JCB

Gradient tracking in mating yeast depends on Bud1 inactivation and actin-independent vesicle delivery

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March 31, 2022

Re: JCB manuscript #202203004

Dr. David E Stone
University of Illinois at Chicago
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Molecular Biology Research Building
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Chicago, Illinois 60607

Dear David - we have now received two external reviews of your manuscript "Mobility of the gradient tracking machine in mating yeast depends on Bud1 inactivation and actin-independent vesicle delivery." Both referees acknowledge interest in the data and proposed mechanisms, but each also notes significant issues that would have to be addressed by further experiments. For this reason we are unfortunately unable to accept the manuscript in its present form. However, we would be willing to consider a suitably revised manuscript that addresses the key points raised in the reviews.

In particular, reviewer #1, who was more supportive, notes that the use of the exo70- DdC is inappropriate because it has multiple defects (with Rho3 and Cdc42 as well as with Bem1), and they recommend the use of a more specific mutant such as Exo70-M30 (Liu & Novick). The same point is also raised by the second reviewer, who suggests using the same mutant. Reviewer #1 also asks for quantitative analysis of Figure 3, and a consideration of the effects of dilution of polarity factors by vesicle fusion events, as they might be an important factor in moving the GTM during actin-independent vesicle delivery.

Reviewer #2 is concerned about conceptual novelty, but also raises a number of specific issues with the data. They note, for example, that the phenotypes described in panel 2D show that the ratio of cells that mate from their default site to those that show a mobile site is quite similar in WT and cells expressing constitutively active Bud1, which does not argue for the default site being stuck. Figure 2G reports the pause time in WT vs bud1Δ, showing that pause is shorter or inexistent in bud1Δ. This quantification is problematic and impossible to interpret without a clear explanation for how pause time is measured. Overall, this reviewer argues that the provided data do not convince that Bud1 inactivation is required for site mobility. Other comments concern the actin-independence, and there are several issues raised with the data that would need to be addressed, or that appear to conflict with previous observations, in addition to questions about conceptual novelty of this process. This reviewer also feels that the manuscript does not acknowledge other groups working in this area, or provide a more balanced view of current models.

Overall, we feel that the study is of potential interest but will require substantial additional experimental work, in addition to modifications to the text. If you choose to submit a suitably revised version, we will need a point-by-point response to each of the reviewer comments, and the manuscript will be re-evaluated by the two external reviewers.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

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Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

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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,

Ian Macara, Ph.D.
Editor

Andrea L. Marat, Ph.D.
Senior Scientific Editor

Journal of Cell Biology

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

This manuscript uses careful time-lapse microscopy to visualize the assembly of the gradient tracking machine (GTM) at the designated polarity site followed by movement away from the DS towards the mating partner independent of Myo2 and actin. Previously, the Stone lab used this method to visualize the assembly and distribution of Ste2 and other components of the GTM before and during chemotropism and mating. In the current manuscript, Wang, Pai, and Stone build on their recent paper to more precisely characterize the steps and machinery driving polarization of the GTM from the DS to the CS.

The study presents three mechanisms that explain how GTM localization is driven away from the DS. First, Bud1 inactivation is required to leave the DS. Bud5 disappears from the neck and is not detectable at the DS, whereas Bud2 polarizes to the DS and tracks with the GTM to the CS. Second, markers for actin-dependent vesicle delivery do not track with, and are not required for, GTM movement towards the CS. Interestingly, Myo2 repolarizes later at the established CS. Third, direct interaction between Bem1 and Exo70 is important for tracking and suggests that actin-independent vesicle delivery mediated by the exocyst is required for moving the GTM. If fully explored, these results would be a milestone in understanding how polarization of trafficking machinery responds to external cues.

Overall, the manuscript is clearly written and most of the conclusions are well supported by the data. My main concern with this work is the analysis of the Bem1-exocyst interaction in actin-independent vesicle delivery that the authors propose is critical to the gradient-dependent movement of the GTM. In particular the authors have chosen an allele of exo70 (exo70- C) for their analyses which has been reported to have possible defects in interactions with both Rho3 and Cdc42 GTPases in addition to possible defects in interaction with the Bem1 scaffold. Besides the fact this mutant contains a large deletion in the middle of structure, the effects of this deletion are controversial as to their effects on Rho/Cdc42 binding. In contrast, in their 2014 JCB paper Liu and Novick identify an allele of Exo70 (exo70-M30) that would be a substantial improvement for these studies. Rather

than a large deletion, the exo70-M30 mutant contains several charged-to-alanine point mutations which have no effect on Rho GTPase or PI(4,5)P2 binding, but shows almost no detectable binding to Bem1 in vitro. Importantly, unlike the exo70- C mutant, this allele shows no synthetic lethality with the sec3- N allele.

Given the likely pleiotropic nature of the exo70- C mutant defects, I do not think the authors can conclude that the defects observed in gradient tracking in this mutant are due solely to defects in Bem1-exocyst interactions (which is the most important conclusion of the paper). Therefore I would strongly suggest that they repeat this analysis with the exo70-M30 mutant which specifically targets the Bem1 interaction but appears to retain the other roles of the Exo70 C-domain. Furthermore, this allele can be used in combination with a sec3- N mutant to test their hypothesis of redundancy between Sec3 and Exo70 in driving AI-VD -an experiment that the authors nicely describe in the discussion.

Additional points:
1. One of the strengths of this work is the quantitative analysis of these results. However, Figure 3 lacks quantitation.
2. In the Figure 8 model and the discussion, the effect of dilution of polarity factors by vesicle fusion events (Ghose &Lew, MBoC, 2020) should be considered as they could play an important role in moving the GTM during actin-independent vesicle delivery.
3. It appears from the materials and methods that the authors have re-created many of the mutant alleles first described by other labs. I just wanted to point out (especially for the exo70-M30 allele) that the other labs in this field are supported by NIH grants which requires them to adhere to a resource sharing plan to share all plasmids, mutants, strains generated from this support. In addition, the yeast community is normally exceedingly generous in this regard and they should not hesitate to ask for these reagents rather than wasting time and resources remaking them.

