JCB

The Novel Role of Midbody-Associated mRNAs in Regulating Abscission

Trey Farmer, Katherine Vaeth, Ke-Jun Han, Raeann Goering, Matthew Taliaferro, and Rytis Prekeris

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August 14, 2023

Re: JCB manuscript #202306123

Dr. Rytis Prekeris
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University of Colorado Denver School of Medicine, Department of Cell and Developmental Biology 12801 East 17th Ave., Mail Stop 8108, P.O. Box 6511
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Dear Dr. Prekeris,

Thank you for submitting your manuscript entitled "The Novel Role of Midbody-Associated mRNAs in Regulating Abscission". The manuscript was assessed by expert reviewers, whose comments are appended to this letter.

Given the overall positive comments of the reviewers, we invite submission of a revised manuscript. Please specifically address all of the reviewers comments, most of which can be addressed with revision of text and figures. In particular, two of the reviewers comment that the manuscript is long (10 figures) and presents somewhat redundant data, showing several sets of experiments that basically show similar results. They recommend condensing some of this by showing the strongest data in support of a point in the main figures and moving some of the additional supporting data to the supplement. Please consider this in preparing your revised manuscript. The reviewers also noted that there is a recently published paper by the Skop lab that reports similar findings. We agree with their request that the results in this published paper must be mentioned and discussed relative to your results. Given that time is of the essence, additional experiments should focus on bolstering critical points already in the manuscript rather than on extending the work.

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.

GENERAL GUIDELINES:

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

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The typical timeframe for revisions is three to four months. While most universities and institutes have reopened labs and allowed researchers to begin working at nearly pre-pandemic levels, we at JCB realize that the lingering effects of the COVID-19 pandemic may still be impacting some aspects of your work, including the acquisition of equipment and reagents. Therefore, if you anticipate any difficulties in meeting this aforementioned revision time limit, please contact us and we can work with you to find an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

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

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

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

Sincerely,

Karen Oegema, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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

In this manuscript, Farmer et al. investigate the contribution of mRNAs translated within the midbody to the process of cytokinetic abscission. Research over the last two decades has pointed to the existence of translation machinery in the midbody, but to this reviewer's knowledge there has been no detailed study describing a role for it in abscission. Recent work showing a) midbodies can be released into the extracellular milieu post-abscission and b) be engulfed by neighbouring cells and alter their state (stimulating proliferation, determining polarity etc.) has made the question of translation within midbodies even more pertinent and interesting. Work on this topic would undoubtedly be of interest to cell division and mRNA targeting researchers and the broader JCB readership.

Timely as it may be, the submitted manuscript has some deficiencies. The most fundamental is that the presented experiments merely suggest active translation in the midbody occurs - active translation in the MB is never unambiguously shown. Likewise, a mechanism of mRNA MB recruitment is put forward and, in my view, at best weakly supported by the data provided. In its current state, I feel the paper best serves as a resource detailing mRNAs enriched/depleted in the MB.

Comments
- The authors report an astounding number of midbody-enriched mRNAs in the text related to Figure 1 (>16000 mRNAs detected in MB; ~700 increased in MB, ~500 decreased in MB). They should report in the text what fold enrichment and FDR was used to determine this.

- The manuscript lacks an easily digestible figure highlighting some of the interesting abscission-related and other cellular process related mRNAs found enriched in the MB. Perhaps a bubble plot showing highly enriched MB mRNAs clustered according to their GO term annotations.

- A lot of abscission research has focused on CHMP4B, yet as the authors acknowledge, it functions in conjunction with CHMP2A. CHMP2A mRNA is markedly increased in MB (appears >3-fold) whereas CHMP4B is just weakly increased (~1.5 fold). Why wasn't CHMP2A chosen for follow up study? Or at least studied at least initially in parallel to further support findings.

- The purification protocol for MBs should be expanded and the earlier reference to their protocol rather the subsequent citation they use included. To convince readers of the purity of their MB preparations, a low magnification micrograph of purified MBs stained with a MB marker (like MKLP1) and an exosome marker should be supplied. Likewise to clearly exclude the presence of EVs, as there are some reports of ribosomal proteins and translation machinery being enriched in EVs.

