A novel coordinated function of Myosin II with GOLPH3 controls centralspindlin localization during cytokinesis in *Drosophila*

Stefano Sechi, Anna Frappaolo, Angela Karimpour-Ghahnavieh, Roberta Fraschini and Maria Grazia Giansanti

DOI: 10.1242/jcs.252965

Editor: David Glover

Review timeline

Original submission:	13 August 2020
Editorial decision:	3 September 2020
First revision received:	28 September 2020
Accepted:	7 October 2020

Original submission

First decision letter

MS ID#: JOCES/2020/252965

MS TITLE: An intimate liaison between non-muscle Myosin II and GOLPH3 during early events of cytokinesis.

AUTHORS: Stefano Sechi, Anna Frappaolo, Angela Karimpour-Ghahnavieh, Roberta Fraschini, and Maria Grazia Giansanti
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this manuscript, Sechi et al showed that celibe (cbe), a missense allele of Drosophila zipper affected contractile ring structure in both mitotic and meiotic cytokinesis. Although the cbe mutant protein Zip/non-muscle myosin II (NMII) failed to bind Sqh/myosin regulatory light chain (MRLC), it still localised to the cell division site, suggesting a novel Rho-independent Zip localisation pathway during cytokinesis. The cbe mutation also impaired localisation of a PI(4)P binding protein GOLPH3 to the cell division site. Since GOLPH3 was also associated with both Sqh and Pav, accordingly the cbe mutant spermatocytes failed to form functional contractile rings and central spindles. Taken all these data together, the authors proposed that the reciprocal dependence between Myosin and PI(4)P-GOLPH3 regulated centralspindlin stabilization at the invaginating plasma membrane and contractile ring assembly. Since this novel pathway, together with the conventional Rho-dependent pathway, may contribute for providing cytokinesis with a certain level of robustness, this study is potentially important to be considered for publication in Journal of Cell Science.

Comments for the author

The authors need to address the following major and minor points in the revised manuscript.

Major comments:
1. Main aim of this study is unclear.
Based on the authors' statements such as "However, the molecular mechanisms underlying the recruitment of NMII to the cleavage site are not fully understood" (Page 2, lines 7-8) or "However, the molecular mechanisms that regulate initial accumulation of NMII to the contractile ring require further investigation" (Page 3, line 7-9), the main aim of this study is supposed to elucidate molecular mechanisms of NMII recruitment to the cell division site. However, the contents are mainly focused on the coordinated function of NMII and GOLPH3 in cytokinesis as described in the summary statements, "We show that during cytokinesis non-muscle Myosin II controls centralspindlin maintenance at the cleavage furrow and actomyosin ring constriction by recruiting the Phosphatidylinositol 4-phosphate binding protein GOLPH3". What is the main aim of this study, recruitment mechanisms of NMII to the cell division site or novel function of NMII in cytokinesis? If it is the former case, the authors need more insights into the initial recruitment mechanisms of NMII to the cell division site. In the proposed model (Fig. 8), initial recruitment of NMII depends on its direct binding with the membrane. Does Zip directly bind to membrane lipid just the same as mammalian NMII (Liu et al., JBC 2016)? What is the specificity of Zip-binding lipids at the cleavage furrow (e.g. PI(4,5)P2)? Is the substitution of Isoleucine with Phenylalanine in the cbe mutant indeed affecting lipid binding affinity of Zip (as discussed in Page7, line16)? Alternatively, if the main aim was to describe the novel coordinated function of NMII with GOLPH3 in cytokinesis, the manuscript as well as the title (currently too vague) need to be amended so that the object of this study becomes more specific.

