors do not combine cercosporamide with deletion of Mpk1. Inhibition of Pkc1 concomitant to deletion of Mpk1 might lead to even higher lysis (Fig 1B) and/or less dispersal of Ste5 (Fig 1D) than cercosporamide alone.

A reasonably easy way to support an Mpk1 independent action of Pkc1 would be to activate Mkp1 using a constitutively active component downstream of Pkc1 (such as the alleles Bck1-20 or Mkk1-DD) and then show that these alleles cannot rescue the effect of cercosporamide. In fact, using these alleles might allow the authors to delete PKC1 altogether, obviating the need of using cercosporamide.
4- The role of Ste5-S185 phosphorylation during mating is unclear to me. The authors say that Pkc1 acts on Ste5 during fusion, due to the mechanical stress that mating involves, to dislodge Ste5 quickly after mating. However, their data does not support this conclusion. Ste5 doesn't seem to "rapidly disperses to the cytoplasm concomitant with cell-cell fusion". Actually, Fig 6E and F show that Ste5 continues to increase for about 10' after fusion. Only then it drops. Moreover, the effect of Ste5-S185A on the timing of Ste5 dispersal after mating is minimal, only a few minutes (Fig 1G). Thus, to me, something else, post fusion, maybe unrelated to mechanical stress causes Ste5 dispersal.
5- In the discussion, the authors speculate that because phosphorylation of a single amino acid completely blocks signaling, this modification could act as an on-off switch, and they contrast that to a putative rheostat like role of multiple phosphorylation. It seems to me that this speculation is incorrect.
First, this switch would turn off signaling only if ALL Ste5 molecules in a cell are phosphorylated. If, say, 50% are phosphorylated, the remaining would be able to bind to Ste4 OK, and the response is predicted to be about 50% (ie, not a switch). In fact, the authors detected this phosphosite in pheromone-stimulated and responding cells, clearly showing that this phosphorylation only existed in some fraction of the Ste5 molecules.
Second, it is multisite phosphorylation one way to accomplish high ultrasensitivity in responses, helping cells evolve molecular switches. Although not every multisite phosphorylation event necessarily exhibits ultrasensitivity, some do. A well-known example is the case of Sic1 at the G1 to S transition.
6- A general comment is that some aspects of the text seem to be in draft state (see some comments in the minor critique part).

Minor comments

1- The strains used in each experiment are not spelled out. For example, from the table, there are a number of "WT" strains to choose from, some BY some W303, most BAR1, except for one used in the proximity assay, which is bar1 delete.
2- In general, the concentration of alpha factor used is not spelled out. In some places the concentration seems incorrect. For example, in Fig1A it says that the concentration used was 2.7 nM. It seems too low, I assume it is 2.7 micromolar.
3- In the introduction, there is a reference to Zhang 2014 which seems to be incorrect ("Construction of conditionally replicating adenovirus...").
4- Fig 1C lacks a control without mechano-stress. Among other things, that control would allow us to know if cercosporamide relieves all inhibition of Fus3 activity caused by compression or not.
5- In Fig 1D legend is unclear as to when NaPP1 was added. Was it applied after the 100 minutes of alpha factor (like cercosporamide) or together with alpha factor? This is relevant, since blocking Fus3 from the beginning will prevent proper shmoo formation.
6- In Fig 1E, would it be possible to show a field with several yeast? Not just one selected example?
7- This statement in the discussion seems to be missing a reference: "Recently it was shown that these and other sites in the PH domain are also phosphorylated by Fus3, constituting a negative feedback mechanism that regulates membrane-turnover of Ste5 and thus down-tunes its signaling activity".

8- What is the dominant active Pkc1 allele used (the info is not in the strain table, or elsewhere)? It needs to be explained and referenced.
9- In the two hybrid assay (Fig S4) , the low lacZ signal displayed by the D mutants is still above what one sees with an empty AD vector? If so, it might explain why the Far1-3D strain does not orient completely randomly in the pheromone gradient.

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

This manuscript describes cross-talk between the cell wall integrity (CWI) signaling pathway and the pheromone response pathway. It has been known for many years that the CWI pathway is important for survival during pheromone-induced morphogenesis. The current work does a very nice job of demonstrating that a key aspect of this requirement is the down-regulation of pheromone response signaling by Pkc1 during cell fusion through phosphorylation of the scaffold protein Ste5. In vivo and in vitro data support the direct phosphorylation by Pkc1 of Ste5 at Ser185, which is within the RING-H2 domain and which blocks recruitment of Ste5 to Gβγ at the plasma membrane. The action of Pkc1 induces rapid dispersal of Ste5 from the mating projections at the time of cell-cell fusion.

The paper is well-written and the data implicating Pkc1 in the regulation of Ste5 are compelling. However, the data do not similarly support the regulation of Far1 by Pkc1. Although Ste5 and Far1 share some sequence similarity and have some overlapping roles in the pheromone response, they are not largely redundant. More importantly, the Ser residue in Far1 that is analogous to Ste5 Ser185 (Far1 Ser 210) is most decidedly not at a consensus Pkc phosphorylation site. There is not a basic residue anywhere near this site. Therefore, although the authors report MS data revealing that this residue (as well as neighboring residues S208 and S211) is phosphorylated, it seems highly unlikely that Pkc1 is responsible for these events. Indeed, the authors do not present any evidence that Pkc1 regulates Far1, but rely on extrapolation from their data on Ste5 and the observation that a mutation of those residues in Far1 to Ala exacerbates the viability defect of the Ste5 S185A mutation (Fig. 6C). This is not nearly enough to conclude that Pkc1 acts on Far1. I would recommend either removal of the Far1 data, or greatly softening the conclusions with regard to Pkc1 and Far1.

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

Introductory comment: I reviewed an earlier version of this manuscript two years ago for a different journal. Both then and now, my impression is that the core findings of the study are informative and valuable. However, my earlier critique identified numerous examples of missing information, unjustified statements, and outright errors. Unfortunately, the vast bulk of these cases remain uncorrected in the current manuscript. This apparent indifference to scientific accuracy is concerning; it is difficult to comprehend why authors would refuse to fix mistakes and oversights. I hope the current editor will help ensure that these issues are taken seriously.

Summary:

This manuscript reports new and interesting findings suggesting that two important signaling

proteins in the yeast pheromone response pathway, Ste5 and Far1, are regulated by inhibitory phosphorylation via yeast PKC (Pkc1). These effects are proposed to allow signaling to be coordinated with cell wall remodeling events that ultimately trigger cell-cell fusion of two gametes. Strengths of the study include straightforward analyses of phosphorylation site mutants (Fig 3 and Fig 4A/B) and their rescue by membrane targeting (Fig 4C), plus convincing NMR evidence for phosphorylation of Ste5 Ser185 in vitro (Fig 2B-E and Supp Fig S3) and for how Ser185 mutations affect Gbetagamma binding (Fig 4E).