- Localizing RPL3 to the MB suggests translation machinery is present there but given how weakly it localizes further corroboration is warranted. Staining for at least one other ribosomal component would bolster that argument.

- If there are mRNAs in the MB, it should show signal with a polyA FISH probe.

- In many cases the p values shown would normally be considered barely significant, yet strong definitive statements and conclusion are made in the manuscript.

- Shouldn't CHMP4B mRNA be enriched in the MB relative to the whole cell? The FISH images in Figure 3 do not suggest this and consequently work against the prior statements that the mRNA is enriched that were drawn from the mRNA analysis. Further, the MB as a distinct substructure of the intercellular bridge is proposed to be the site of mRNA recruitment and translation. The analysis in Figure 3B should be expanded to compare CHMP4B mRNA FISH speckles that overlap with GFP-MKLP1 signal to the rest of the cell body.

- Figure 4CD: How is the ICB mask or ROI generated for these panels? Is it the same size across all cells? The methods are not descriptive enough on this point. Intensity measurements should be restricted to the MB and not the ICB as a whole and it is unclear if this is how the analysis was performed.

- How do RFP-CHMP4B protein levels compare to endogenous CHMP4B?

- Supp Fig 4F: If I understand this analysis correctly, it implies that the endogenous Chmp4B mRNA level is quite low while the RFP-constructs are in every case much higher. This would confound claims about active transport of mRNAs to the MB as it could just be a feature of overexpression.

- Strong statements are made that the 3'UTR of Chmp4B contains the targeting information. However, the images and data do not support such an unequivocal statement. It could be argued that the coding sequence of Chmp4B could also contribute to the enhanced localization. Whilst, traditional studies have focused on 5' and 3'UTRs possessing targeting information, there is increasing evidence that coding sequence can all play a part, for example Ash1.

- In the latter figures a focus switches to neurons and Net1. I think this is distracting and the study would be better kept focused on the MB and intercellular bridge; mapping the targeting information in Chmp4B and determining if this is conserved throughout other MB/intercellular bridge targeted transcripts. Analogies to Net1 could be restricted to the discussion and the potential for future more in-depth studies.

Minor comments:
- Labels above the main and supplemental figure pages would be appreciated.
- The intercellular bridge is referred to as the 'intracellular' bridge throughout the manuscript.
- Line 216: Heading states a bold conclusion that the paragraph beneath pokes some holes in. Heading title should be toned down in accordance with sober interpretation of the data.
- Page 12: Puromycin experiments do not conclusively show that active translation occurs in midbody. Tone down the claim.
- Supp Fig1: Are error bars for missing for Cep55 and Chmp4B knockdowns?

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

The study by Farmer et al. describes a new role for midbody RNA localization and local translation in regulating abscission. Specifically, they identify that a specific subset of mRNAs for ESCRT-III (and other abscission-relevant factors) mRNAs localize at midbodies where they are translated locally. Importantly, they show that local translation of CHMP4B promotes protein accumulation and is required for efficient abscission. Finally, they identify GA-rich localization elements within the 3' UTR of CHMP4B mRNA that are required for mRNA targeting to the midbody.

Overall, this is an important new contribution to our understanding of midbody biology and abscission regulation. The study is quite comprehensive in supporting every step of the model - i.e., RNA enrichment, the presence of translational machinery, the localization requirement for efficient abscission, etc., and using different approaches including protein and RNA imaging, PCR, abscission assays, etc. (and it even felt a bit repetitive to this reviewer - the authors should consider whether they can streamline the presentation of the highly complementary data presented in Figs. 3-8). The work also appears to have been carefully performed. Overall, I found the story quite convincing, although effects are often rather modest (often just 2-3 fold, albeit statistically significant, in key experiments in Figs. 3-7), and the work presents an interesting and novel mechanism by which cells increase the concentration of relevant abscission factors at the midbody.

Points that should be addressed (all minor)
1) The authors make the point, in several places including the abstract, that it is "conceptually hard to imagine how large protein complexes, such as the ESCRT-III complex, can successfully diffuse into the midbody from the cytosol in a rapid and highly regulated manner". I think the authors should de-emphasize this point since most ESCRT-III subunits are small (20-30 kD) and have well characterized autoinhibited states that keep them soluble and monomeric until they are activated for polymerization at sites of ESCRT activity. This doesn't negate the main point of the paper (i.e., that local midbody translation of some ESCRT-III proteins occurs and is important for abscission), but it sounds naïve to describe ESCRT-III as a large, non-diffusible complex.

