FAZ27 cooperates with FLAM3 and ClpGM6 to maintain cell morphology in *Trypanosoma brucei*

Tai An, Qing Zhou, Huiqing Hu, Harshini Cormaty and Ziyin Li
DOI: 10.1242/jcs.245258

Editor: David Stephens

Review timeline
Original submission: 18 February 2020
Editorial decision: 1 April 2020
First revision received: 8 April 2020
Editorial decision: 14 April 2020
Second revision received: 15 April 2020
Accepted: 21 April 2020

Original submission

First decision letter

MS ID#: JOCES/2020/245258

MS TITLE: The flagellum attachment zone protein FAZ27 cooperates with FLAM3 and ClpGM6 to regulate cell morphogenesis in Trypanosoma brucei

AUTHORS: Tai An, Qing Zhou, Huiqing Hu, Harshini Cormaty, and Ziyin Li
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 raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We may then return it to the reviewers.

In particular, I agree with the reviewers request for much improved presentation of the proteomics data and deposition of the raw data in a recognised public repository eg. PRIDE (https://www.ebi.ac.uk/pride/). You should also be absolutely clear on experimental replicates in all figures. I hope that you can make these revisions without any further lab work.

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 study, An et al., identified and studied an uncharacterized FAZ protein. By expressing tagged and truncation mutants, they showed that localisation to the flagellar side of the FAZ depended on each of the two TPR domains but not the IQ domain or LPOL domain. Ablation of transcripts by RNAi generated an epimastigote morphology, which was however not stable over time, leading to failures of cell division at later time points. BioID identified a list of 60 proteins in the vicinity of FAZ27 and a combination of tagging and RNAi allowed the dissection of dependencies between FAZ27 and the previously characterised FAZ proteins ClpGM6 and Flam3. Taken together the results are consistent with a FAZ27 being in a functional complex with GlpGM6 and Flam3. The data add additional detail to the growing understanding of the flagellar attachment zone in trypanosomes and support the previously proposed mechanism of FAZ assembly in a proximal to distal orientation.

Comments for the author

Overall the data, which are very nicely presented, support the main claims. Clarification of the following points would be helpful.

(1) p.3 Intro, “These life cycle developmental forms differ by…” there are also important metabolic differences, changes to surface coat, and functional differentiation (e.g. gametes), not just morphology.

(2) Results, first paragraph p. 4-5, the preliminary characterisation of FAZ27 should also mention whether homologous proteins are found in other kinetoplastids or outside the kinetoplastid lineage.

(3) p.6, final sentence: “the result suggests that cell division was inhibited after prolonged FAZ27 RNAi induction” – could be more nuanced: rather than an “inhibition” of cell division, this may reflect an increase in the failure rate for attempted divisions, where the morphologically abnormal cells seen early on generate progressively more progeny that are unable to generate further progeny. As mentioned in the discussion, this is in apparent contrast to the relatively stable phenotype seen with loss of ClpGM6. The reason for this difference remains unclear, but could potentially be important, regarding their functional interplay.

(4) p.8 end of first paragraph “caused the detachment of the new flagellum” – this suggests a previously attached flagellum became untethered, which is not what the data suggests. Rather, most of the newly growing flagellum is never being attached to the cell body.

(5) p8. Please state more clearly what specific criteria were used to define the 60 putative binding partners of FAZ27. Presence/absence, enrichment score above a certain threshold?
The mass spec data should be deposited in a recognised repository.

(6) Methods: under what conditions does FA27 become soluble? Results of the BioID experiments show that FAZ27 was in the “cytoskeleton fraction” defined as “pellet” after 0.5% NP40 extraction, then solubilised with 0.4% SDS for gel electrophoresis. The IP experiments were done on lysates in IP buffer (1% NP40, 150 mM NaCl…). It is not clear whether these conditions quantitatively remove all FAZ27, or whether a portion remains associated with the cytoskeleton. Establishing the conditions under which FAZ27 can be removed from the cytoskeleton vs. the conditions that dissociate GlpGM6 and Flam3 may help the dissection of their “functional interplay” discussed on p.14.