The main weaknesses include: (i) No evidence that Ser185 phosphorylation in vivo depends on Pkc1 or mechanical stress. (ii) Several experiments lack basic controls (i.e., unstressed cells) to support the conclusion that the results depend on mechanical stress. (iii) Numerous cases of missing information about reagents or methods, unjustified statements, and abundant errors; as mentioned above, most of these were noted in an earlier critique of this paper yet remain uncorrected.

On balance, the general story would warrant a positive recommendation if the experimental weaknesses were addressed adequately. Moreover, the manuscript itself should receive a thorough and attentive overhaul to ensure that it is clear, complete, and accurate.

Specific comments:

1. Several experiments quantify cell lysis that is claimed to be induced by mechanical stress (Figs. 1B, 1D, 5C). Yet, none of these experiments includes a simple control that quantifies lysis in un-stressed cells. When measuring the effect of a specific treatment, it is standard practice to include un-treated cells as a control. Currently, the only data relevant to this is the un-quantified picture in Fig 1A. At least one (and preferably all) of the quantified experiments needs to report the quantified values from this important negative control.

2. The efforts to document that the in vivo phenotypes are due directly to Pkc1 rather than one of the other kinases in its downstream cascade are quite minimal. Only Fig 1F deals with this issue. At the very least, to support the current interpretation this figure should include an additional control showing that cercosporamide still has the same inhibitory effect even in the mpk1Δ mutant. It would be even better to test a strain lacking the kinase immediately downstream of Pkc1 (i.e., bck1Δ), given that there are two other kinases between Pkc1 and Mpk1.

3. The only evidence of Ser185 is phosphorylated in vivo comes from mass spectrometry data (summarized in Figs 2A and S2A). A few points should be addressed:
(a.) These MS data do not provide any information about whether Ser185 phosphorylation depends on Pkc1 or mechanical stress. The absence of this dependency information should be stated explicitly and openly. (E.g., "One caveat of the in vivo phosphorylation data is that we did not establish whether or not Ser185 phosphorylation depends on Pkc1 or mechanical stress....")
(b.) In these MS data, it is unclear if the Ser185 phosphorylation is promoted by pheromone treatment (as opposed to being present in all conditions, regardless of treatment). In particular, it is unclear if the analysis includes comparison of pheromone-treated cells to untreated cells. This should be clarified in the Methods, and the Results text should include a clear statement of whether or not there is evidence for pheromone-dependence.
(c.) The length of pheromone treatment in the MS experiment(s) is also unclear; the Methods section states that treatment was "for times indicated", but I could not find where it was "indicated". The treatment time is relevant to the possible involvement of Pkc1, which it expected

to become more activated after longer treatment times and the consequent morphological responses. Thus, the authors should clarify the treatment time and cite relevant prior evidence for whether Pkc1 activity can be expected to be stimulated under these conditions.

4. The NMR data provide sound in vitro evidence for Ser185 phosphorylation. However, the way the experiments are described in the text is misleading. They are said to use "recombinant Pkc1" (line 175) or "purified Pkc1 in vitro (line 372). But neither is true. The in vitro reactions used a metazoan PKC relative (Xenopus PKCalpha), which is not the same enzyme as yeast Pkc1. Thus, these descriptions do not represent the experiments honestly and accurately. The experiments are fine, but it is unacceptable to obscure the source of enzyme.

5. The authors claim that the Ser185 site in Ste5 (and analogous site in Far1) conforms to the established PKC consensus site (lines 163-164 and 371-372). As far as I know, tested PKC isoforms (and other members of the larger AGC group, including PKA, PKB, PKC, PKD, etc.) show a strong preference for positively-charged side chains at positions -3 and -2, (N-terminal to the phospho-acceptor site). References include PMID # 15782149, 8995387, 20159853, 9678592. By those criteria, the sites studied here do NOT conform to the PKC consensus. This disagreement should be noted and discussed.

6. The two-hybrid data in Supp Fig S4 are a bit of a mess:
(a.) The Figure legend (lines 638-640) states that Ste4 is fused to the DBD, and Far1/Ste5 are fused to the AD. But reagents in the strain list suggest the opposite: that Ste4 is fused to the AD and Far1 to the DBD. Obviously, the conflict needs to be resolved, but in addition the arrangement suggested by the strain list raises an important issue about negative controls: It is well known that some DBD fusions can show strong transcriptional activation even in the absence of a partner AD fusion. Therefore, a negative control (i.e., AD vector) needs to be tested for EACH DBD fusion - including DBD fusions to mutant and WT proteins. This is necessary to demonstrate that the measured interaction signal requires the AD partner, and that any changes in interaction signal caused by mutations are not due to changes in basal auto-activation by the DBD fusion.
(b.) Table I or II does not appear to list any Ste5 reagents for these two-hybrid experiments.
(c.) The legend should clarify if the fusions use full-length proteins or fragments.
(d.) The number of replicates (n) is not stated.

7. Misleading or unsubstantiated statements:
(a.) Line 373-374: "...a constitutively-activate Pkc1 mutant interferes with FIG1-qV reporter expression in wild-type and mpk1Δ cells". In fact, there are no such tests performed in mpk1Δ cells; hence, the statement is unsubstantiated and should be deleted.
(b.) Line 351-352: "...phosphorylation of S185 located in its RING-H2 domain directly interferes with Gβγ binding". This is an overstatement. It was not shown that S185 phosphorylation interfered with Gβγ binding, only that the phospho-mimetic S185D mutation interfered. The statement should accurately fit the actual results. The rest is extrapolation.
(c.) Lines 342-343: "...phosphorylation of multiple sites within distinct membrane-binding domains are required to induce negative feedback". This is unsubstantiated. There is no evidence that this is "required" for negative feedback.
(d.) Lines 324 and 328: "Fus3 allosterically regulates its activation by phosphorylating Ste5 on T287" and "It is thus possible that the allosteric activation mechanism of Ste5 also involves phosphorylation of these MAP kinase consensus sites". These comments do not make sense. There is no evidence that these phosphorylations regulate Ste5 by an "allosteric" mechanism.
(e.) Lines 247-250: The "increase of total peak number" is taken to suggest "asymmetric oligomerization while binding to Ste4". But the Methods section states that Ste4/Ste18 is purified

as a GST fusion, which is known to homo-dimerize. Therefore, a simpler interpretation would be that two separate Ste5 RING-H2 molecules bind to a linked pair of Ste4/Ste18 heterodimers. (I.e., rather than implying any induced oligomerization.) If there is a good reason to exclude this simpler explanation, the authors should describe their logic.
(f.) Line 96: The authors state that Fus3/Kss1 phosphorylate Gpa1. I am not aware of such evidence. If it is correct, provide a citation.