2) p. 4, line 155 (and elsewhere). It seems strange to group CHMP4B/2A and CHMP3/6, since the canonical ordering of ESCRT-III recruitment, which is best worked out in yeast but appears similar in mammals, is ESCRT-II/CHMP6/CHMP4/CHMP3/CHMP2.
3) p. 4, line 160-166. It's true that ESCRT-III proteins are thought to act late in the abscission process, but the same is true for VPS4 (whose mRNA is not enriched), so I don't think one can make a strong case that mRNA localization occurs preferentially for late-acting abscission factors.
4) The authors should discuss how their results relate to a rather different model of vesicle-mediated transport of ESCRT-III proteins to the midbody presented recently by Pust, Hogland, and colleagues (https://pubmed.ncbi.nlm.nih.gov/37523003/).
5) The authors should consider moving the first paragraph of the Discussion to the end of the Discussion. It seems odd to start the Discussion with the open-ended concept of MBsome signaling, which really isn't addressed in this study (and this is conceded by the statement that further research will be needed to fully define the role of MBsome-dependent mRNA delivery). These points would seem better made after the basics of the study have been discussed (i.e., starting with paragraph 2, p. 12, line 562).
6) If the authors want to make the point that CEP55 isn't present in non-vertebrates and that it isn't clear how ESCRT factors are recruited in those systems, then they should also note that centralspindlin has been shown to participate in this process in drosophila (https://pubmed.ncbi.nlm.nih.gov/31607533/).

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

In the submitted manuscript Farmer et. al, provide evidence to support a role for the midbody in local mRNA translation of proteins including proteins of the ESCRT-III complex that drive abscission and midbody release. They further identify the elements in CHMP4B mRNA that are responsible for midbody targeting. Overall, I find the data solid, convincing, and considerably advancing our knowledge in the field. Yet, I have some concerns regarding the novelty of the findings given the preprint by Skop and colleagues that was deposited to BioRXiv on Nov. 2022 (doi: https://doi.org/10.1101/2022.11.01.514698). While the submitted work include new results that are not included in the BioRXiv preprint, unless coordinating publication, the claim of novelty should be toned down. Additionally, the results reported by Skop and colleagues should be mentioned and discussed thoroughly in the submitted manuscript. That said, I find the fact that two laboratories independently found evidence for active mRNA translation at the midbody to significantly strengthen the findings reported in the manuscript. Therefore, once this issue is resolved I think the manuscript is suitable for publication with relatively minor revisions (detailed below).

Specific comments:
1. The manuscript is relatively long (10 figures), but sometimes include several sets of experiments that basically show similar results that were obtained with more accurate, sophisticated assays. I recommend showing the key experiments in the main figures and moving some of the redundancies to the sup. figures.
2. Some of the effects described are relatively mild (p values ~0.02). In these cases, it will be good to increase the statistical significance by increasing the sample size in order to show that the phenotype reported indeed represent the overall cellular response.
3. In Fig 9 the authors describe the use of doxycycline inducible constructs. Gene expression in this system is relatively slow (48 hours in the experiments described). Therefore, the advantage of using the inducible plasmid is not fully clear to me. The authors should better communicate the motivation for using this plasmid in the text.

Reviewer #1:

Comments

1) The authors report an astounding number of midbody-enriched mRNAs in the text related to Figure 1 (>16000 mRNAs detected in MB; ~700 increased in MB, ~500 decreased in MB). They should report in the text what fold enrichment and FDR was used to determine this.

As requested, we added all this information to the Method section of the manuscript. We used an FDR cutoff of 0.05 and a log2 fold change cutoff of > 0.

2) The manuscript lacks an easily digestible figure highlighting some of the interesting abscission-related and other cellular process related mRNAs found enriched in the MB. Perhaps a bubble plot showing highly enriched MB mRNAs clustered according to their GO term annotations.

As requested, we changed Figure 2A to a bubble plot.