Reviewer 2

Advance summary and potential significance to field

Review of "The Flagellum attachment zone protein FAZ27 cooperates with FLAM3 and ClpGM6 to regulate cell morphogenesis in Trypanosoma brucei."

My recommendation is acceptance of the paper with a major revision and minor revisions.

Trypanosoma brucei is an extracellular protozoan parasite transmitted by the bite of the tsetse fly. It is the causative agent of sleeping sickness in humans and of nagana in cattle in sub-Saharan Africa. There are no efficient vaccines against trypanosomes, and sleeping sickness is challenging to treat, considering the toxicity and complex administration of the drugs currently available for treatment.

T.brucei has a complex life cycle characterized by multiple developmental stages with distinct cell morphology, but they are all flagellated cells. The flagellum is an essential organelle. It is involved in motility, cell division, morphogenesis, attachment to the salivary glands and infectivity of T. brucei.
As the authors wrote, “the molecular mechanisms underlying the transition from the trypomastigote form to the epimastigote form remain poorly understood”. The adhesion of the flagellum to the cell body is vital to trypanosomes and is mediated by the flagellum attachment zone (FAZ).

In this report, the authors identified a novel FAZ flagellum domain protein named FAZ27 that they localized on the FAZ sub-domain inside the flagellum. They demonstrated that the two tetratrico-peptide repeats(TPR1 and 2) are required for FAZ27 trafficking and localization to the FAZ. To investigate FAZ27 function, the authors successfully generated an inducible knockdown using RNAi. While FAZ27 depletion causes moderate growth defects, the authors observed major changes in cell morphology resulting in the production of epimastigote like cells after RNAi induction for 24h.

The authors utilized immunofluorescence microscopy to characterize the epimastigote-like cells by measuring the length of the FAZ, flagellum, cell body and unattached flagellum as well as the distance of the kinetoplast to the nucleus and posterior. Also, they took advantage of 2K2N cells to study the role of FAZ27 in FAZ assembly and maintenance, and demonstrated that RNAi of FAZ27 does not affect the old cell. The authors concluded that the depletion of FAZ27 “disrupted the elongation of the new FAZ and abolished the migration of the newly formed kinetoplast and flagellum-associated structures, and caused the detachment of the new flagellum.”

Interestingly, the authors demonstrated that FAZ27 forms a complex with FLAM3 and ClpGM6, which are two flagellar proteins known to play a role in Trypanosome morphogenesis.
The depletion of FAZ27 24 h post-RNAi induction disrupts the assembly/localization of FLAM3 and ClpGM6 as well as their destabilization after 2-3 days of RNAi induction.

Finally, the authors monitored the incorporation of an ectopic FAZ27 tagged protein at 2, 4 and 6 hours post-induction and concluded that the assembly of the FAZ flagellum domain occurs at the proximal end of the new FAZ.

Comments for the author

The major revision concerns the BioID experimental design.
To my understanding, no biological replicate has been conducted to validate the 60 proteins identified as biotinylated.
The Table 1 that shows the mass spectrometry data does not contain the raw data obtained for the minus Tet condition and the table lacks a detailed description of the columns; for instance what does "matches" mean?.
The Materials and Methods section lacks the method used to calculate the "score" used to rank the biotinylated proteins.

The result of this BioID experiment should not be reduced to a supplementary table, but an overview of the top proteins pulled down should be represented in Figure 5.
On a minor note, the authors might want to present a better representative cell for panel F. The current cell has a body length and K-N distance that are similar to some 1N1K cells after 24 hours of FAZ27 RNAi.
Finally, the authors do not discuss the absence of KIN-E in their BioID pull down, and they do not review their choice to study ClpGM6 (ranked 27) versus FAZ19 and Hook complex protein, respectively ranked 6 and 8.