8. Important information missing:
(a.) Fig 5A-B: There are no reagents listed that would allow for expression of Pkc1 (WT and dominant active) as claimed .
(b.) Fig 5A-B: The number of replicates (n) is not stated, and no error bars are shown. Are these single experiments (n = 1)? There should be some indication that the difference between WT and S185A in Fig 5B is reproducible.
(c.) Regarding Figs 2B-D and S3: The Methods section does not describe how long these in vitro phosphorylation reactions proceed. It also does not describe how the "yeast extract" was prepared (for Fig S3). Also, the Fig S3 legend does not describe the nature of the "activator".
(d.) Fig 4C: There is practically no information about how these experiments were performed. Table I lists no strains that would allow for these experiments; i.e., strains with an estradiol induction system. There is no information on the estradiol induction time. How many replicates (n) were performed? Finally, the results for Ste5-TMD are obscured and not visible.
(e.) There is confusion about how Gβγ (Ste4/Ste18) was purified. The Methods section (lines 799-804) and Fig 4D both imply that a GST fusion was used, but Table II lists a plasmid suggesting the tag is 6His, not GST. Which is correct? (Also, is the tag on both Ste4 and Ste18, or only on one?)
(f.) Fig. 2B: What is denoted by the RED spectra peaks? I.e., it seems there is an overlay of red and black for the 3 peaks other than "pSer". What is this meant to imply? It is not described. Is it an overlay of two distinct conditions?
(g.) There is no Methods information describing how the FIG1-qV reporter assays were conducted in Figures 3C, 4C, 5A-B.
(h.) Fig 6A: The Methods section does not describe how these mating assays were performed.
(i.) Fig 6C: There is no Methods description for how viability was scored in these mating/fusion assays.
(j.) Fig 5D: The legend does not state the number of replicates (n) or define the error bars.
(k.) Fig 3C: There is no statement of replicates; is this a single experiment?

9. Other errors, oversights, or issues needing clarification:
(a.) Line 334: "Recently it was shown...". Where? These comments need a citation.
(b.) Fig 2A and Line 479: The labeling of the Ste5 domain as a "VWD" domain is incorrect. Ste5 has been reported to contain a VWA domain (type A) not a VWD domain (type D); these are distinct types of domains.
(c.) Fig S2 and legend: The description of colors in the legend are inconsistent with the Figure. The PH region is not "green" and VWA is not "dark grey". (It seems the descriptions were copied from the Fig 2A legend without correcting for changes.)
(d.) Line 403: "Using a powerful microfluidic chip...". Probably the authors do not really intend to say that the chip is powerful. Re-write.
(e.) Line 440-441: Comments on "in cell NMR experiments" and "using Xenopus extracts" are likely typographical errors.
(f.) Fig 2A: Presumably the label "L. par" is erroneous and should be "S. par". (There is a mushroom named L. paradoxus, but that is different from the yeast S. paradoxus.)
(g.) The text uses at least 4 different ways to refer to the heterodimer "beta gamma": Gβγ, Gβ/γ, βγ,

and β/γ. Better to pick one and use it consistently.
(h.) Table I contains a lot of non-standard nomenclature (e.g., "Leu", "Ura", rather than "LEU2", "URA3"; alternating uppercase and lowercase for recessive markers in BY4741, etc.).

May 9, 2019

Re: JCB manuscript #201808161R

Prof. Matthias Peter
ETH Zurich
ETH Zurich Institute of Biochemistry
Otto-Stern-Weg 3
Zurich 8093
Switzerland

Dear Prof. Peter,

Thank you for submitting your revised manuscript entitled "Mechano-stress inhibits pheromone signaling via Pkc1-dependent regulation of the MAPK scaffold Ste5". 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.

You will see that the reviewers have outlined numerous remaining issues that must be attended to before publication, though the majority of these can likely be addressed through improvements to the text and data presentation. Our general policy is that papers are considered through only one revision cycle; however, given that the remaining comments do not question your main conclusions, we are open to one additional short round of revisions to carefully and thoroughly address all remaining concerns. Please note that I will expect to make a final decision without additional reviewer input upon resubmission.

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,

Ian Macara, Ph.D.
Editor
Journal of Cell Biology

Andrea L. Marat, Ph.D.
Scientific Editor
Journal of Cell Biology

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

Review of resubmitted version of Van Drogen et al.

Most of the major concerns have been addressed. However, there are of outstanding issues.

The most important is that there is no statistical analysis of the data, in contrast to what is said in the text and mentioned in the rebuttal. What is standard is ANOVA followed by post-test comparisons, in a way that takes into account the number of comparisons made and the number of replicates. This should be described with enough detail in the method section.

Fig 1G: There is a missing control of mechanical stress alone in WT cells. From panel to panel the number of WT cells that lyse after mechano-stress varies a lot: in Fig1B is 25%, in Fig 1C is 12%, in 1F seems to be less than 10%.

In Supple Fig 2G: That the Bck1-20 constitutively active mutant is actually working as such (for example a blot showing highly phosphorylated Slt2.
This point is relevant to be able to assert, as the authors do, that the effect of Pkc1 is not mediated by Slt2.

About the scoring of Ste5 dispersal. The methods section presents two methods.
a) A direct method: "In order to do that, the intensities of 4x4 pixel (16 pixels square) in the shmoo and brightest area elsewhere were compared." How was the scoring done? How much dispersal there has to be for a cell to be considered as "dispersed"? In the images provided in Supple Fig 2, more than dispersal, what I see is relocation of Ste5 to other areas away from the shmoo.
This difference (dispersal vs relocation) is important since the claim of the authors is that Pkc1 phosphorylates Ste5 and thus prevents it from localizing to the PM. But the images show that Ste5 is still able to accumulate at the PM, maybe in another location, where a new shmoo would form.

b) An indirect method: "When Ste5 dispersal was analyzed in a 96-well plates, the arrest in shmoo growth was also considered as dispersal due to focus issue."
First, it is not clear to which experiments this alternative criterion was applied. Second, to equal lack of growth to Ste5 dispersal is not demonstrated, and it might not be the same. I doubt that data analyzed this way could be considered a proxy of Ste5 dispersal.

Minor:

Fig 1E: it seems that the graph is backwards, since the protected cells show higher Fus3 activity than the lysing...