2. Mitotic cytokinesis defects The authors claimed that 100% of cbe mutant neuroblasts failed to form fully constricted rings (Page 5, lines 27-29). However, cbe mutant neuroblasts in late telophase shows rather normal constriction with good accumulation of a contractile ring component Anillin (Figure 3D). What are the consequences of defected cytokinesis in these mutant neuroblasts? Are they eventually failing to divide and regressing the cleavage furrow? The authors also claimed that "cbe/Df revealed failure to maintain Zip protein at the cleavage furrow" (Page 5, line 25-26). However, Zip is not localised to the cleavage furrow in early telophase neuroblasts (Figure 3B), suggesting a possible failure of the initial recruitment of Zip to the cleavage furrow. To clarify these points, the authors need to analyse phenotype of wild type and mutant neuroblasts at different stages of cell division (from metaphase to cytokinesis) using immunofluorescence microscopy or live cell imaging.

3. Is NMII part of physical linkers between the contractile ring and the central spindle?
In this study, the authors identified that GOLPH3 was associated with both Sqh and Pav. Since NMII binds to Sqh and a contractile ring component Anillin (Straight et al., MBC 2005), it is possible that Anillin-NMII-Sqh-GOLPH3 axis may serve as a physical linker between the cleavage furrow and the

central spindle. Indeed, in telophase cbe mutant spermatocytes, the contractile ring (anillin) and the central spindle (RacGAP50C) are dissociated, but their respective structures looked relatively normal (Figure 5B). The authors need to show the overall localisation profiles of RacGAP50C and Anillin during cytokinesis including earlier stages (metaphase and anaphase/early telophase) to address this possibility and describe and discuss about the results in the revised manuscript.

Minor comments:
1. Improved F-actin staining protocol
Details of improved F-actin staining protocol (Figure S2, Page 5, line 17-19) should be described in the Materials and Methods section, if it is different from the method previously described in Frappaolo et al., 2017a (Page 10, line 3-4).

2. Reference For the sentence "Phosphoinositides bind NMII subunits" (Page 3, line 28-29), corresponding reference should be cited. It may be Liu, X., Shu, S., Billington, N., Williamson, C. D., Yu, S., Brzeska, H., Donaldson, J. G., Sellers J. R. and Korn, E. D. (2016). Mammalian Non muscle Myosin II Binds to Anionic Phospholipids With Concomitant Dissociation of the Regulatory Light Chain. J. Biol. Chem. 291, 24828-24837. DOI: 10.1074/jbc.M116.739185.

Reviewer 2

Advance summary and potential significance to field

In this paper, Giansanti and co-workers report the characterization of the Drosophila male-sterile mutant celibe (ceb), which turned out to be an allele of zipper, the gene that encodes the non-muscle myosin II heavy chain. They carry out a detailed molecular analysis and found that the ceb mutant has a substitution of an isoleucine into a phenylalanine within a highly conserved region near the IQ motif. They then show that ceb mutant spermatocytes and neuroblasts fail to fully ingress the furrow and display abnormal localization of a number of cytokinesis proteins, including the myosin regulatory light chain Spaghetti Squash (Sqh), the Golgi phosphoprotein 3 (GOLPH3), and centralspindlin. Importantly, they show that in ceb mutants Zipper does not associate with Sqh and that Sqh binds to GOLPH3, which in turn bids to the Pavarotti component of centralspindlin, thus providing a molecular explanation for the cytokinesis defects observed in ceb mutants.
From these results, the authors conclude that the association of myosin with GOLPH3 is necessary for both stabilizing the invaginating the membrane at the cleavage furrow and promoting the association of centralspindlin with the membrane.

In my opinion, this a novel and interesting study that provide some important insights into the mechanisms the regulate the association of Zipper with the invaginating membrane and its role in cytokinesis. The manuscript is generally well written and the data are convincing and support the authors' conclusions. I recommend its publication in JCS. I have only some minor comments that the authors may want to consider for a revised version.

Comments for the author

Specific comments:

1. I think that "intimate liaison" might not be the best choice of words as they bring to mind a sexual relationship. I would suggest "close relationship" instead.

2. I think that figure 1 would work better if panels D and E become B and C instead, which is more logical and also follows that narrative in the text.

3. The first letter of amino acids should not be capitalized.

4. The sentence: "resulting in the substitution of Phenylalanine for a conserved Isoleucine" is incorrect and should instead read "resulting in the substitution of a conserved isoleucine into a phenylalanine".

