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Review COMMONS

Interactions of cytosolic termini in the major facilitator superfamily member Jen1 are critical for trafficking and transport activity

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Reviewer 1

Evidence, reproducibility and clarity

SUMMARY

In this manuscript, Barata-Antunes investigate the effect of truncating wither the N, C or N and C termini of Jen1 on plasma membrane expression levels. They use cell lines that are deficient in certain alpha-arrestins to probe questions regarding what arrestins might be interacting with and with which termini. The authors then use split YFP to demonstrate that the N and C domain come within proximity of each other throughout the transport cycle.

MAJOR COMMENTS

- Deletion of the entire N and C termini resulted in non-functional Jen1. This implies that the entire sequences are important for plasma membrane (PM) localization of Jen1. Can the authors please justify why shorter truncations provide meaningful insight as to how the N and C termini control PM localization?

- Fig. 4: and Lines 290-316: Could the authors please provide more information about how they are extracting crude protein for Western blot analysis, e.g. are total cell proteins isolated or just PM proteins? This is critical for understanding these experiments as internalized proteins will still give off a GFP signal. Along the same line, this reviewer is confused as to why internalized GFP can still be seen in epifluorescence microscopy but not on Western - it would make sense that only PM proteins are being isolated for western however this does not appear to be not detailed in the methods.

- Lines 302-305 and 311-316: The authors claim that Jen1 localization and stability is similar between ROD1+ BUL1/2+ and the strain lacking Bul1/2 proteins and conclude that Bul1/2 participate little, if at all, in the endocytosis of the wild-type transporter. This reviewer does not quite see this reflected in the data. Rather, it appears that the deltaCT33 is not internalized by the strain lacking Bul1/2 proteins. This discrepancy should be to be addressed and re-analyzed.
This reviewer has an issue with claims regarding the sequential action of rod1 and bul1/2(e.g. lines 344-346), as these come across as vague and non-quantitative, and do not appear to be substantiated by the western blot analysis.

- This reviewer strongly feels that this manuscript would be improved if a more quantitative approach to data analysis was taken. In the absence of this many of the claims made are hand-wavy and solid conclusions cannot be reliably made.

- The authors mention specific lysine residues that are important for Jen1 ubiquitylation. However, no specifics regarding these residues seem to be mentioned, and/nor do the authors investigate the effect of mutating these residues on their system. This reviewer views this as a missed opportunity that would provide substantial insight into the molecular determinants required for Rod1/Bul1&2 ubiquitylation and feels that the paper would benefit from a more in-depth discussion and investigation of these residues.

- Time points for YFPn-Jen1-YFPc should also be provided in the absence of lactic acid as a control.

- The major aims and conclusions of this paper are never clearly defined. Saying a region of a protein has a "cryptic role" in modifying transporter function doesn't, in the Reviewer's opinion, quite suffice. The authors should use quantitative measures and focus on more specific regions or amino acids within these terminal regions in order to reach meaningful conclusions regarding which alpha-arrestins interact with what regions of the termini.

MINOR COMMENTS

- The introduction provides an overwhelming amount of information regarding the different types of alpha-arrestins in Saccharomyces cerevisiae, Aspergillus nidulans, and mammals (lines 69-19). Most of these are rarely mentioned again throughout the paper. This reviewer questions the relevance of these broad summaries of different types of arrestins in different organisms and finds it of little objective utility. Lines 125-136 (currently in the results section) would be a more suitable and relevant introduction, and this reviewer suggests cutting back on the current introduction's discussion of different alpha-arrestins to only include those that are directly relevant to understanding the results presented in this manuscript, and instead incorporating lines 125-136 into the introduction.

- Line 126 - a "model cargo" doesn't make much sense to this reviewer as cargo is typically the substrate of the transporter, not the transporter itself.

- Line 152-158: did the reviewers use alpha-fold to assist in construct design? This reviewer feels it would be a very useful tool and it could even be used for visualization.

- Fig. 3 and lines 262-265: Is expression accounted for in substrate transport assays? The authors should be cautious when describing kinetic properties of the truncated proteins as substrate binding and transport cannot be clearly distinguished in this type of assay. Additionally, a log10 scale on the x-axis would provide a much clearer visualization of the data distribution.

- Fig. 5A: Concentration response curves should be provided, preferably with a log10 scale on the x-axis - as is, the claim that they reached saturable xanthine import is not substantiated.