Fig 1H: when was "dispersal" measured. In the legend it seems that it was measured continuously over 30'. So, how fast is "rapidly"? The text says: "Venus-tagged Ste5 (Ste5-tV) accumulated at the tips of mating projections in absence of stress, Ste5-tV was rapidly dispersed upon mechanical pressure (Fig. 1H)."

In line 160, it seems to me that the panel to be called should be Fig 1H, not Fig 1G.

Suppl Fig2C: images are not clear. Please improve. And accompanying brightfield images would help.

About Far1 phosphorylation, the text says:
"MS/MS analysis of purified Far1 confirmed that S210 and possibly also S208 and S211 in its RING-

H2 domain were phosphorylated in cells exposed to □-factor".

Since there were no analysis of cells NOT treated with alpha factor, this should be said in such a way as to not lead to the notion that alpha factor stimulates the phosphorylation of these sites. Note that this comment also applies to Ste5.

Fig 5D, in the y-axis there is a mistake in the numbers: it should be 15 instead of 50.

Fig 6C, there are no error bars in the measurements...

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

This revised manuscript includes several welcome improvements that significantly buttress the original claims and strengthen the overall story. They include the following:
• inclusion of appropriate "no-stress" controls into assays of cell lysis, Ste5 dispersal, etc.
• additional controls to suggest that the relevant in vivo target of cercosporamide is Pkc1.
• phosphorylation of Ste5 and Far1 RING domains by yeast Pkc1 purified from cell lysates.
• explicit discussion of the difficulties and uncertainties relating to the quantification of Ste5-S185 phosphorylation.

Some points in my original critique were not addressed adequately. These do not involve central issues, and they certainly are not grounds for rejection. But I think they should be fixed, because some create ambiguity in interpretation and others involve errors that are rather embarrassing. It should be easy, even trivial, to fix these (though in some cases I am raising them for the third time). There are also a few new errors that arose as a result of the revisions. All of these are detailed below, and they are briefly summarized here:
• In Figure S5A, the relevant controls should be shown rather than kept secret, or the figure should be removed.
• The authors seem rather confused about certain aspects of the relevant literature, both simple (i.e., the difference between Gpa1 and Ste18) and complex (i.e., effects of phosphorylation at Ste5 T287).
• The authors misunderstood my point about interpreting the Ste4/18-induced doubling of NMR peaks for the Ste5 RING domain, and the implications for oligomerization.

Overall, I think the revised manuscript is considerably improved, and the remaining errors and ambiguities should be easily fixable.

Specific points.

** REGARDING PREVIOUS CRITIQUES and the author responses:

A. Regarding my original point 6, involving the two-hybrid assays in Figure S5A:
First, The authors have still not fixed the direct conflict between the Figure S5A legend and the strain list in Table I. The Figure legend says Ste4 is fused to the DBD and Far1 is fused to the AD, whereas Table I implies the reverse. This is now the third time I have pointed this out.

Second, the authors respond by stating: "We have performed the two-hybrid assays with all controls, and it is clear that the effect is not caused by transcriptional activation of the DBD fusion." I am unsure which "effect" the authors are commenting on here, but I certainly cannot agree that anything has been made "clear" by data that is not shown. Instead, I am skeptical. The key concern about these data is as follows: If the basal signal (i.e., without any AD fusion) for the Far1-DBD fusion was half as as strong for 3D mutant as it is for wild-type, then the similar fold drop in the signal observed with the AD-Ste4 fusion (in Fig S5A) *cannot* be interpreted as a change in Far1-Ste4 interaction, and instead it can just reflect a change in the starting basal value. So, if the assays truly included "all controls", as the authors state, then it would be a trivial matter to include those controls in the figure (i.e., legibly show the signal measured for each DBD fusion with no AD-Ste4). I suggest one of two options: either add the controls, or remove these data. Omitting relevant controls is unacceptable, especially given the ongoing uncertainties about how the experiment was performed, which the authors have repeatedly failed to clarify.

Third, further compounding the murkiness, none of the relevant strains in Table I list any reporter for detecting the interaction signal (i.e., a lacZ gene under control of the lexA operator), and it is also unclear how the DBD-Far1 and AD-Ste4 fusions are introduced (i.e., on plasmids? integrated? with what markers?).

B. Regarding my original point 7d, the authors provide this respons:
"The paper by (Bhattacharyya et al., 2006) claims that Fus3-dependent phosphorylation of T287 located within the MAP-kinase docking site allosterically activates Ste5."

No, the Bhattacharyya paper does not claim this. This statement is wrong in multiple ways, and the authors seem to be conflating distinct phenomena. First, that paper does not study allosteric activation of Ste5, but rather allosteric activation of Fus3 (by Ste5). Second, the paper explicitly states that T287 phosphorylation is not required for the allosteric activation of Fus3 by Ste5 (see the "conclusion" statement in the legend to their Figure S9). Third, they do claim that T287 phosphorylation has a regulatory effect, but the effect is *inhibitory*, which is the *opposite* of the authors' claim that it "activates Ste5". Fourth, the mechanism of this inhibition was not determined by Bhattacharyya et al, who did NOT claim that it was an "allosteric" effect but instead speculated that it might be due to effects on "turnover" or "trafficking".

If the authors want to relate their results to those of the Bhattacharyya paper, I suggest that a statement along the following lines would be accurate: "Our mass spectrometry uncovered phosphorylation at four sites near the MAPK-docking site in Ste5 that were previously proposed to be targeted by Fus3 as part of a negative feedback circuit (Bhattacharyya 2006, Malleshaiah 2010)." (However, the authors might want to note that recent findings question whether these phosphorylations are truly inhibitory; see PubMed ID # 30726174.)

C. Regarding the authors' response to my original point 7e:
"In this experiment (Fig. 4.E), we only observe 15N-labelled Ste5. This construct of Ste5 was prepared without a GST-tag and the His-tag used for the purification was removed. GST-tagged Ste4/18 heterodimers were produced in an NMR-invisible manner (no labeling with 15N-containing salts). Therefore, all the changes detected in the NMR spectra originate from Ste5. The overall reorganization of the peaks in the spectrum suggests prominent conformational rearrangement, however the number of peaks is roughly doubled which we attribute to the formation of the so-called asymmetric dimer."

This response missed the point. The factual statements are true (and I understood them before), but they do not address the issue raised. As I explained previously, because the GST moiety on Ste4/Ste18 can homo-dimerize, it is possible for two Ste5 RING-H2 domains to bind to two adjacent Ste4/Ste18 complexes (i.e., that are artificially linked together by a GST-GST interaction). As a consequence, this could allow interactions between two adjacent RING-H2 domains (and hence the observed asymmetric conformational changes) that would not ordinarily occur when a single RING-H2 domain binds to a single Ste4/Ste18 complex. Therefore, the observed asymmetric peak doubling of the Ste5 RING-H2 domain does not necessarily occur "while binding to Ste4", as stated, but instead it could be an artificial consequence of binding to a ligand that has been forcibly dimerized.