3) A lot of abscission research has focused on CHMP4B, yet as the authors acknowledge, it functions in conjunction with CHMP2A. CHMP2A mRNA is markedly increased in MB (appears >3-fold) whereas CHMP4B is just weakly increased (~1.5 fold). Why wasn't CHMP2A chosen for follow up study? Or at least studied at least initially in parallel to further support findings.

The main reason we chose CHMP4B is because it is better studied for its involvement in abscission. As a result, we have good antibodies that work for western blotting and microscopy. We (and others) also used tagged CHMP4B to study its subcellular localization. Finally, we have siRNAs that are confirmed to knock-down CHMP4B in HeLa cells. Since our goal was to test whether local translation of ESCRT-III components, we were looking for a best studied ESCRT-III subunit with most available tools to do that. Thus, we chose CMHP4B instead of CHMP2 for that reason. As suggested by reviewer we do include qPCR data for all ESCRT subunits to provide some evidence that mRNA of other subunits is also targeted to the midbody.

4) The purification protocol for MBs should be expanded and the earlier reference to their protocol rather the subsequent citation they use included. To convince readers of the purity of their MB preparations, a low magnification micrograph of purified MBs stained with a MB marker (like MKLP1) and an exosome marker should be supplied. Likewise, to clearly exclude the presence of EVs, as there are some reports of ribosomal proteins and translation machinery being enriched in EVs.

As suggested, we added an image of purified MBs labeled with GFP-MKLP1 (see Supplemental Figure 2). It is important to point out that this type of MB purification (essentially using a modification of the same protocol) has now been published by at least three labs, including ours (all cited in this manuscript; PMIDs: 31320617, 32321914, 33767328). Importantly, one of them (PMID: 33767328) specifically compared MBs to exosomes and have shown that these are distinct organelles. We added short discussion to the manuscript to make that clear.

5) Localizing RPL3 to the MB suggests translation machinery is present there but given how weakly it localizes further corroboration is warranted. Staining for at least one other ribosomal component would bolster that argument.

As suggested, we have added staining for another ribosomal marker, RPS6. The images are shown in Supplemental Figure 2G.

6) If there are mRNAs in the MB, it should show signal with a polyA FISH probe.

As suggested we added images stained using polyA probe (see Supplemental Figure 2H).

7) In many cases the p values shown would normally be considered barely significant, yet strong definitive statements and conclusion are made in the manuscript.

As suggested, we toned-down some of the statements in the text.

8) Shouldn't CHMP4B mRNA be enriched in the MB relative to the whole cell? The FISH images in Figure 3 do not suggest this and consequently work against the prior statements that the mRNA is enriched that were drawn from the mRNA analysis. Further, the MB as a distinct substructure of the intercellular bridge is proposed to be the site of mRNA recruitment and translation. The analysis in Figure 3B should be expanded to compare CHMP4B mRNA FISH speckles that overlap with GFP-MKLP1 signal to the rest of the cell body.

We do not necessarily expect for an MB-enriched RNA you do not expect a higher number of an RNA at the MB, but for it to make up a higher proportion of the RNAs at the MB than in the cytoplasm. CHMP4B has many functions outside the midbody and it is obviously also translated in the whole cell cytosol. That is also likely true for just about any MB-localized mRNA. Our RNAseq, qPCR, and to some extend smFISH data demonstrate that multiple mRNAs (including CHMP4B mRNA) are more abundant at the MB as would be expected if they are simply randomly localized there (such as TSG101 or GAPDH mRNAs). That indicates the presence of an active mechanism to target some of these mRNAs to the MB. Indeed, our analysis of Net1-3'UTR later in the manuscript identifies the localization element that drives Net1 mRNA targeting. We re-wrote some of the text to make that clear. We also changed phrase "MB-enriched RNAs" to "MB-localized RNAs" or "MB-associated RNAs".

Regarding translation/targeting of mRNAs specifically to the GFP-MKLP1-labeled midbody, it is difficult to be that precise. We did RNAseq on MBsomes, the released MBs after abscission. During abscission, intercellular bridge is cut on both sides of the MB (in most cases) but how far it cuts from MB can vary quite a bit. Indeed, we and others have shown that post-mitotic MBsomes (or MR remnants) clearly have part of the intercellular bridge still attached (often that is referred to as "MB stalk"). The MB-stalk does not have MKLP1 in it since MKLP1 specifically accumulates at the zone of anti-parallel microtubules. Consequently, in all our studies we extended localization analysis to MB and the intercellular bridge. In short, we do not know

whether mRNA and translation only occurs in MB proper or is also occurring in the intercellular bridge.