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

This manuscript by David Stone and colleagues examines the mechanisms of chemotropism during mating in the budding yeast, when cells reorient their pheromone sensing and polarity site towards a pheromone gradient source. The manuscript is an extension of previous work by the Stone lab, who had proposed that yeast cells assemble a gradient tracking machine first at a default site next to the previous division site, which pauses there until deterministic tracking starts towards the end point where growth projection (shmoo) occurs. The manuscript makes two main claims. First, it claims that initiation of tracking requires inactivation of the Ras-like GTPase Bud1/Rsr1. Second, it claims that mobility of the site requires actin-independent delivery of secretory vesicles. Overall, the provided data are interesting and largely supportive of the second claim (though this needs to be strengthened - see below) but fail to convince about the first one.

Bud1 inactivation:
Figure 1 clearly shows presence of Bud1 GAP Bud2 at the polarity site, but absence of detection of the Bud1 GEF Bud5. A marker for Bud1-GTP would be more convincing, but this is suggestive that Bud1 is inactive at the mobile site. However, absence of signal is not proof of absence. The real question is whether constitutively active Bud1 compromises mating, which is addressed in Figure 2, and here the data is not in line with how it is described in the text:
- Even though the text states that "Bud1 must be inactivated to enable tracking", the phenotypes described in panel 2D show that the ratio of cells that mate from their default site to those that show a mobile site is quite similar in WT and cells expressing constitutively active Bud1, which does not argue for the default site being stuck.
- In panel 2C, 70% of cells expressing constitutively active Bud1 do mate. Of the 30% that do not mate, the authors explain that two distinct phenotypes are observed, shown in Figure 2A and 2B. The 2B example would be a clear example of cells with defect in orientation. By contrast, in Figure 2A, the example shows a cell that keeps budding instead of shmooing (and so does the WT partner). If the cell buds, it has progressed from G1 to S phase in the cell cycle, and is thus in a phase that is not permissive to pheromone production. This indicates that the Bud1-G12V-expressing cell does not arrest in G1 phase, and therefore cannot differentiate for mating, which is a very different phenotype from one affecting growth position. The authors do not report the frequency of each of these two phenotypes, but they cannot be aggregated.
- Figure 2E-F comparing Ste2 expression levels are not comparing the same time point in WT and mutant. For a valid comparison, shmooing time should be used for both (or fusion time), not tracking time vs shmooing time.
- Figure 2G reports the pause time in WT vs bud1Δ, showing that pause is shorter or inexistent in bud1Δ. This quantification is problematic and impossible to interpret without a clear explanation for how pause time is measured. Is there a marker for the different stages? From the examples shown in Fig 1, it seems very arbitrary. The comparison is further complicated by the fact that bud1Δ cells do not assemble a default site.
Thus, the provided data does not convince that that Bud1 inactivation is required for site mobility.

Actin-independent vesicle delivery:
Figures 3 and 4 show complementary evidence that deleting a region of Bem1 or Exo70 that binds the other protein compromises site mobility. This is an interesting finding, which should be strengthened to make it compelling.
- First, the mutants used are rather coarse ones, removing a large chunk of the protein, which may affect other interactions. For

instance, the exo70ΔC allele also blocks binding to Rho3. Use of more specific mutants, characterized to specifically block the Bem1-Exo70 interaction would be more convincing. Such mutants have been described by Liu and Novick, JCB 2014 (for instance exo70M26 or better exo70M30).
- In Fig 3B, shmoo formation is not evident. Could you please show the whole mating process up to the point of fusion? This figure also lacks quantifications (equivalent to Figure 4C-D).
- The data shows a role for Exo70, but, at this point in the manuscript, there is no evidence that it is actin-independent. This should be rephrased (end of section on Figure 4).

Figures 5 and 6 examine the actin-independence of the process, showing that the type V myosin Myo2 is absent from mobile sites, that the actin cable marker Abp140 is also poorly detectable, and that inactivation of Myo5 in the myo2-16 allele does not abrogate site mobility. The Myo2 localization is clear, though the authors should be careful is their statement, as absence of evidence is not evidence of absence. The myo2-16 phenotype is a bit more difficult to interpret, as many cells do not display a polarity site, though it is clear that a substantial fraction of cells still display site mobility. To probe the function of Myo2 specifically during tracking, a good approach would be to change temperature on the microscope after site assembly.

The claimed absence of actin cables is less convincing. The Abp140 images only have cortical dots pretty much all around the cell. It is hard the deduce anything about the assembly of actin cable from these images. This should be substantiated by investigating localization and function of formins (of which there are very tight ts mutants).

Though I don't think there were strong previous claims that secretory vesicle delivery depends on actin during site motility, the data here seems to conflict with previous observations. For instance, the Lew lab showed that Spa2 (a component of the formin-associated polarisome) leads the mobile patch in pheromone-exposed cells (McClure et al, 2015). Ghose et al 2020 further showed colocalization of Spa2 with Bni1 formin. The Martin lab showed that both exocyst and type V myosin are present on mobile patches in the fission yeast cells (Bendezu et al, 2013). These differences should be at least discussed. Because the myo2-16 phenotype is a bit difficult to interpret and because blocking Bem1-Exo70 interaction does not completely block site mobility, it is also possible that exocyst-based tethering and actin-based vesicle delivery both contribute to polarity site movement.

More globally, I find regrettable that the introduction and discussion do not present a more balanced view of chemotropism. There are strong disagreements in the field, on how deterministic vs more stochastic the search for a partner may be. Disagreement can be a strong motor of scientific progress, but unfortunately neither side appears to acknowledge the other. This manuscript does not cite a single paper from the Lew lab on mating, nor any mention of similar phenomenon in the fission yeast. This is highly detrimental to an open discussion.