I suggest that an accurate description of the results would include a statement along the following lines: "...it is unknown whether the asymmetric RING-H2 oligomer is bound to a single Ste4/18 complex or to two complexes that are linked by the homodimerizing GST moiety."

D. Regarding the response to point 7f:
"We have now included the citation that describes phosphorylation of Gpa1 by Fus3/Kss1 (Choudhury et al., 2018)."

No, that is incorrect. The Choudhury paper reports phosphorylation of Ste18, not Gpa1. It should be obvious that these are different proteins. Even the most cursory glance at that paper should have allowed the authors to recognize their error - it's stated directly in the Title!
Also, Ste18 is a membrane-tethered protein, so it is not a "cytoplasmic target" (as stated on line 100).

E. Regarding the response to point 8e:
"We have corrected the relevant information in the revised Material and Method section and Table II. Briefly, Gβγ (Ste4/Ste18) was purified as a GST-fusion..."

In fact, Table II still erroneously lists the Ste18/Ste4 plasmid as a 6His fusion, rather than a GST fusion. It really should not be so difficult to correct such simple errors.

** NEW ISSUES arising in the revised version:

F. In Tables I-II, the column listing the Figure numbers is helpful. But what are we to make of the entries without any Figure number listed (1 plasmid and 11 strains)? It appears that the authors simply did not finish constructing the table.

G. Lines 145-146: "...the lysis defect in Pkc1-inhibited cells...". This is potentially confusing because the phrase "lysis defect" seems to imply a defect in lysis, but I think the authors mean the reverse. It would probably be clearer to replace "defect" with "observed", so that it reads "... the lysis observed in Pkc1-inhibited cells...".

H. Lines 159-160: "This phenotype was blocked by addition of cercosporamide but independent of Mpk1 (Fig. 1G, Suppl. Fig. 2C)...". There are two issues here that need fixing:
(i) The phrase "this phenotype" appears to refer to the Pkc1 and Mpk1 phenotype in the preceding

sentence, but I don't think that is the intention. Instead, this phrase should say something like "The Ste5 dispersal phenotype was blocked...".
(ii) I believe the figure citation should refer to Fig 1H, not Fig 1G.

I. Line 164: The Figure citation should refer to Supp Fig 2E and 2F (not 2D and 2E).

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Institute of Biochemistry

Zürich, May 31st, 2019

Dear Ian,

Please find enclosed the final version of our manuscript JCB #201808161R entitled "Mechanostress inhibits pheromone signaling via Pkc1-dependent regulation of the MAPK scaffold Ste5". We were happy that you and all reviewers agree that the revised manuscript has considerably improved, the major concerns have been addressed and the additional data substantiate the original claims. Nevertheless, two reviewers spotted some remaining and a few new issues and ambiguities that we have now fixed in this final version. Indeed, we apologize that a few errors remained uncorrected in our revised text, due to an unfortunate mix-up of corrected versions at the time of submission. In addition, we extended the statistical analysis and addressed the remaining issues by changing some of the Figures, Tables and Suppl. Material and including text changes in most cases as proposed by the reviewers. A detailed point-by-point response describing the changes and our response to the remaining reviewer comments is included below. We would like to state that we perceive some of the statements rather opinionated and with an unnecessary undertone.

The revised manuscript is now approximately 65 000 characters in the main text and includes 6 Figures, 5 Supplemental Figures and two Supplemental tables. We are convinced that these final changes further improved the manuscript, which we hope is now acceptable for publication in *Journal of Cell Biology*. All three reviewers agree that the reported results are highly relevant for a broad audience of scientists as they provide a novel and functionally important molecular mechanism showing how cells respond to external or internal mechanical stress conditions.

We appreciate the time and efforts that you and the reviewers spent to help improve the manuscript, and are looking forward to your reply. If you require further information, please do not hesitate to contact us.

With best regards,

Matthias and Frank

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Point-by-point response:

Reviewer 1:

1) The most important is that there is no statistical analysis of the data, in contrast to what is said in the text and mentioned in the rebuttal. What is standard is ANOVA followed by post-test comparisons, in a way that takes into account the number of comparisons made and the number of replicates. This should be described with enough detail in the method section.

As requested by the reviewer, we have included additional statistical analysis in the final Fig. 1, 5 and 6 and Suppl. Fig. 2, to demonstrate that the relevant changes are significant. Statistical significance was tested using the two-tailed Student's t-test in Microsoft Excel, and the results are shown in the Figure panels with *p*-values (***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$). In addition, we performed one way ANOVA (Analysis of Variance) for the data in Fig. 6D and G, where the data points are broadly spread. As the reviewer commented, it is followed by post-test comparison – All Pairwise Multiple Comparisons Procedures (Dunn's Method). The results are comparable to the ones from the t-test: the differences in the median values among the groups are greater than would be expected by chance - there is a statistically significant difference.

2) Fig 1G: There is a missing control of mechanical stress alone in WT cells. From panel to panel the number of WT cells that lyse after mechano-stress varies a lot: in Fig1B is 25%, in Fig 1C is 12%, in 1F seems to be less than 10%.

The "mechanical stress alone" wild type control relevant for Fig. 1G was in fact shown in Figure 1B. However, to avoid any confusion, we have now included an additional "mechanical stress alone" experiment performed along-side with the other conditions shown in the final Fig. 1G and combined with corresponding data from Fig. 1B. The number of wild type cells that lyse upon mechanical stress is affected by the genetic background (compare Fig. 1B and F) and the used growth conditions. For example, cells are more prone to lysis if grown in the presence of 2% glucose compared to those grown in 2% raffinose followed by 2% galactose used to induce expression of proteins from the *GAL1*-promoter (compare Fig. 1B and C). However, within comparable growth conditions and the same genetic background the % lysis of wild type cells remains within narrow margins and is highly reproducible (Fig. 1B and G).

3) In Supple Fig 2G: That the Bck1-20 constitutively active mutant is actually working as such (for example a blot showing highly phosphorylated Slt2.
This point is relevant to be able to assert, as the authors do, that the effect of Pkc1 is not mediated by Slt2.

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The Bck1-20 allele was characterized previously and shown to suppress a *PKC1* deletion phenotype (Lee and Levin, 1992). It has been widely used since then, but none of the papers demonstrated Mpk1 activation. In any event, it remains difficult to rigorously exclude that the strength of the Bck1-20 allele compared to $Pkc1^{R398A}$ contributes to the observed difference in Ste5-dispersal, and we have thus carefully worded our conclusions (lines 170-172). However, together with the experiments comparing Ste5 dispersal in wild type and *mpk1Δ* cells (revised Fig. 1H) and the Ste5-S185 mutant analysis (Fig. 5C and D), we think it is amply justified to conclude that this Pkc1-dependent Ste5 regulation is direct and not mediated by Mpk1.