5. I wonder if perhaps it might be better to merge the two sections about phenotypic analyses in spermatocytes and neuroblast into one (page 5).

6. As it is presented, the characterization of the interaction between GOLPH3 and Pavarotti seems like an afterthought. It would be easy to explain its rationale by linking it to the mis-localization of RacGAP50C observed in ceb mutants (Fig. 5)

7. Could the authors speculate in the discussion why ceb mutants are only male sterile?

8. A general comment about statistical analyses. The student's t-test can only be used for normally distributed samples, which is unlikely to be the case for the data shown in Figs 6-7. I suggest to use a non-
parametric test such as the Mann–Whitney U test.

First revision

Author response to reviewers' comments

Point-by-point responses to reviewers' comments. (The reviewers' comments are in Italics. Our responses are non-italicized)
Reviewer 1 Comments for the author

The authors need to address the following major and minor points in the revised manuscript.
Major comments:
1) Main aim of this study is unclear.
Based on the authors' statements such as "However, the molecular mechanisms underlying the recruitment of NMII to the cleavage site are not fully understood" (Page 2, lines 7-8) or "However, the molecular mechanisms that regulate initial accumulation of NMII to the contractile ring require further investigation" (Page 3, line 7-9), the main aim of this study is supposed to elucidate molecular mechanisms of NMII recruitment to the cell division site. However, the contents are mainly focused on the coordinated function of NMII and GOLPH3 in cytokinesis as described in the summary statements, "We show that during cytokinesis non-muscle Myosin II controls centralspindlin maintenance at the cleavage furrow and actomyosin ring constriction by recruiting the Phosphatidylinositol 4-phosphate binding protein GOLPH3". What is the main aim of this study, recruitment mechanisms of NMII to the cell division site or novel function of NMII in cytokinesis?
If it is the former case, the authors need more insights into the initial recruitment mechanisms of NMII to the cell division site. In the proposed model (Fig. 8), initial recruitment of NMII depends on its direct binding with the membrane. Does Zip directly bind to membrane lipid just the same as mammalian NMII (Liu et al., JBC 2016)? What is the specificity of Zip-binding lipids at the cleavage furrow (e.g. PI(4,5)P2)? Is the substitution of Isoleucine with Phenylalanine in the cbe mutant indeed affecting lipid binding affinity of Zip (as discussed in Page7, line16)? Alternatively if the main aim was to describe the novel coordinated function of NMII with GOLPH3 in cytokinesis, the manuscript as well as the title (currently too vague) need to be amended so that the object of this study becomes more specific.

Our study is mainly focused on the coordinated function of NMII with GOLPH3 in the early stages of cytokinesis. Investigating whether *Drosophila* NMII directly binds to membrane lipids just the same as mammalian NMII, is beyond the scope of this paper. Following the reviewer's suggestion, we have amended the manuscript as well as the title so that the main aim of this study is clear. Specifically, the new title of the manuscript "A novel coordinated function of Myosin II with GOLPH3 controls centralspindlin localization during cytokinesis" describes, in a more detailed way, the object and the main results of our study. To clarify that our article does not aim at elucidating molecular mechanisms of NMII recruitment to the cell division site, we have deleted the statements *"However, the molecular mechanisms underlying the recruitment of NMII to the cleavage site are not fully understood"* and *"However, the molecular mechanisms that regulate initial accumulation*

of NMII to the contractile ring require further investigation". In addition, we have changed the introduction (Page 3) and the discussion (Pages 7-10) to better illustrate the main focus of our study.