From: jcellbiol@msubmit.net
Subject: JCB Manuscript - Editorial Decision 202203004

Date: March 31, 2022 at 5:13 PM **To:** dstone@uic.edu

Dear Drs. Macara and Marat,

Once again, thank you for providing us the opportunity to resubmit our manuscript about gradient tracking in budding yeast (#202203004). We would also like to thank the reviewers for their careful and helpful evaluation of our work. In answering their comments, we believe we have made considerable improvements to our study. Please see the point-by-point responses below.

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

This manuscript uses careful time-lapse microscopy to visualize the assembly of the gradient tracking machine (GTM) at the designated polarity site followed by movement away from the DS towards the mating partner independent of Myo2 and actin. Previously, the Stone lab used this method to visualize the assembly and distribution of Ste2 and other components of the GTM before and during chemotropism and mating. In the current manuscript, Wang, Pai, and Stone build on their recent paper to more precisely characterize the steps and machinery driving polarization of the GTM from the DS to the CS.

The study presents three mechanisms that explain how GTM localization is driven away from the DS. First, Bud1 inactivation is required to leave the DS. Bud5 disappears from the neck and is not detectable at the DS, whereas Bud2 polarizes to the DS and tracks with the GTM to the CS. Second, markers for actin-dependent vesicle delivery do not track with, and are not required for, GTM movement towards the CS. Interestingly, Myo2 repolarizes later at the established CS. Third, direct interaction between Bem1 and Exo70 is important for tracking and suggests that actin-independent vesicle delivery

Exo70 is important for tracking and suggests that actin-independent vesicle delivery mediated by the exocyst is required for moving the GTM. If fully explored, these results would be a milestone in understanding how polarization of trafficking machinery responds to external cues.

Overall, the manuscript is clearly written and most of the conclusions are well supported by the data. My main concern with this work is the analysis of the Bem1-exocyst interaction in actin-independent vesicle delivery that the authors propose is critical to the gradient-dependent movement of the GTM. In particular the authors have chosen an allele of exo70 (exo70-1C) for their analyses which has been reported to have possible defects in interactions with both Rho3 and Cdc42 GTPases in addition to possible defects in interaction with the Bem1 scaffold. Besides the fact this mutant contains a

large deletion in the middle of structure, the effects of this deletion are controversial as to their effects on Rho/Cdc42 binding. In contrast, in their 2014 JCB paper Liu and Novick identify an allele of Exo70 (exo70-M30) that would be a substantial improvement for these studies. Rather than a large deletion, the exo70-M30 mutant contains several charged-to-alanine point mutations which have no effect on Rho GTPase or PI(4,5)P2 binding, but shows almost no detectable binding to Bem1 in vitro. Importantly, unlike the exo70-2C mutant, this allele shows no synthetic lethality with the sec3-2N allele.

Given the likely pleiotropic nature of the exo70-2C mutant defects, I do not think the authors can conclude that the defects observed in gradient tracking in this mutant are due solely to defects in Bem1-exocyst interactions (which is the most important conclusion of the paper). Therefore I would strongly suggest that they repeat this analysis with the exo70-M30 mutant which specifically targets the Bem1 interaction but appears to retain the other roles of the Exo70 C-domain. Furthermore, this allele can be used in combination with a sec3-2N mutant to test their hypothesis of redundancy between Sec3 and Exo70 in driving AI-VD -an experiment that the authors nicely describe in the discussion.

Response: We fully agree with this point and greatly appreciate the reviewer's suggestion. In the revised manuscript, we analyze the effects of *exo70^{M30}*, *sec3$^{\Delta N}$*, and *exo70^{M30} sec3$^{\Delta N}$* double mutation on gradient sensing. The results, which strongly support our claim that AI-VD is essential for tracking, are reported in Fig. 4 and the corresponding text.

Additional points:
1. One of the strengths of this work is the quantitative analysis of these results. However, Figure 3 lacks quantitation.

Response: We've added the requested quantitation in new Fig. 3 panels C and D, as well as the following statement in the corresponding text. "Considering both the cells that ignored potential partners and those that formed zygotes, about 2% of the *bem1$^{\Delta CPX}$* mutants exhibited Ste2-GFP tracking (Fig. 3C and D)." This value was calculated by multiplying the percent of cells that mated (38.8) by the percent of mating cells that tracked (5.9%).

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2. In the Figure 8 model and the discussion, the effect of dilution of polarity factors by vesicle fusion events (Ghose &Lew, MBoC, 2020) should be considered as they could play an important role in moving the GTM during actin-independent vesicle delivery.

Response: Having carefully considered this comment, we respectfully disagree with the reviewer. In the revised manuscript, we describe the biased wandering models of yeast gradient sensing proposed by Lew and colleagues and Peter and colleagues, and we compare them to each other and to our deterministic model. We also discuss the evidence on which these models are based (see paragraph 2 on p. 4, the following paragraph on p. 5, and the paragraph that begins on p. 16). Lew et al. have focused on

what causes the polarity complex to move, and particularly, what causes it to move persistently in one direction. Their conclusion – that the polarity complex is driven forward by vesicles arriving just behind its center – is largely based on following Bem1-GFP and Spa2-mCherry in cells treated with various doses of isotropic pheromone, or in cells whose pheromone response is activated internally by Ste5-CTM. In contrast, we have focused on the redistribution of the sensory apparatus – the receptor and its regulators (Yck1/2, Sla2), its G protein, a G-protein regulator (Sst2), a G-protein effector (Far1), and an exocyst component (Sec3) – in mating cells (i.e., cells responding to physiological pheromone gradients). What happens at the level of the polarity complex and what happens at the level of the receptor are different questions. Notably, when we follow Bem1-GFP in mating cells (Pai and Stone, unpublished data), we see the same biased wandering reported in the Lew lab papers. Conversely, I expect that Lew et al. would see the deterministic tracking we report if they followed sensory-level reporters in mating cells.