As a minor revision, both panel A from figure 6 and 7 must be supplementary data, and both panel B from figure 6 and 7 combined into a single figure. The authors themselves wrote in the discussion section that the data presented in both panels A are " not surprising". Then, it would help the fluidity of the result section by reducing the redundancy in the description of the data, while giving additional space to emphasize the beautiful result on FLAM3 and ClpGM6 RNAi cell lines showing the remaining signal of FAZ27 at the distal end of the FAZ

Finally, in the discussion section, I regret that the authors did not take the opportunity to share their thoughts on FAZ27 and calcium signaling.

First revision

Author response to reviewers' comments

Response to reviewers' comments

MS ID#: JOCES/2020/245258
MS TITLE: FAZ27 cooperates with FLAM3 and ClpGM6 to maintain cell morphology in *Trypanosome brucei*

Authors: Tai An, Qing Zhou, Huiqing Hu, Harshini Cormaty, and Ziyin Li

We very much appreciate the insightful comments and the useful suggestions made by the two experts. We have revised the manuscript accordingly. The following is a point-by-point response detailing how we have dealt with the points raised by the reviewers.

Reviewer 1:
Overall the data, which are very nicely presented, support the main claims. Clarification of the following points would be helpful.

(1) p.3 Intro, "These life cycle developmental forms differ by…" there are also important metabolic differences, changes to surface coat, and functional differentiation (e.g. gametes), not just morphology.

ANSWER:
Thanks for pointing this out. We were focusing on the morphology differences, and apparently missed other differences between these forms. We have revised the sentence accordingly.

(2) Results, first paragraph p. 4-5, the preliminary characterisation of FAZ27 should also mention whether homologous proteins are found in other kinetoplastids or outside the kinetoplastid lineage.

ANSWER:
FAZ27 has close homologs in other kinetoplastids. This information has been included in the revised manuscript. Thanks.

(3) p.6, final sentence: "the result suggests that cell division was inhibited after prolonged

FAZ27 RNAi induction" – could be more nuanced: rather than an "inhibition" of cell division, this may reflect an increase in the failure rate for attempted divisions, where the morphologically abnormal cells seen early on generate progressively more progeny that are unable to generate further progeny. As mentioned in the discussion, this is in apparent contrast to the relatively stable phenotype seen with loss of ClpGM6. The reason for this difference remains unclear, but could potentially be important, regarding their functional interplay.

ANSWER:
This is a great point that is very well taken. The generation of epimastigote-like cells after FAZ27 RNAi suggests that these cells were derived from the division of the 2N2K cells containing a detached new flagellum. However, those multi-nucleated cells all contained multiple detached flagella, suggesting that the epimastigote-like cells were not able to divide further. We have deleted this statement in the revised manuscript.

As for the distinction in cell division between FAZ27 and ClpGM6, as we have discussed in paragraph 2 on page 13, we believe that it is due to the difference in the length of the new FAZ. As to why ClpGM6 RNAi produced the epimastigote-like cells with a FAZ longer than the cells generated by FAZ27 RNAi and by FLAM3 RNAi, it is possible that it is due to the lower efficiency of ClpGM6 RNAi.

(4) p.8 end of first paragraph "caused the detachment of the new flagellum" – this suggests a previously attached flagellum became untethered, which is not what the data suggests. Rather, most of the newly growing flagellum is never being attached to the cell body.

ANSWER:
This is a great point. We have revised the sentence to make it clear that the new flagellum was unable to be attached to the cell body. Thanks.

(5) p8. Please state more clearly what specific criteria were used to define the 60 putative binding partners of FAZ27. Presence/absence, enrichment score above a certain threshold? The mass spec data should be deposited in a recognised repository.

ANSWER:
We have done a parallel BioID experiment using the non-induced control cell line (Table S2). We then exclude the proteins that were also identified in the control BioID experiment and the known contaminants, such as the ribosomal proteins.

The raw mass spectrometry data is now included as Supplementary Tables S4 and S5.