Although our study does not investigate whether Zip protein directly binds to membrane lipids, Liu and collaborators (J. Biol. Chem. 291, 24828-24837. DOI: 10.1074/jbc.M116.739185) demonstrated that binding of NMII proteins to negatively charged liposomes [containing either PS, PI(4,5)P2, or PI(3,4,5)P3], occurs predominantly through the interaction of the liposomes with the regulatory light chain (RLC) binding site of the heavy chain (HC). According to their work the short sequence of the HC involved in binding to anionic phospholipids contains a high percentage of hydrophobic amino acids (~55%) and basic amino acids (~24%). Similarly, *Acanthamoeba* myosin IC binds to acidic phospholipids *in vitro* through a short sequence of basic/hydrophobic amino acids, named the BH site, located in the non-helical tail (Brzeska et al. 2008. J. Biol. Chem. 283, 32014 –32023). Moreover, a BH site in the tail of *Dictyostelium* myosin IB, was shown to mediate binding to PI(4,5)P2/ PI(3,4,5)P3 enriched regions of the plasma membrane *in vivo* (Brzeska et al. 2012. J. Biol. Chem. 287. 14923-14936). Taken together these studies suggest that substitution of isoleucine for phenylalanine in the *cbe* mutant allele might affect binding of Zip protein to the plasma membrane anionic phospholipids. Since PI(4,5)P2 is enriched at the cleavage furrow of dividing spermatocytes (Wong et al. 2005. Curr. Biol. 15, 1401-1406; Sechi et al. 2014. PLoS Genet. 10(5): e1004305.), it is likely that wild type Zip protein binds to this acidic phospholipid at the furrow plasma membrane.

2. Mitotic cytokinesis defects
The authors claimed that 100% of cbe mutant neuroblasts failed to form fully constricted rings (Page 5, lines 27-29). However, cbe mutant neuroblasts in late telophase shows rather normal constriction with good accumulation of a contractile ring component Anillin (Figure 3D). What are the consequences of defected cytokinesis in these mutant neuroblasts? Are they eventually failing to divide and regressing the cleavage furrow?

With regards to the immmunofluorescence analysis of neuroblast telophases, we have included two panels to better illustrate the different pattern of Anillin localization in *cbe/Df* mutants compared to wild type (Figure 3D). The pattern of Anillin localization in late telophase changes from compact ring-like structures in wild-type to large, unconstricted rings in *cbe/Df* mutants.

The authors also claimed that "cbe/Df revealed failure to maintain Zip protein at the cleavage furrow" (Page 5, line 25-26). However, Zip is not localised to the cleavage furrow in early telophase neuroblasts (Figure 3B), suggesting a possible failure of the initial recruitment of Zip to the cleavage furrow. To clarify these points, the authors need to analyse phenotype of wild type and mutant neuroblasts at different stages of cell division (from metaphase to cytokinesis) using immunofluorescence microscopy or live cell imaging.

To meet the reviewer's request, we included images of wild type and *cbe/Df* mutant neuroblasts immunostained for Zip during early stages of cell division (Figure 3A,B). Our analysis demonstrated that Zip localization during early stages of telophase is less robust in *cbe/Df* mutants than in wild type.

3. Is NMII part of physical linkers between the contractile ring and the central spindle?
In this study, the authors identified that GOLPH3 was associated with both Sqh and Pav. Since NMII binds to Sqh and a contractile ring component Anillin (Straight et al., MBC 2005), it is possible that Anillin-NMII-Sqh- GOLPH3 axis may serve as a physical linker between the cleavage furrow and the central spindle. Indeed, in telophase cbe mutant spermatocytes, the contractile ring (anillin) and the central spindle (RacGAP50C) are dissociated, but their respective structures looked relatively normal (Figure 5B). The authors need to show the overall localisation profiles of RacGAP50C and Anillin during cytokinesis including earlier stages (metaphase and anaphase/early telophase) to address this possibility and describe and discuss about the results in the revised manuscript.

We have added panels to Figure 5A and Figure 5B, showing RacGAP50C localization during earlier stages of meiotic divisions. Moreover, we have included a new Figure (Figure S4) to illustrate the overall localization of Anillin in wild type and *cbe/Df* mutant spermatocytes during early and late stages of meiotic division.