In the Ghose and Lew paper referenced by the reviewer, the authors are once again interested in "spontaneous polarity site movement" in cells activated by Ste5-CTM (i.e., not by pheromone). Based almost entirely on output from a computational model, they conclude that tight spatial localization of exocytosis might enhance the directional persistence of polarity site movement and that vesicular delivery of Cdc42 GAP proteins might increase the distance moved in each step. These conclusions are in no way at odds with ours. In fact, our model also calls for focused positioning of the exocyst and controlled Cdc42 activity during GTM tracking. We simply feel that, given the great differences in experimental approach, the Ghose & Lew paper is not immediately relevant to the work we describe in this manuscript. If the reviewer remains unconvinced, we can add a few sentences to our Discussion citing Ghose & Lew.

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3. It appears from the materials and methods that the authors have re-created many of the mutant alleles first described by other labs. I just wanted to point out (especially for the exo70-M30 allele) that the other labs in this field are supported by NIH grants which requires them to adhere to a resource sharing plan to share all plasmids, mutants, strains generated from this support. In addition, the yeast community is normally exceedingly generous in this regard and they should not hesitate to ask for these reagents rather than wasting time and resources remaking them.

Response: The reviewer's point is well taken. By way of explanation, we like to keep our genetic background constant, and for the cost of some primers and sequencing, it is sometimes more efficacious to knock-in the desired mutations than to wait for the shipment of strains and plasmids. That said, our request for the *exo70-M30* allele from Dr. Novick was answered within 12 hours and we had the plasmid within a week.

Reviewer #2 (Comments to the Authors (Required)):
This manuscript by David Stone and colleagues examines the mechanisms of chemotropism during mating in the budding yeast, when cells reorient their pheromone sensing and polarity site towards a pheromone gradient source. The manuscript is an extension of previous work by the Stone lab, who had proposed that yeast cells

assemble a gradient tracking machine first at a default site next to the previous division site, which pauses there until deterministic tracking starts towards the end point where growth projection (shmoo) occurs. The manuscript makes two main claims. First, it claims that initiation of tracking requires inactivation of the Ras-like GTPase Bud1/Rsr1. Second, it claims that mobility of the site requires actin-independent delivery of secretory vesicles. Overall, the provided data are interesting and largely supportive of the second claim (though this needs to be strengthened - see below) but fail to convince about the first one.

Bud1 inactivation:
Figure 1 clearly shows presence of Bud1 GAP Bud2 at the polarity site, but absence of detection of the Bud1 GEF Bud5. A marker for Bud1-GTP would be more convincing, but this is suggestive that Bud1 is inactive at the mobile site. However, absence of signal is not proof of absence.

Response: To our knowledge, there is no published reporter for active Bud1. I confirmed this with Dr. Hay-Oak Park of Ohio State University, whose research focusses on the yeast BUD genes.

The real question is whether constitutively active Bud1 compromises mating, which is addressed in Figure 2, and here the data is not in line with how it is described in the text:
- Even though the text states that "Bud1 must be inactivated to enable tracking", the phenotypes described in panel 2D show that the ratio of cells that mate from their default site to those that show a mobile site is quite similar in WT and cells expressing constitutively active Bud1, which does not argue for the default site being stuck.

Response: First, the Results section describing our analysis of BUD1^{G12V}/BUD1 cells starts with the statement that the Bud2 and Bud5 localization data "*suggested* to us that Bud1 inactivation is required for gradient tracking (emphasis added)." After discussing the data presented in Fig. 2A-F, we conclude that the results "*support our hypothesis* that Bud1 must be inactivated to allow GTM tracking (emphasis added)." It is only in the Discussion, when we synthesize the data shown in Figures 1, 2A-F, and 2H, that we conclude Bud1 must be inactivated and remain inactive to permit tracking.

Second, we disagree that the ratio of cells that default mate to those that track is similar in the BUD1^{G12V}/BUD1 and WT strains. For WT cells, the ratio of trackers to default maters is 77/23 = 3.35, whereas in for BUD1^{G12V}/BUD1 cells, the ratio is 56.6/43.4 = 1.3 (2.58-fold smaller than this ratio in WT cells). These ratios do not include the "ignored partner" cells. When the cells that ignored a potential partner with which they were in direct contact (Fig. 2C) are considered, the inhibitory effect of BUD1^{G12V} on tracking is even more apparent. We now note this in the Results: "Considering both the cells that ignored potential partners and those that formed zygotes, about 40% of the *BUD1^{G12V}*/*BUD1* cells exhibit Ste2-GFP tracking as compared to 75% of the WT cells (Fig. 2C and D)." And in the Discussion: "Ste2-GFP tracking was seen in about half as many *BUD1^{G12V}*/*BUD1* cells as in WT cells ($p < 0.0001$)."

- In panel 2C, 70% of cells expressing constitutively active Bud1 do mate. Of the 30% that do not mate, the authors explain that two distinct phenotypes are observed, shown in Figure 2A and 2B. The 2B example would be a clear example of cells with defect in orientation. By contrast, in Figure 2A, the example shows a cell that keeps budding instead of shmooing (and so does the WT partner). If the cell buds, it has progressed from G1 to S phase in the cell cycle, and is thus in a phase that is not permissive to pheromone production. This indicates that the Bud1-G12V-expressing cell does not arrest in G1 phase, and therefore cannot differentiate for mating, which is a very different phenotype from one affecting growth position. The authors do not report the frequency of each of these two phenotypes, but they cannot be aggregated.