9) Figure 4CD: How is the ICB mask or ROI generated for these panels? Is it the same size across all cells? The methods are not descriptive enough on this point. Intensity measurements should be restricted to the MB and not the ICB as a whole and it is unclear if this is how the analysis was performed.

We added a better description of how ROI was generated (see method section, "Immunofluorescence and Quantification"). Briefly, ROI included both, MB and the intercellular bridge (see our comments in #8). Since ROIs were different sizes, we divided intensity by a size of the ROI and plotted data as intensity/square micron.

10) How do RFP-CHMP4B protein levels compare to endogenous CHMP4B?

RFP-CHMP4B is overexpressed about 10-15 fold (see qPCR data in Supplemental Figure 5B). Importantly, however, all exogenous constructs are expressed at about the same level. If anything, RFP-CHMP4B-3'UTR mRNA expresses a bit lower, yet is present in MB at higher levels.

11) Supp Fig 4F: If I understand this analysis correctly, it implies that the endogenous Chmp4B mRNA level is quite low while the RFP-constructs are in every case much higher. This would confound claims about active transport of mRNAs to the MB as it could just be a feature of overexpression.

We certainly cannot fully discount this possibility (and state that in the text). Please note, however, that all exogenous constructs are expressed at about the same level (see Supplemental Figure 5B). If anything, RFP-CHMP4B-3'UTR mRNA expresses a bit lower, yet, is present in MB at higher levels. Similarly, our MB and intercellular bridge-associated PLA signal (see Figure 8) is much higher in CHMP4B-3'UTR construct as compared to no 3'UTR constructs despite the fact that they are expressed in similar levels.

12) Strong statements are made that the 3'UTR of Chmp4B contains the targeting information. However, the images and data do not support such an unequivocal statement. It could be argued that the coding sequence of Chmp4B could also contribute to the enhanced localization. Whilst, traditional studies have focused on 5' and 3'UTRs possessing targeting information, there is increasing evidence that coding sequence can all play a part, for example Ash1.

We totally agree with the reviewer that, in the case of CHMP4B, coding sequence appears to be required (our data in Fig. 7G). We edited text in the Results section to make that clear. What is interesting is that in some mRNAs (like Net1, see figures 9&10) 3'UTR is sufficient to target mRNA to the MB, while in others (such as CHMP4B) you need both, coding sequence and 3'UTR. Since similar observations were made in other mRNA targeting contexts (targeting to axon or lamellipodia), it is clear that there are probably several mechanisms mediating targeting of mRNA to the MB. In this paper we identify one localization element (required and sufficient to

target Net1, but further studies will be needed to identify other targeting mechanisms. The goal of this manuscript is to set a paradigm that we and others can then further investigate in the future.

13) In the latter figures focus switches to neurons and Net1. I think this is distracting and the study would be better kept focused on the MB and intercellular bridge; mapping the targeting information in Chmp4B and determining if this is conserved throughout other MB/intercellular bridge targeted transcripts. Analogies to Net1 could be restricted to the discussion and the potential for future more in-depth studies.

We did not shift the focus on neurons. We simply used comparison to mRNA targeting in neurons and epithelial cells to identify candidate localization elements that may be involved in targeting Net1 mRNA to the MB. The reason we switched to Net1 is that, unlike CHMP4B, it does not need the coding sequence for targeting. That allowed us to use our reporter-gene constructs with the previously identified Net1 targeting sequence (see Figures 9-10). As we mentioned in the manuscript, it is becoming clear that there are probably several ways (some dependent on coding sequence, some not) to target mRNA to the MB. We identified one of them using Net1 mRNA. We are certainly interested in identifying CHMP4B mRNA targeting mechanisms but that is way beyond the scope of this manuscript.

Minor comments:
1) Labels above the main and supplemental figure pages would be appreciated.

As suggested, we added labels to each figure.

2) The intercellular bridge is referred to as the 'intracellular' bridge throughout the manuscript.

Our apologies for a mistake. It was fixed.