To precisely classify each stage of meiotic division, dividing spermatocytes were simultaneously stained for tubulin and either RacGAP50C or Anillin. Moreover, based on these results, we discuss the possibility that the Anillin-Zipper-Sqh- GOLPH3 axis might serve as a physical linker between the cleavage furrow membrane and the central spindle.
Minor comments:
1. Improved F-actin staining protocol
Details of improved F-actin staining protocol (Figure S2, Page 5, line 17-19) should be described in the Materials and Methods section, if it is different from the method previously described in Frappaolo et al., 2017a (Page 10, line 3-4).
The new protocol is the one described in Frappaolo et al., 2017. We added the indicated reference.
2. Reference
For the sentence "Phosphoinositides bind NMII subunits" (Page 3, line 28-29), corresponding reference should be cited. It may be Liu, X., Shu, S., Billington, N., Williamson, C. D., Yu, S., Brzeska, H., Donaldson, J. G., Sellers J. R. and Korn, E. D. (2016). Mammalian Non muscle Myosin II Binds to Anionic Phospholipids With Concomitant Dissociation of the Regulatory Light Chain. J. Biol. Chem. 291, 24828-24837. DOI: 10.1074/jbc.M116.739185.

We cited the suggested reference for the sentence in the introduction (Page 3, line 29).

Reviewer 2
Comments for the author Specific comments:
I think that "intimate liaison" might not be the best choice of words as they bring to mind a sexual relationship. I would suggest "close relationship" instead.
Following the reviewer's suggestion, we changed the title of the manuscript.
I think that figure 1 would work better if panels D and E become B and C instead, which is more logical and also follows that narrative in the text.
Panels D and E become B and C in the new figure 1.
The first letter of amino acids should not be capitalized.
The first letter of amino acids is not capitalized in the revised Manuscript.
The sentence: "resulting in the substitution of Phenylalanine for a conserved Isoleucine" is incorrect and should instead read "resulting in the substitution of a conserved isoleucine into a phenylalanine".
The sentence has been changed as indicated by the referee
I wonder if perhaps it might be better to merge the two sections about phenotypic analyses in spermatocytes and neuroblast into one (page 5).
The two sections about phenotypic analyses in spermatocytes and neuroblasts are now merged into one section.
As it is presented, the characterization of the interaction between GOLPH3 and Pavarotti seems like an afterthought. It would be easy to explain its rationale by linking it to the mislocalization of RacGAP50C observed in ceb mutants (Fig. 5).
To meet the reviewer's suggestion, we changed the text on page 7. The following sentence has been added: "Mislocalization of RacGAP50C at the cleavage site of *cbe* mutants led us to further investigate GOLPH3-Pav interaction."
Could the authors speculate in the discussion why ceb mutants are only male sterile?
To meet the reviewer's suggestion, we have added a paragraph in the discussion to speculate why *cbe* mutants are only male sterile (First paragraph of the discussion). The underlying cause of the different effects of the *cbe* mutation on female and male fertility may depend on sex specific regulatory mechanisms and different expression patterns of the non-muscle Zipper isoforms that remain to be clarified. In this context MYH10, the heavy chain of NMIIB, is required for meiotic cytokinesis in male but not in female mice (Yang et al., 2012. Dev. Biol. 369, 356-361).

A general comment about statistical analyses. The student's t-test can only be used for normally distributed samples, which is unlikely to be the case for the data shown in Figs 6-7. I suggest to use a non- parametric test such as the Mann-Whitney U test.
As suggested by the referee we analyzed the data in Figs 6-7 using the non-parametric Mann–Whitney U test. Since the data in former Fig. 6B, using this test, were not significant, we removed the panel 6B in the revised manuscript.

Second decision letter

MS ID#: JOCES/2020/252965

MS TITLE: A novel coordinated function of Myosin II with GOLPH3 controls centralspindlin localization during cytokinesis

AUTHORS: Stefano Sechi, Anna Frappaolo, Angela Karimpour-Ghahnavieh, Roberta Fraschini, and Maria Grazia Giansanti
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.