Response: We thank the reviewer for bringing this point to our attention, as we can see how it might be confusing. It is certainly correct that a cell cannot differentiate for mating (i.e., assemble a GTM and track) unless it is arrested at START late in the G1 phase of the cell cycle. However, the *MAT***a** cell in Fig. 2A is clearly arrested in G1. By referring to Fig. 1B, we can see that the interval between cytokinesis and bud emergence under our standard growth and mating conditions is about 30 minutes; the full cell cycle (cytokinesis to cytokinesis) takes about 2 hours. In contrast, the BUD1^{G12V}/BUD1 *MAT***a** cell in the Fig. 2A took 135 minutes to bud (between 105 and 140 minutes in the representative time-lapse images). Moreover, this cell elongated by about 36% at what appears to be its distal default site, although it budded before beginning to shmoo. In the revised manuscript, we have explained the differences between the phenotypes represented in Figs. 2A and 2B as follows: “These *BUD1*^{G12V}/*BUD1* cells either showed a prolonged G1 arrest before ultimately budding (Fig. 2A), or they polarized the receptor and shmooed at the DS without regard to the positions of their potential partners (Fig. 2B).” The distinction is also described in the Fig. 2 legend, which also indicates the relative proportion of cells in each category: “(A) a *BUD1*^{G12V}/*BUD1* cell that arrested and elongated but ultimately resumed budding (white asterisks). (B) a *BUD1*^{G12V}/*BUD1* cell that polarized its receptor and shmooed but failed to orient toward and mate with its potential partner.…Of the *BUD1*^{G12V}/*BUD1* cells that ignored partners, two thirds behaved as shown in (A) and one third behaved as shown in (B).”

- Figure 2E-F comparing Ste2 expression levels are not comparing the same time point in WT and mutant. For a valid comparison, shmooing time should be used for both (or fusion time), not tracking time vs shmooing time.

Response: Again, we appreciate that the reviewer has alerted us to a potential source of confusion. In fact, the cells are being compared at the same point in the process – the end of GTM Assembly. At the next time point, cells either begin to shmoo at the DS or the GTM begins to track. What we are showing here is that the decreased ability of *BUD1*^{G12V}/*BUD1* cells to track cannot be attributed to insufficient receptor accumulation at the DS at the end of the Assembly phase. To clarify this point, we added a few words in the text (emphasis added): “In fact, the mean and total Ste2-GFP intensities were significantly higher in *BUD1*^{G12V}/*BUD1* cells one time point before shmooing **at the DS** than in WT cells one time point before tracking **from the DS** (Fig. 2E and F).”

- Figure 2G reports the pause time in WT vs bud1Δ, showing that pause is shorter or inexistent in bud1Δ. This quantification is problematic and impossible to interpret without a clear explanation for how pause time is measured. Is there a marker for the different stages? From the examples shown in Fig 1, it seems very arbitrary. The comparison is further complicated by the fact that bud1Δ cells do not assemble a default site.
Thus, the provided data does not convince that that Bud1 inactivation is required for site mobility.

Response: This is our bad. We originally labeled the time-lapse images of mating mixtures in a way that did indeed make it difficult to see how we measure pause time. Pause time is quite simply the interval between the time point when a polarized GTM reporter such as Ste2-GFP is first detected (aka "polarity established," PE) at the Assembly site (the DS in WT cells) and the first time point that tracking is observed. In the revised manuscript, we have changed Figs 1C-D, 4B-E, 5A-C and 7D&F so that PE is appropriately indicated as a single time point, which we label as the beginning of Assembly, while the beginning of Tracking corresponds with the end of Assembly. The interval between PE and the initiation of tracking is the pause time. We have also added a cartoon that illustrates pause time (Fig. 2G).

It is true that mating *bud1*Δ cells do not assemble a single GTM at the default site (i.e., the axial bud site). However, they do assemble multiple GTMs at random positions, just as *bud1*Δ cells bud at random positions during vegetative growth. We showed in Wang et al. (2019) that the GTMs in *bud1*Δ cells are functional. Therefore, the significantly reduced pause time in *bud1*Δ cells is consistent with the idea that Bud1 must be inactivated to enable tracking.

Actin-independent vesicle delivery:
Figures 3 and 4 show complementary evidence that deleting a region of Bem1 or Exo70 that binds the other protein compromises site mobility. This is an interesting finding, which should be strengthened to make it compelling.
- First, the mutants used are rather coarse ones, removing a large chunk of the protein, which may affect other interactions. For instance, the exo70ΔC allele also blocks binding to Rho3. Use of more specific mutants, characterized to specifically block the Bem1-Exo70 interaction would be more convincing. Such mutants have been described by Liu and Novick, JCB 2014 (for instance exo70M26 or better exo70M30).

Response: Done. See new Fig. 4 and the corresponding text.

- In Fig 3B, shmoo formation is not evident. Could you please show the whole mating process up to the point of fusion? This figure also lacks quantifications (equivalent to Figure 4C-D).

Response: Fig. 3B shows an example of a *bem1*$^{\Delta CPX}$ cell that polarizes the receptor at the DS but does not track or mate despite physically contacting a potential partner. This was not previously noted but is now indicated in the figure legend. We agree that the cell of interest (labeled *MAT***a**) in Fig. 3B is not obviously shmooing in the DIC images;

however, its polarized growth and robust polarization of the receptor to the growth site is apparent in the fluorescent images (e.g., see the 60' and 70' time points). Quantifications have been added (Fig. 3C-D). We summarized the quantifications in the text with this statement: “Considering both the cells that ignored potential partners and those that formed zygotes, about 2% of the *bem1*$^{\Delta CPX}$ mutants exhibited Ste2-GFP tracking (Fig. 3C and D).”

- The data shows a role for Exo70, but, at this point in the manuscript, there is no evidence that it is actin-independent. This should be rephrased (end of section on Figure 4).

Response: Fixed. With the addition of the *sec3*$^{\Delta N}$ mutant and *exo70*^{M30} *sec3*$^{\Delta N}$ double mutant data, this section has been substantially revised.