3) Line 216: Heading states a bold conclusion that the paragraph beneath pokes some holes in. Heading title should be toned down in accordance with sober interpretation of the data.

As suggested, we toned-down the heading. It now says "Active translation contributes to accumulation of CHMP4B at the MB during late telophase".

- Page 12: Puromycin experiments do not conclusively show that active translation occurs in midbody. Tone down the claim.

*As suggested, we toned down the statement. It now states "*We also use anti-puromycin antibodies to suggest that active translation is occurring in the MB*".*

- Supp Fig2: Are error bars for missing for Cep55 and Chmp4B knockdowns?

Error bars are not present because qPCR was done once (with 3 technical repeats). We also

wanted to point out that these siRNAs sequences were listed in previous studies from other labs, thus, we only needed to confirm that we also see knock-down using these siRNAs.

Reviewer #2:

Points that should be addressed (all minor)
1) The authors make the point, in several places including the abstract, that it is "conceptually hard to imagine how large protein complexes, such as the ESCRT-III complex, can successfully diffuse into the midbody from the cytosol in a rapid and highly regulated manner". I think the authors should de-emphasize this point since most ESCRT-III subunits are small (20-30 kD) and have well characterized autoinhibited states that keep them soluble and monomeric until they are activated for polymerization at sites of ESCRT activity. This doesn't negate the main point of the paper (i.e., that local midbody translation of some ESCRT-III proteins occurs and is important for abscission), but it sounds naïve to describe ESCRT-III as a large, non-diffusible complex.

As suggested, we removed these statements.

2) p. 4, line 155 (and elsewhere). It seems strange to group CHMP4B/2A and CHMP3/6, since the canonical ordering of ESCRT-III recruitment, which is best worked out in yeast but appears similar in mammals, is ESCRT-II/CHMP6/CHMP4/CHMP3/CHMP2.

We edited text to remove CHMP4B/2A and CHMP3/6 grouping.

3) p. 4, line 160-166. It's true that ESCRT-III proteins are thought to act late in the abscission process, but the same is true for VPS4 (whose mRNA is not enriched), so I don't think one can make a strong case that mRNA localization occurs preferentially for late-acting abscission factors.

We removed statements about late acting factors and translation at the MB.

4) The authors should discuss how their results relate to a rather different model of vesicle-mediated transport of ESCRT-III proteins to the midbody presented recently by Pust, Hogland, and colleagues (https://pubmed.ncbi.nlm.nih.gov/37523003/).

As suggested, we added this to discussion and cited the paper.

5) The authors should consider moving the first paragraph of the Discussion to the end of the Discussion. It seems odd to start the Discussion with the open-ended concept of MBsome signaling, which really isn't addressed in this study (and this is conceded by the statement that further research will be needed to fully define the role of MBsome-dependent mRNA delivery).

These points would seem better made after the basics of the study have been discussed (i.e., starting with paragraph 2, p. 12, line 562).

As suggested, we changed order of paragraphs.

6) If the authors want to make the point that CEP55 isn't present in non-vertebrates and that it isn't clear how ESCRT factors are recruited in those systems, then they should also note that centralspindlin has been shown to participate in this process in drosophila (https://pubmed.ncbi.nlm.nih.gov/31607533/).

We added a couple sentences referring to this finding.

Reviewer #3:
In the submitted manuscript Farmer et. al, provide evidence to support a role for the midbody in local mRNA translation of proteins including proteins of the ESCRT-III complex that drive abscission and midbody release. They further identify the elements in CHMP4B mRNA that are responsible for midbody targeting. Overall, I find the data solid, convincing, and considerably advancing our knowledge in the field. Yet, I have some concerns regarding the novelty of the findings given the preprint by Skop and colleagues that was deposited to BioRXiv on Nov. 2022 (doi: https://doi.org/10.1101/2022.11.01.514698). While the submitted work include new results that are not included in the BioRXiv preprint, unless coordinating publication, the claim of novelty should be toned down. Additionally, the results reported by Skop and colleagues should be mentioned and discussed thoroughly in the submitted manuscript. That said, I find the fact that two laboratories independently found evidence for active mRNA translation at the midbody to significantly strengthen the findings reported in the manuscript. Therefore, once this issue is resolved I think the manuscript is suitable for publication with relatively minor revisions (detailed below).