(6) Methods: under what conditions does FA27 become soluble? Results of the BioID experiments show that FAZ27 was in the "cytoskeleton fraction" defined as "pellet" after 0.5% NP40 extraction, then solubilised with 0.4% SDS for gel electrophoresis. The IP experiments were done on lysates in IP buffer (1% NP40, 150 mM NaCl…). It is not clear whether these conditions quantitatively remove all FAZ27, or whether a portion remains associated with the cytoskeleton. Establishing the conditions under which FAZ27 can be removed from the cytoskeleton vs. the conditions that dissociate GlpGM6 and Flam3 may help the dissection of their "functional interplay" discussed on p.14.

ANSWER:
FAZ27 was not soluble in PEME buffer containing 0.5% NP40. In the IP experiments, cells were lysed in IP buffer containing 1% NP40 by sonication, which resulted in the solubilization of ~80% of FAZ27 protein.

We feel that the experimental conditions under which FAZ27 can be removed from the cytoskeleton are irrelevant to the functional interplay between FAZ27 and its interacting proteins (i.e. ClpGM6 and FLAM3). The former experiments lysed the cells and used detergent to remove the prior assembled FAZ27 protein from the FAZ, whereas the latter experiments (immunofluorescence microscopy, Figs. 6A, C and 7A, C) used intact cells to investigate the assembly of the newly synthesized proteins (FAZ27, FLAM3 or ClpGM6) into the new FAZ when their partner protein was depleted by RNAi. Please note that the stability of these proteins in the

old FAZ, which were prior assembled into the old FAZ, was not affected (see the 1N2K and 2N2K cells located at the bottom of Figs. 6A, C and 7A, C).

Reviewer 2:
The major revision concerns the BioID experimental design. To my understanding, no biological replicate has been conducted to validate the 60 proteins identified as biotinylated. The Table 1 that shows the mass spectrometry data does not contain the raw data obtained for the minus Tet condition and the table lacks a detailed description of the columns; for instance what does "matches" mean? The Materials and Methods section lacks the method used to calculate the "score" used to rank the biotinylated proteins. The result of this BioID experiment should not be reduced to a supplementary table, but an overview of the top proteins pulled down should be represented in Figure 5.

ANSWER:
This is a great point that is very well taken. We did BioID experiments for both the "+Tet" and "-Tet (non-induced control)" cells, and the 60 putative FAZ27 proximal proteins were obtained by excluding the proteins identified in the "-Tet" samples and the ribosomal proteins that are known as common contaminants. Our plan was to identify FAZ27-interacting proteins that cooperate with FAZ27 to regulate cell morphogenesis; therefore, we chose BioID to identify putative FAZ27-interacting proteins and then verified the interaction by co-IP. When we found that FLAM3 and ClpGM6 were identified by BioID, we just focused on these two proteins because they have been previously reported to regulate cell morphogenesis (Hayes et al., 2014; Rotureau et al., 2014; Sunter et al., 2015).

We have provided the detailed description of the columns in Table S1. The "matches" actually mean "the total peptides that match the identified proteins". We have changed it in the table. Also for the method used to calculate the "score", we believe the score was generated automatically when the Mascot search was performed. We have changed the "score" to "Mascot score" in the table, and included the following sentence in the M&M section: "The Mascot score for a protein is the logarithmic score for the individual peptides, e.g. peptide masses and peptide fragment ion masses, for all peptides matching a given protein."

We have included a table in Figure 5D, which lists putative FAZ27-proximal proteins that localize to the vicinity of FAZ27, such as the FAZ flagellum domain, the FAZ filament domain, the PFR and the flagellum.

On a minor note, the authors might want to present a better representative cell for panel F. The current cell has a body length and K-N distance that are similar to some 1N1K cells after 24 hours of FAZ27 RNAi. Finally, the authors do not discuss the absence of KIN-E in their BioID pulled down, and they do not review their choice to study ClpGM6 (ranked 27) versus FAZ19 and Hook complex protein, respectively ranked 6 and 8.