Figures 5 and 6 examine the actin-independence of the process, showing that the type V myosin Myo2 is absent from mobile sites, that the actin cable marker Abp140 is also poorly detectable, and that inactivation of Myo5 in the myo2-16 allele does not abrogate site mobility. The Myo2 localization is clear, though the authors should be careful is their statement, as absence of evidence is not evidence of absence. The myo2-16 phenotype is a bit more difficult to interpret, as many cells do not display a polarity site, though it is clear that a substantial fraction of cells still display site mobility. To probe the function of Myo2 specifically during tracking, a good approach would be to change temperature on the microscope after site assembly.

Response: We agree that absence of evidence is not evidence of absence; however, our conclusion that AD-VD is not required during tracking is based on multiple lines of evidence, not only on the absence of the Myo2-GFP signal at the PM in the tracking phase (Figs. 5A and 6C).

The reviewer comments that it is difficult to interpret the *myo2-16* phenotype. We disagree. At restrictive temperature, about 40% of the *myo2-16* cells were unable to stably polarize the receptor at the DS; the remaining 60% polarized the receptor at the DS and tracked normally (Fig. 7G-H). These data suggest that AD-VD contributes to GTM assembly but is dispensable during tracking. We agree with the reviewer that this could be demonstrated more cleanly by permitting Myo2 function while the GTM is assembled (i.e., at permissive temperature), then inactivating Myo2 (restrictive temperature) at the onset of tracking. This is a great idea in principle but is not workable in practice. Unless the *MAT***a** cells are synchronized in early G1 when we set up the mating mixtures, there will always be cells at all stages of the process – GTM assembly not yet started, assembly ongoing, assembly completed – regardless of when we shift to the restrictive temperature. Even if we were to start with G1-synchronized *MAT***a** cells, they would initiate assembly and tracking at different times due to local differences in pheromone gradients. Moreover, it is only by observing cytokinesis that we know the position of the DS. This cannot be determined in G1 cells.

The claimed absence of actin cables is less convincing. The Abp140 images only have cortical dots pretty much all around the cell. It is hard the deduce anything about the assembly of actin cable from these images. This should be substantiated by investigating localization and function of formins (of which there are very tight ts mutants).

Response: Although it is difficult to see actin cables in our images of mating yeast cells expressing Abp140-RFP, we are confident that our scoring is meaningful. We scored cells as showing actin cables oriented towards the receptor if we could see one or more lines of Abp140-RFP dots converging on and contacting the Ste2-GFP crescent on the PM. This double-label pattern can be most easily seen in the 40' and 50' time points of the Fig. 5B. Our quantitative analysis of this phenotype is shown in Fig. 5E. It should be noted that the detection of Abp140-RFP dots all around the PM is not unexpected: 1) Abp140-GFP lights up cortical actin patches in addition to actin cables, although to a lesser degree[1]; 2) Using Abp1-GFP, cortical actin patches can be seen forming and disappearing all around the cell cortex of shmooing cells (see Fig. 1A-B of Smith et al, 2001)[2].

Inactivating temperature-sensitive formin alleles in mating cells is another way of asking whether AD-VD is required for tracking, but one we do not think is necessary. In addition to finding that Myo2 is not required for tracking in the original manuscript, we have added strongly supportive evidence in the revised manuscript: *exo70^{M30} sec3$^{\Delta N}$* double mutant cells, which can polarize secretion by AD-VD but not by AI-VD, are almost entirely unable to track. See paragraph 1 of the new Discussion section, “Actin-independent vesicle delivery is necessary and sufficient for GTM tracking.”

Though I don't think there were strong previous claims that secretory vesicle delivery depends on actin during site motility, the data here seems to conflict with previous observations. For instance, the Lew lab showed that Spa2 (a component of the formin-associated polarisome) leads the mobile patch in pheromone-exposed cells (McClure et al, 2015). Ghose et al 2020 further showed colocalization of Spa2 with Bni1 formin. The Martin lab showed that both exocyst and type V myosin are present on mobile patches in the fission yeast cells (Bendezu et al, 2013). These differences should be at least discussed. Because the myo2-16 phenotype is a bit difficult to interpret and because blocking Bem1-Exo70 interaction does not completely block site mobility, it is also possible that exocyst-based tethering and actin-based vesicle delivery both contribute to polarity site movement.

Response: The apparent contradictions between our claims and previous findings is addressed in paragraph 2 of the new Discussion section, “Actin-independent vesicle delivery is necessary and sufficient for GTM tracking,” and in the new Discussion section, “Switching modes of vesicle delivery: stability vs. mobility.” Our deterministic tracking model is also compared to the biased wandering models in paragraphs 3 and 4 of the revised Introduction. Our reasons for not discussing the Ghose et al (2020) paper are explained in our response to Review 1, comment #2.

More globally, I find regrettable that the introduction and discussion do not present a more balanced view of chemotropism. There are strong disagreements in the field, on how deterministic vs more stochastic the search for a partner may be. Disagreement can be a strong motor of scientific progress, but unfortunately neither side appears to acknowledge the other. This manuscript does not cite a single paper from the Lew lab on mating, nor any mention of similar phenomenon in the fission yeast. This is highly detrimental to an open discussion.

Response: We find it regrettable that we disappointed the reviewer in this way, especially as scientific integrity demands that we cite the relevant work of other groups. We have published three previous papers on gradient sensing in yeast. In Wang et al. (2019), we fully described the biased wandering models in two long Discussion paragraphs, citing Dyer et al. (2013), McClure et al. (2015), Hegemann et al. (2015), and Hegemann and Peter (2017) multiple times. In Abdul-Ganiyu et al. (2021), we also described the biased wandering models, although more briefly, referencing these same four papers. In Ismael et al. (2016), we described biased wandering and cited Dyer et al. (2013), McClure et al. (2015), and Hegemann et al. (2015). We agree with the reviewer that the Lew lab has never appropriately cited or described our model of yeast gradient sensing, but that has nothing to do with why we did not include the requisite citations here. In writing the first version of this manuscript, we simply did not think that biased wandering was relevant. We sought to explain how the GTM is released from the DS; the Lew model does not recognize that polarity is established at the DS. Upon reflection, prompted by the reviewer, we realized that this reasoning was faulty. A central tenant of the Lew model is that mobility of the polarity site is driven by AD-VD, which does indeed conflict with the second major claim of our study. We now fully agree that discussion of the other models is relevant and that it enhances our manuscript.