Reviewer is absolutely correct that Skop and colleagues has pre-printed the study showing that MB contains RNA and that this RNA maybe translated at the MB. Thus, we toned down some statements about novelty.

Please note, that we pre-printed our study in October 2022 (and updated with new data in April 2023) (https://www.biorxiv.org/content/10.1101/2022.10.27.514111v2).

In any case, Skop's paper was just published (PMID:37552987) and I am happy to include citation and discussion of this paper, especially since it is fully consistent with our findings (see Discussion section). We also wanted to add a figure that shows a comparison between our and Skop's RNAseq data, but unfortunately, since they only have a single repeat of RNAseq, we could not perform such an analysis.

We also want to point out that there are some significant differences between our and Skop's paper. First, we used different purification methods to isolate MBs. Second, we show that 3'UTR

is involved in mRNA targeting and identify 3'UTR localization element that is required for mRNA targeting. Third, we do bioinformatic comparison between MB-associated RNA and RNAs associated with other cellular components (axon, mitotic spindle, cells in different cell cycle stages). Fourth, we investigate the consequences of inhibiting translation of some of these mRNAs, such as CHMP4B, on cell division. Fifth, our MB RNAseq data set consists of multiple repeats and whole cell controls, thus, statistical analyses can be done on MB-associated RNA enrichment. Because of all these differences I strongly believe that our and Skop's studies nicely complement each other.

Specific comments:
1) The manuscript is relatively long (10 figures), but sometimes include several sets of experiments that basically show similar results that were obtained with more accurate, sophisticated assays. I recommend showing the key experiments in the main figures and moving some of the redundancies to the sup. figures.

As suggested, we moved some of the data to Supplemental Figures and decreased the number of main figures.

2) Some of the effects described are relatively mild (p values ~0.02). In these cases, it will be good to increase the statistical significance by increasing the sample size in order to show that the phenotype reported indeed represents the overall cellular response.

Many image quantifications do have p value of about 0.01-0.02. Image analysis is notoriously variable, so p value of 0.02 is actually pretty good. I am not certain that adding more images would change it much.

3) In Fig 9 the authors describe the use of doxycycline inducible constructs. Gene expression in this system is relatively slow (48 hours in the experiments described). Therefore, the advantage of using the inducible plasmid is not fully clear to me. The authors should better communicate the motivation for using this plasmid in the text.

We used lox-integrated dox-inducible system to better control levels of expression between different cells and different plasmids. It is especially key when comparing mRNA localization since we wanted to eliminate the effects of differential expression on mRNA distribution. In principle, dox-induction is not absolutely needed. However, that is the system that we have set up and we figured it would not hurt to have an additional way of controlling expression (by titrating various dox concentrations if needed. We edited the text to make that clear.

October 7, 2023

RE: JCB Manuscript #202306123R

Dr. Rytis Prekeris
University of Colorado Anschutz Medical Campus
University of Colorado Denver School of Medicine, Department of Cell and Developmental Biology 12801 East 17th Ave., Mail Stop 8108, P.O. Box 6511
Bldg. RC1, Room L18-12402
Aurora, CO 80045

Dear Dr. Prekeris:

Thank you for submitting your revised manuscript entitled "The Novel Role of Midbody-Associated mRNAs in Regulating Abscission". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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

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

2) Figures limits: Articles may have up to 10 main 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 highly discourage the use of red/green color schemes.

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 use of the term "novel" needs to be removed from your title as per JCB policy

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. While we can allow an extension if essential, please try to reduce your SI and change the callouts in the text accordingly. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

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

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

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. Please note that ORCID IDs are now *required* for all authors. At resubmission of your final files, please be sure to provide your ORCID ID and those of all co-authors.

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

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

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

Journal of Cell Biology now requires a data availability statement for all research article submissions. These statements will be published in the article directly above the Acknowledgments. The statement should address all data underlying the research presented in the manuscript. Please visit the JCB instructions for authors for guidelines and examples of statements at (https://rupress.org/jcb/pages/editorial-policies#data-availability-statement).

B. FINAL FILES:

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

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

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

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel.

Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

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

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Please contact the journal office with any questions at cellbio@rockefeller.edu.

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

Sincerely,

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