4) About the scoring of Ste5 dispersal. The methods section presents two methods.

a) A direct method: "In order to do that, the intensities of 4x4 pixel (16 pixels square) in the shmoo and brightest area elsewhere were compared. "How was the scoring done? How much dispersal there has to be for a cell to be considered as "dispersed"? In the images provided in Supple Fig 2, more than dispersal, what I see is relocation of Ste5 to other areas away from the shmoo.
This difference (dispersal vs relocation) is important since the claim of the authors is that Pkc1 phosphorylates Ste5 and thus prevents it from localizing to the PM. But the images show that Ste5 is still able to accumulate at the PM, maybe in another location, where a new shmoo would form.

b) An indirect method: "When Ste5 dispersal was analyzed in 96-well plates, the arrest in shmoo growth was also considered as dispersal due to focus issue."
First, it is not clear to which experiments this alternative criterion was applied.
Second, to equal lack of growth to Ste5 dispersal is not demonstrated, and it might not be the same. I doubt that data analyzed this way could be considered a proxy of Ste5 dispersal.

We have intentionally used a direct and indirect method to establish that expression of $Pkc1^{R398A}$ but not Bck1-20 interferes with polarized Ste5 localization and shmoo formation (Suppl. Fig. 2, panels E, F and G). We believe that the two approaches using different phenotypic readouts strongly complement each other, although we do not have formal proof that the observed Ste5 dispersal and lack of shmoo formation are indeed caused by the same mechanism. But in light of the comprehensive results described in this manuscript, it is the most likely explanation.

The direct method: Ste5-GFP dispersal was quantified as described by reduced Ste5 intensity at shmoo tips compared to cytoplasmic regions, and we expanded the method description in the revised Method section. Upon mechanical stress, it is correct that in a fraction of cells, Ste5 accumulates in patches at other regions of the cell cortex (Suppl. Fig. 2C). We now highlighted such patches with arrow head and explicitly mention this fact in the revised legend. Moreover, we marked dead cells to clarify their absence in the

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GFP images. We do not think however that this observation speaks against a Pkc1-dependent mechanism. First, unlike Ste5 at shmoo tips, these cortical Ste5 patches are transient and assemble and disassemble within minutes, in accordance with previously published data (Hegemann et al., 2015) (Wu et al., 2015). Second, we don't know whether this membrane recruitment is dependent on the interaction of Ste5 with Gbeta gamma. Third, we have shown earlier (Mishra et al., 2017) that Pkc1-GFP relocalizes from shmoo tips to a more uniform but somewhat patchy cortical distribution upon mechanical stress. We have no information of the S185 phosphorylation status of Ste5 recruited to these secondary sites, which may well accumulate at sites with low Pkc1 activity. Finally, in cells overexpressing Pkc1^{R398A} before addition of pheromones, Ste5-tV is rarely found at other cortical sites and shmoo formation is strongly inhibited (Suppl. Fig. 2E-F). Taken together, we strongly believe that the effects of Ste5 dispersal triggered by mechanical stress or overexpression of Pkc1^{R398A} are consistent with the reported findings.

The indirect method: We found that overexpressed Pkc1^{R398A} but not Bck1-20 prevents efficient shmoo formation, which is consistent with the fact that it inhibits localization of Ste5 to the site of polarization. As indicated by the reviewer, we have no experimental proof that the observed Ste5 dispersal and lack of shmoo formation are indeed caused by the same mechanism, but we consider this possibility as the most likely explanation. To avoid any confusion, we have clarified the data underlying Fig. 1G, which quantified the number of cells that show Ste5-dispersal after application of mechano-stress determined by fluorescence microscopy. In this analysis, the intensities of 4x4 pixel (16 pixels square) in the shmoo and brightest area elsewhere in the cell body were compared and the cells that exhibit lower intensities in the shmoo within 30 minutes of applying mechano-stress were scored as Ste5 dispersed cells. In contrast, in Suppl. Fig. 2D, cells with pronounced enrichment of Ste5-tV at shmoo tips were monitored every 10 minutes for 2 hours. Cells that lose their polarized Ste5-tV enrichment within 2 hours of Pkc1^{K398A} induction were scored manually as cells with Ste5 dispersal. Loss of Ste5-tV from the shmoo tip was further confirmed by the fact that these cells stop polarized growth. Finally, Suppl. Fig. 2F and G quantifies the shmooing/polarization defect caused by expression of Pkc1^{R398A} but not Bck1-20 using morphology analysis based on phase-contrast imaging. We have now explained the rational and detailed description of these methods in the expanded Material and Methods section.

Minor:

1) *Fig 1E: it seems that the graph is backwards, since the protected cells show higher Fus3 activity than the lysing...*

Thanks - we have corrected the labeling in Fig. 1E.

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2) Fig 1H: when was "dispersal" measured. In the legend it seems that it was measured continuously over 30'. So, how fast is "rapidly"? The text says: "Venus-tagged Ste5 (Ste5-tV) accumulated at the tips of mating projections in absence of stress, Ste5-tV was rapidly dispersed upon mechanical pressure (Fig. 1H)."

Ste5 dispersal was monitored over 30 minutes, but typically observed already 10 - 20 minutes after application of mechano-stress. In fact, dispersal starts already after 10 minutes, but complete dispersal takes a bit longer. Besides, the response is heterogeneous between cells. In our analyses, we have counted the fractions of cells that disperse Ste5-tV within 30 minutes of mechano-stress. To avoid any misunderstandings, we have now removed the word “rapidly” in the final sentence.

3) *In line 160, it seems to me that the panel to be called should be Fig 1H, not Fig 1G.*

We have changed the sentence to unequivocally refer to the correct panels.

4) Suppl Fig2C: images are not clear. Please improve. And accompanying brightfield images would help.

We have now labeled cells that die during the experiment with a white dot and also highlight cells where Ste5-tV accumulates transiently at cortical sites other than the shmoo tips (arrow). Finally, we adjusted the Figure legend accordingly.

5) *About Far1 phosphorylation, the text says:*
"MS/MS analysis of purified Far1 confirmed that S210 and possibly also S208 and S211 in its RING-H2 domain were phosphorylated in cells exposed to α-factor". Since there were no analysis of cells NOT treated with alpha factor, this should be said in such a way as to not lead to the notion that alpha factor stimulates the phosphorylation of these sites. Note that this comment also applies to Ste5.