1. Yang, H.C. & Pon, L.A. Actin cable dynamics in budding yeast. *Proc Natl Acad Sci U S A* **99**, 751-756 (2002).
2. Smith, M.G., Swamy, S.R. & Pon, L.A. The life cycle of actin patches in mating yeast. *J Cell Sci* **114**, 1505-1513 (2001).

August 24, 2022

RE: JCB Manuscript #202203004R

Dr. David E Stone
University of Illinois at Chicago
Biological Sciences
Molecular Biology Research Building
900 South Ashland
Chicago, Illinois 60607

Dear Dr. Stone:

Thank you for submitting your revised manuscript entitled "Mobility of the gradient tracking machine in mating yeast depends on Bud1 inactivation and actin-independent vesicle delivery". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below). In your final revision, please be sure to address reviewer #2's final concerns with appropriate text edits.

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

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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2) Figures limits: Articles 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. In order to accommodate readers with red-green color blindness, we ask that you please change the red/green color scheme used in graphs, or to include an additional distinguishing feature.

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.

The following edited title is suggested: Gradient tracking machine mobility in mating yeast depends on Bud1 and actin-independent vesicle delivery

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

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

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:

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

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

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

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

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

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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.

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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,

Ian Macara, Ph.D.
Editor

Andrea L. Marat, Ph.D.
Senior Scientific Editor

Journal of Cell Biology

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

With the new data using the EXO70-M30 allele, the authors have nicely responded to my concerns about the specificity of the EXO70-deltaC results. The manuscript is much improved as a result.

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

Bud1 inactivation:
I thank the authors for their addition of quantifications and their explanations. I agree that a reduction from 75% of cells to 40% of cells upon ras1G12V expression is clear, but it is also a relatively mild phenotype.

I also still find measurement of pause time arbitrary in absence of an independent marker, and especially difficult to compare in cells that assemble the polarity patch at different locations and in different numbers. I carefully examined the examples of timing now highlighted in Figure 1. For example in Figure 1D, at timepoint 38' (first one annotated as tracking), the Ste2 signal seems to have expanded more away from the future shmoo site than towards it. At the next 44' timepoint, it seems to have contracted rather than progressed towards the future shmoo site (and the peak signal is displaced away from the shmoo site relative to the previous timepoint, as measured in FIJI). At the 50' timepoint, it is displaced towards the future shmoo site. Thus, one could argue that tracking starts at the 50' mark, instead of the 38' mark. Without a more consistent reference, I stand by my comment that the measure of pause time is arbitrary and subject to bias, and so not convincing to be meaningful.

For these reasons, I also still think that the claim that Bud1 must be inactivated is too strong. The wordings in the introduction and results are careful but they are very strong in the title and abstract. For instance, "Here we describe two mechanisms that are essential for tracking. First, the Ras GTPase Bud1 must be inactivated", should be re-phrased.

Actin-independent vesicle delivery:
The new experiments provided have strongly strengthened this part of the manuscript and the authors now make a convincing case that actin-independent vesicle delivery promotes patch mobility. I also thank the authors for adding discussion comparing their results with previous ones.

August 31, 2022

RE: JCB Manuscript #202203004R

Dear Drs. Macara and Marat,

Thank you for the prompt review of our revised manuscript entitled “Mobility of the gradient tracking machine in mating yeast depends on Bud1 inactivation and actin-independent vesicle delivery,” and for your provisional plan to publish our work in JCB. We hope that our responses to reviewer 2 (see below) will resolve any remaining issues.

Under point #5 of the *Manuscript Organization and Formatting* list, you suggested that we change the title from “Mobility of the gradient tracking machine in mating yeast depends on Bud1 inactivation and actin-independent vesicle delivery” to “Gradient tracking machine mobility in mating yeast depends on Bud1 and actin-independent vesicle delivery.” We believe that your intention is to qualify our claim that Bud1 must be inactivated to enable tracking. However, leaving out the word "inactivation" does not qualify our claim – it switches our meaning to its opposite. At the risk of over explaining, this is like changing the title “Traveling to the moon requires escaping the earth’s gravity” to “Traveling to the moon requires the earth’s gravity.”

To stay under the character limit, we have changed the title to “Gradient tracking in mating yeast depends on Bud1 inactivation and actin-independent vesicle delivery.”

Responses to reviewer #2

Bud1 inactivation:
I thank the authors for their addition of quantifications and their explanations. I agree that a reduction from 75% of cells to 40% of cells upon ras1G12V expression is clear, but it is also a relatively mild phenotype.

Response: Although the reviewer considers the *BUD1*^{G12V}/*BUD1* phenotype to be mild, the increased incidence of *BUD1*^{G12V}/*BUD1* cells that ignored potential partners in direct contact was highly significant ($p \leq 0.0001$; $n \geq 109$), as was the decrease in *BUD1*^{G12V}/*BUD1* cells that formed zygotes but failed to track ($p \leq 0.0001$; $n \geq 76$).

Some additional considerations: It is likely that the native Bud1 partially rescues gradient tracking by competing with Bud1^{G12V} for localization to the GTM assembly site. In other words, Bud1^{G12V} is a dominant negative for tracking that is not fully penetrant. It is also important to note that *BUD1*^{G12V} is carried on a centromeric plasmid. The mitotic stability of such plasmids depends on the insert and the strain but can be as low as 89% per cell division, even when the cells are grown on selective media (Larionov et al. Curr Genet 1985 10:15-20). Thus, as many as 10% of the cells in any *BUD1*^{G12V}/*BUD1* culture may not carry the *BUD1*^{G12V} plasmid.