ANSWER:
We have replaced the representative cell in panel F. We discussed why KIN-E was not identified by FAZ27 BioID. We think that the reason behind the absence of KIN-E was due to the different localization of KIN-E, which was enriched in the distal tip of the flagellum, whereas FAZ27 is mainly localized to the FAZ domain. As mentioned above, FLAM3 and ClpGM6 have been previously reported to regulate cell morphogenesis; so it makes sense to study the functional relationship between FAZ27 and them. FAZ19 has not been functionally characterized so far, and the hook complex protein is unlikely involved in regulating cell morphogenesis. So far it appears that only the FAZ flagellum domain proteins are involved in cell morphogenesis.

As a minor revision, both panel A from figure 6 and 7 must be supplementary data, and both panel B from figure 6 and 7 combined into a single figure. The authors themselves wrote in the discussion section that the data presented in both panels A are "not surprising". Then, it would help the fluidity of the result section by reducing the redundancy in the description of the data, while giving additional space to emphasize the beautiful result on FLAM3 and ClpGM6 RNAi cell lines showing the remaining signal of FAZ27 at the distal end of the FAZ

ANSWER:
We apologize for making a confusing statement when we said “not surprising” in that sentence. The effect of FAZ27 knockdown on the stability of FLAM3 and ClpGM6 has never been investigated and nobody knew the results. The data presented in Figures 6A and 7A are not something that we have expected, or one can predict. We actually meant that since FLAM3 and ClpGM6 form a complex and are interdependent for stability (reported previously by Sunter et al., 2015) and our results showed that FAZ27 knockdown destabilized FLAM3 and ClpGM6 (Figs. 6A and 7A), it is “not surprising” that if the stability of either FLAM3 or ClpGM6 is affected by FAZ27 knockdown, the stability of another subunit of the FLAM3-ClpGM6 complex is also affected, because FLAM3 and ClpGM6 are interdependent. As such, we don’t think that these data should be presented in Supplemental data. We have rephrased the sentence to make our points clear. Thanks for this great comment.

Finally, in the discussion section, I regret that the authors did not take the opportunity to share their thoughts on FAZ27 and calcium signaling.

ANSWER:
Thanks for bringing this up. We briefly commented in the Results section about the potential involvement of the IQ motif in FAZ27 in calcium signaling. We didn’t expand the discussion on this point further for the following reasons. 1) There is no evidence that the IQ motif in FAZ27 is capable of binding to calmodulin and, hence, is involved in calcium signaling. 2) Calmodulin has not been reported to be involved in cell morphogenesis in *T. brucei*. Therefore, we feel that it is too speculative to discuss the potential relationship between FAZ27 and calcium signaling.

Second decision letter

MS ID#: JOCES/2020/245258

MS TITLE: FAZ27 cooperates with FLAM3 and ClpGM6 to maintain cell morphology in Trypanosoma brucei

AUTHORS: Tai An, Qing Zhou, Huiqing Hu, Harshini Cormaty, and Ziyin Li
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

Thanks for your extensive revisions following which I did not consider it necessary to return this to the reviewers.

There remains one outstanding point that you have not addressed and which was both raised by the original reviewers and forms part of our policies. This relates to deposition of the raw proteomics data. The data provided with the manuscript are processed data following your searches, not the raw data from the instrument. Our policy is that primary data should be deposited.
https://jcs.biologists.org/content/journal-policies#data
ProteomeXchange Consortium provides a collective submission point for proteomics data.
Once you include an accession number with your manuscript, we would be happy to accept your paper for publication.

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.

Second revision

Author response to reviewers' comments

There remains one outstanding point that you have not addressed and which was both raised by the original reviewers and forms part of our policies. This relates to deposition of the raw proteomics data. The data provided with the manuscript are processed data following your searches, not the raw data from the instrument.

ANSWER: An accession number (PA01567) has been included in the revised manuscript (lines 10-11 on page 18).

Third decision letter

MS ID#: JOCES/2020/245258

MS TITLE: FAZ27 cooperates with FLAM3 and ClpGM6 to maintain cell morphology in Trypanosoma brucei

AUTHORS: Tai An, Qing Zhou, Huiqing Hu, Harshini Cormaty, and Ziyin Li
ARTICLE TYPE: Research Article

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