We do not claim that the reported sites are alfa factor-dependent as we have not quantitatively compared the phosphorylation pattern in cells treated or not with alfa factor. However, as indicated in the corresponding Figure legends and the Materials and Method section, we have deposited all MS-experiments in at the ProteomeXchange Consortium (http://www.proteomexchange.org) via the PRoteomics IDEntifications (PRIDE) partner repository with the dataset identifier PXD004657 (*Reviewer account details: Username:* reviewer75034@ebi.ac.uk*; Password:* nNyx1Sn0). Thus the reviewer and interested readers are invited to check which sites were detected under both conditions. All sites indicated in the manuscript are found in alfa-factor treated cells, as stated in the text.

6) Fig 5D, in the y-axis there is a mistake in the numbers: it should be 15 instead of 50.

Thanks – this error is now corrected.

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7) Fig 6C, there are no error bars in the measurements...

As requested, we have now included error bars in these image-based measurements.

Reviewer 3:

1) Regarding my original point 6, involving the two-hybrid assays in Figure S5A: First, the authors have still not fixed the direct conflict between the Figure S5A legend and the strain list in Table I. The Figure legend says Ste4 is fused to the DBD and Far1 is fused to the AD, whereas Table I implies the reverse. This is now the third time I have pointed this out.

We apologize for the fact that the discrepancy in the Figure legend and Table I concerning whether Far1 and Ste4 were expressed as DBD or AD fusions remained uncorrected (despite our claims otherwise). As indicated above, this mistake occurred by an undetected mix-up of the revised versions during uploading. This discrepancy is now corrected in the final version.

*2) Second, the authors respond by stating: "We have performed the two-hybrid assays with all controls, and it is clear that the effect is not caused by transcriptional activation of the DBD fusion." I am unsure which "effect" the authors are commenting on here, but I certainly cannot agree that anything has been made "clear" by data that is not shown. Instead, I am skeptical. The key concern about these data is as follows: If the basal signal (i.e., without any AD fusion) for the Far1-DBD fusion was half as strong for 3D mutant as it is for wild-type, then the similar fold drop in the signal observed with the AD-Ste4 fusion (in Fig S5A) *cannot* be interpreted as a change in Far1-Ste4 interaction, and instead it can just reflect a change in the starting basal value. So, if the assays truly included "all controls", as the authors state, then it would be a trivial matter to include those controls in the figure (i.e., legibly show the signal measured for each DBD fusion with no AD-Ste4). I suggest one of two options: either add the controls, or remove these data. Omitting relevant controls is unacceptable, especially given the ongoing uncertainties about how the experiment was performed, which the authors have repeatedly failed to clarify.*

We have now included an image of the two hybrid measurements which also contains the requested controls (final Suppl. Fig. 5A). However, since these low background numbers were subtracted from the actual measurements to correct for autoactivation, we have not included the basal levels in the graph. We have improved the description of how the two-hybrid data were quantified in the Method section to avoid any confusion. Finally, we do not fully understand why the Far1-S3A and S3D mutants should not serve as internal controls (and argue against the interpretation that the positive measurements are solely

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caused by autoactivation of the DBD-RING fusions), unless the introduced D- but not A-mutations would also affect its basal transcriptional activation capacity. Nevertheless, we hope that including the basal controls now validates the two-hybrid measurements. We acknowledge that biochemical assays would be needed to further substantiate our conclusion that a similar Pkc1-dependent mechanism regulates Far1, but this was not the focus of the manuscript.

3) Further compounding the murkiness, none of the relevant strains in Table I list any reporter for detecting the interaction signal (i.e., a lacZ gene under control of the lexA operator), and it is also unclear how the DBD-Far1 and AD-Ste4 fusions are introduced (i.e., on plasmids? integrated? with what markers?).

The used two-hybrid strain with the integrated lacZ gene is now listed in Table I. The plasmids expressing the DBD-Far1 and AD-Ste4 fusion proteins are listed in Table II, together with the selection markers.

4) Regarding my original point 7d, the authors provide this response:
*"The paper by (Bhattacharyya et al., 2006) claims that Fus3-dependent phosphorylation of T287 located within the MAP-kinase docking site allosterically activates Ste5." No, the Bhattacharyya paper does not claim this. This statement is wrong in multiple ways, and the authors seem to be conflating distinct phenomena. First, that paper does not study allosteric activation of Ste5, but rather allosteric activation of Fus3 (by Ste5). Second, the paper explicitly states that T287 phosphorylation is not required for the allosteric activation of Fus3 by Ste5 (see the "conclusion" statement in the legend to their Figure S9). Third, they do claim that T287 phosphorylation has a regulatory effect, but the effect is *inhibitory*, which is the *opposite* of the authors' claim that it "activates Ste5". Fourth, the mechanism of this inhibition was not determined by Bhattacharyya et al, who did NOT claim that it was an "allosteric" effect but instead speculated that it might be due to effects on "turnover" or "trafficking".*

If the authors want to relate their results to those of the Bhattacharyya paper, I suggest that a statement along the following lines would be accurate: "Our mass spectrometry uncovered phosphorylation at four sites near the MAPK-docking site in Ste5 that were previously proposed to be targeted by Fus3 as part of a negative feedback circuit (Bhattacharyya 2006, Malleshaiah 2010)." (However, the authors might want to note that recent findings question whether these phosphorylations are truly inhibitory; see PubMed ID # 30726174.)

We agree with the reviewer that the sentence referring to the Bhattacharyya paper was misleading, and have thus rephrased the section essentially as suggested. We were not aware of the only recently published results that question the negative feedback. We have revised the paragraph and also included the most recent complications. The final section

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now reads: "*Our mass spectrometry uncovered phosphorylation of four sites near the MAPK-docking site in Ste5, including T287 which was previously proposed to be targeted by Fus3 as part of a negative feedback circuit(Bhattacharyya et al., 2006),(Malleshaiah et al., 2010), although this conclusion was recently challenged (Winters and Pryciak, 2019). All four sites are followed by prolines, including S276 located on the same tryptic peptide as T287. It is thus possible that analogous to T287, phosphorylation of these MAP kinase consensus sites may allosterically contribute to negative feedback regulation in the pheromone signaling pathway.*"

5) Regarding the authors' response to my original point 7e:
"In this experiment (Fig. 4.E), we only observe 15N-labelled Ste5. This construct of Ste5 was prepared without a GST-tag and the His-tag used for the purification was removed. GST-tagged Ste4/18 heterodimers were produced in an NMR-invisible manner (no labeling with 15N-containing salts). Therefore, all the changes detected in the NMR spectra originate from Ste5. The overall reorganization of the peaks in the spectrum suggests prominent conformational rearrangement, however the number of peaks is roughly doubled which we attribute to the formation of the so-called asymmetric dimer."