I also still find measurement of pause time arbitrary in absence of an independent

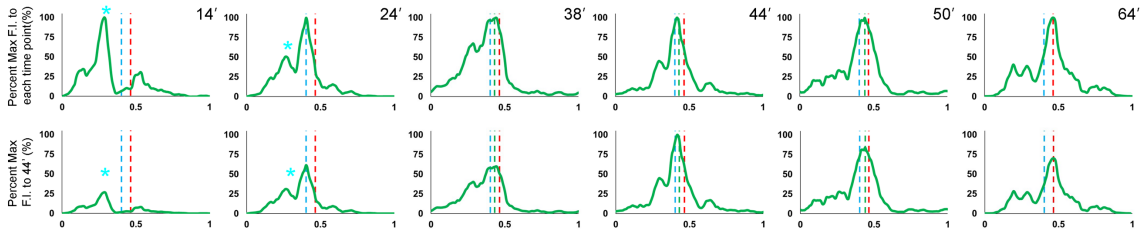
marker, and especially difficult to compare in cells that assemble the polarity patch at different locations and in different numbers. I carefully examined the examples of timing now highlighted in Figure 1. For example in Figure 1D, at timepoint 38' (first one annotated as tracking), the Ste2 signal seems to have expanded more away from the future shmoo site than towards it. At the next 44' timepoint, it seems to have contracted rather than progressed towards the future shmoo site (and the peak signal is displaced away from the shmoo site relative to the previous timepoint, as measured in FIJI). At the 50' timepoint, it is displaced towards the future shmoo site. Thus, one could argue that tracking starts at the 50' mark, instead of the 38' mark. Without a more consistent reference, I stand by my comment that the measure of pause time is arbitrary and subject to bias, and so not convincing to be meaningful.

Response: As described in Wang et al. (J Cell Biol, 2019), pause time is the interval between detectable polarity at the DS of a particular reporter (PE) and the time that reporter begins tracking. (In *bud1*Δ cells, polarity is established at random and usually multiple sites.) We agree with the reviewer that, in principle, the measurement of pause time would be more precise if we had an independent marker indicating the start of GTM assembly. Lacking such a marker, we have measured or indicated pause times as described above for numerous markers (Ste2, Gβ, phosphorylated Gβ, Far1, Sst2, Sec3, and Sla1), and reported our results in eleven figure panels (Wang et al. J Cell Biol 2019), four figure panels (Abdul-Ganiyu et al. Sci Signal 2021), and six figure panels (this manuscript). Most notably, we found that Far1-GFP and GFP-Gβ pause significantly longer than Ste2-GFP, and that Ste2-GFP pauses significantly longer than Sst2-GFP (see Fig. 3H in Wang et al. J Cell Biol 2019). Our point is not that our method for measuring pause time cannot be improved, but rather, that it was accepted as good enough to support our claims in two previous publications.

We also agree with the reviewer that pause time is a key measure, as the decreased pause time in *bud1*Δ cells supports our conclusion that Bud1 must be inactivated to enable tracking. The relevant measurements are summarized in Fig. 2H, where we report that the mean pause time for *bud1*Δ cells is less than one third that observed in WT cells ($p \leq 0.0001$). This difference is large enough to tolerate considerable systematic error in the measurement without leading us to an errant conclusion. Because we did not show images of the *bud1*Δ cells from which we measured pause times, we are confused by the reviewer's comment about their difficulty comparing cells that assemble the polarity patch at different locations and in different numbers (which only happens in *bud1*Δ cells).

All that said, we do understand the reviewer's difficulty in seeing PE and the start of tracking, as indicated in Fig. 1D. This is probably because the tracking distance from DS to CS in this cell is relatively small. We chose this cell to represent the positional relationships between Ste2-GFP and RFP-Bud2 during assembly, tracking, and stabilization – not as an example of how we measure pause time. To help the reviewer resolve the apparent contradictions and to get a better picture of how receptor distribution in this cell changes over time, we have replotted the data showing Ste2-GFP only (top), where each plot (i.e., time point) is normalized to its own maximum intensity value, as in the manuscript. In addition, we have plotted the Ste2-GFP data normalized

to the maximum intensity measured in the time course, which occurs at the 44' time point (bottom). The aqua asterisk indicates the position of the bud neck. Looking at the top set of plots, we can see that the receptor has largely moved from the bud neck and centered at the DS by 24'. At 38', the receptor peak is clearly redistributing upgradient toward the CS. Hence, we indicated PE at 24' and the start of tracking at 38'. We agree with the reviewer that the leading receptor peak does not appear to have advanced toward the CS at 44' but has done so at 50'. In the lower set of plots, however, it is clear the intensity of the leading receptor peak doubled between 38' and 44', after which tracking appears to have resumed. We hope these plots also help the reviewer see that the extension of the Ste2-GFP crescent away from the CS at 38' is attributable to receptor accumulation at the mother-daughter neck between 14' and 38', and that this signal diminishes after 44' (see lower plots).



For these reasons, I also still think that the claim that Bud1 must be inactivated is too strong. The wordings in the introduction and results are careful but they are very strong in the title and abstract. For instance, "Here we describe two mechanisms that are essential for tracking. First, the Ras GTPase Bud1 must be inactivated", should be re-phrased.

Response: We respectfully disagree. The quoted text is from the Discussion. The requirement for Bud1 inactivation is one of our two major claims. Inclusion of a conclusion in a Discussion does not mean that the authors have proven their claim beyond any doubt – only that they have presented persuasive evidence supporting that claim. Our evidence is strong enough to support our conclusions.