This response missed the point. The factual statements are true (and I understood them before), but they do not address the issue raised. As I explained previously, because the GST moiety on Ste4/Ste18 can homo-dimerize, it is possible for two Ste5 RING-H2 domains to bind to two adjacent Ste4/Ste18 complexes (i.e., that are artificially linked together by a GST-GST interaction). As a consequence, this could allow interactions between two adjacent RING-H2 domains (and hence the observed asymmetric conformational changes) that would not ordinarily occur when a single RING-H2 domain binds to a single Ste4/Ste18 complex. Therefore, the observed asymmetric peak doubling of the Ste5 RING-H2 domain does not necessarily occur "while binding to Ste4", as stated, but instead it could be an artificial consequence of binding to a ligand that has been forcibly dimerized.

I suggest that an accurate description of the results would include a statement along the following lines: "...it is unknown whether the asymmetric RING-H2 oligomer is bound to a single Ste4/18 complex or to two complexes that are linked by the homodimerizing GST moiety."

We indeed misunderstood the reviewer's concern, and as suggested changed the final text as follows: *"...it is unknown whether the asymmetric RING-H2 oligomer is bound to a single Ste4/18 complex or to two complexes that are linked by the homodimerizing GST moiety."*

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6) Regarding the response to point 7f:
"We have now included the citation that describes phosphorylation of Gpa1 by Fus3/Kss1 (Choudhury et al., 2018)." No, that is incorrect. The Choudhury paper reports phosphorylation of Ste18, not Gpa1. It should be obvious that these are different proteins. Even the most cursory glance at that paper should have allowed the authors to recognize their error - it's stated directly in the Title!
Also, Ste18 is a membrane-tethered protein, so it is not a "cytoplasmic target" (as stated on line 100).

We have corrected this error, as we have incorrectly referred to phosphorylation of Gpa1 instead of Ste18, which is indeed not a cytoplasmic target. Thus, the corrected sentence now reads as follows: “Activated Fus3 and Kss1 phosphorylate cytoplasmic and cell membrane tethered targets including Ste18 and Far1 to promote cell cycle arrest, and translocate into the nucleus to inactivate the repressors Dig1 and Dig2, thereby liberating the transcription factor Ste12 to initiate a specific transcriptional program.”

7) Regarding the response to point 8e:
"We have corrected the relevant information in the revised Material and Method section and Table II. Briefly, Gβγ (Ste4/Ste18) was purified as a GST-fusion..." In fact, Table II still erroneously lists the Ste18/Ste4 plasmid as a 6His fusion, rather than a GST fusion. It really should not be so difficult to correct such simple errors.

As described above, we apologize for this repeated error that occurred again because of a last-minute switch back to a previous version. The error has been corrected.

8) In Tables I-II, the column listing the Figure numbers is helpful. But what are we to make of the entries without any Figure number listed (1 plasmid and 11 strains)? It appears that the authors simply did not finish constructing the table.

These strains served as parental strains for further strain constructions such as integration or transformation of plasmids, and this is now explicitly mentioned in Table I. We now list all strains with integrated plasmids separately and refer to the corresponding Figures where they were used. We also expanded the list of experiments that the different strains and plasmids were used.

9) Lines 145-146: "...the lysis defect in Pkc1-inhibited cells...". This is potentially confusing because the phrase "lysis defect" seems to imply a defect in lysis, but I think the authors mean the reverse. It would probably be clearer to replace "defect" with "observed", so that it reads "... the lysis observed in Pkc1-inhibited cells...".

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We have corrected this potential confusion, and the sentence now reads as suggested: "….the lysis observed in Pkc1-inhibited cells ….".

10) Lines 159-160: "This phenotype was blocked by addition of cercosporamide but independent of Mpk1 (Fig. 1G, Suppl. Fig. 2C)...". There are two issues here that need fixing:
(i) The phrase "this phenotype" appears to refer to the Pkc1 and Mpk1 phenotype in the preceding sentence, but I don't think that is the intention. Instead, this phrase should say something like "The Ste5 dispersal phenotype was blocked...".
(ii) I believe the figure citation should refer to Fig 1H, not Fig 1G.

We have adjusted the sentence as suggested and changed the Figure citation.

11) Line 164: The Figure citation should refer to Supp Fig 2E and 2F (not 2D and 2E).

We have changed the Figure citation accordingly.

References:

Bhattacharyya, R.P., A. Remenyi, M.C. Good, C.J. Bashor, A.M. Falick, and W.A. Lim. 2006. The Ste5 scaffold allosterically modulates signaling output of the yeast mating pathway. *Science*. 311:822-826.

Hegemann, B., M. Unger, S.S. Lee, I. Stoffel-Studer, J. van den Heuvel, S. Pelet, H. Koeppl, and M. Peter. 2015. A Cellular System for Spatial Signal Decoding in Chemical Gradients. *Dev Cell*. 35:458-470.

Lee, K.S., and D.E. Levin. 1992. Dominant mutations in a gene encoding a putative protein kinase (BCK1) bypass the requirement for a Saccharomyces cerevisiae protein kinase C homolog. *Mol Cell Biol*. 12:172-182.

Malleshaiah, M.K., V. Shahrezaei, P.S. Swain, and S.W. Michnick. 2010. The scaffold protein Ste5 directly controls a switch-like mating decision in yeast. *Nature*. 465:101-105.

Mishra, R., F. van Drogen, R. Dechant, S. Oh, N.L. Jeon, S.S. Lee, and M. Peter. 2017. Protein kinase C and calcineurin cooperatively mediate cell survival under compressive mechanical stress. *Proc Natl Acad Sci U S A*. 114:13471-13476.

Winters, M.J., and P.M. Pryciak. 2019. MAPK modulation of yeast pheromone signaling output and the role of phosphorylation sites in the scaffold protein Ste5. *Mol Biol Cell*. 30:1037-1049.

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Wu, C.F., J.G. Chiou, M. Minakova, B. Woods, D. Tsygankov, T.R. Zyla, N.S. Savage, T.C. Elston, and D.J. Lew. 2015. Role of competition between polarity sites in establishing a unique front. *Elife*. 4.

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June 4, 2019

RE: JCB Manuscript #201808161RR

Prof. Matthias Peter
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Dear Prof. Peter:

Thank you for submitting your revised manuscript entitled "Mechano-stress inhibits pheromone signaling via Pkc1-dependent regulation of the MAPK scaffold Ste5". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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* We suggest a slight edit of your title to: "Mechanical stress impairs pheromone signaling via Pkc1-mediated regulation of the MAPK scaffold Ste5" *

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