Significance

In this reviewers opinion the significance of these results is not as high as it could be should the authors perform more directed and quantitative experiments. The authors make claims that this study is informative for other transporters however aside from saying that the N and C termini are important (which is unsurprising) the study cannot really be extended to other proteins. The conclusions made in this paper need to be more concrete and specific in order for true significance to be assessed.

Readers of the paper in it's current state would have to be in the same field to find the conclusions informative.

This reviewers expertise is membrane protein biochemistry and structural biology.

Reviewer 2

Evidence, reproducibility and clarity

In the present work, Barata-Antunes et al. investigate the potential role of the N- and C- terminal regions of the Saccharomyces cerevisiae Jen1 transporter in the control of the protein endocytosis. For this, they generated truncated versions of Jen1 lacking either or both cytosolic N- and C-terminal fragments, and characterized the corresponding proteins. As confirmed by transport experiments, three out of the five originally obtained Jen1 truncated versions (called ?NT94, ?CT33 and ?NT94-?CT33) were functional, exhibiting even higher substrate affinity to lactate than the wild type counterpart. Interestingly, the stability of these truncated proteins in the plasma membrane (PM) was contrasting: Whereas ?NT94 underwent very rapid internalization and degradation, ?CT33 and ?NT94-?CT33 stably localized at the PM even under conditions known to trigger Jen1 endocytosis, with the latter being even more resistant to internalization than the former. To investigate the potential binding sites of the ?-arrestins Rod1 and Bul1/2, known to mediate Jen1 degradation, the authors then studied the dynamics of the functional Jen1 truncated versions in the presence or absence of either or both Rod1 and Bul1/2. The results suggested that Rod1 and Bul1/2 interact with the C- and the N-termini of Jen1, respectively, to induce the transporter degradation, and that these ?-arrestins act sequentially. The preponderance of Rod1 action via its interaction with the C-terminus of Jen1 was confirmed by studying the dynamics of protein chimeras in which the N- and C-terminal fragments corresponding to the functional Jen1 truncations were added to the Aspergillus nidulans UapA transporter, insensitive to endocytosis when expressed in S. cerevisiae. An interesting observation concerns the role of cER-PM contact points in protein retention in the ER, as their abolishment led to an enhanced protein chimera sorting to the PM. Finally, Barata-Antunes et al. explored the possibility of Jen1 protein turnover being regulated by the direct interaction of its N- and C- termini. BiFC experiments suggest that the two Jen1 tails do interact in cis, and that this interaction is dependent on the conformational changes owed to the protein transport activity, triggering protein endocytosis upon substrate translocation.

The work by Barata-Antunes et al. presents several lines of evidence convincingly supporting the central hypothesis of the work. The experimental design and the controls used are consistent with the research objective, and the data are adequately presented to allow their analysis. This is a well-written and well-structured manuscript which, I believe, would nonetheless benefit from tackling the minor issues raised below:

1. In lines 312-314, the authors say that "the role of Bul1/2 in glucose-triggered endocytosis was found to be more complex, as it seemed to depend on the presence or absence of Rod1". In fact, the western blots in Figure 4E-H are consistent with a negligible role for Bul1/2, as Jen1 levels decrease in a similar fashion in strains with a functional Rod1 (wild type and bul1?bul2?), whereas the protein amount is relatively constant in the strains lacking this arrestin (rod1? and rod1?bul1?bul2?), irrespective of Bul1/2 presence. However, the images in Figure 4A-D convincingly suggest that Bul1/2 do participate in Jen1 endocytosis. The authors should address this apparent contradiction and propose potential explanations for these results.
2. The arrows pointing towards some cell structures in the images in Figure 2C are not described in the figure legend.
3. In line 389, the reference for the value of native Km of UapA measured in A. nidulans is missing, as, if I understood correctly, the first value shown in Figure 5A corresponds to UapA expressed in S. cerevisiae.
4. For consistency, the position of Jen1?CT33 and Jen1?NT94 images in panel A of Figure S3 should be swapped.

Significance

The results of this work advance our understanding on: i) the importance of the N- and C- cytosolic tails of transporters of the MFS superfamily for protein localization, stability and function in yeast, and ii) the central role that the physical interactions between these structural elements play for the regulation of MFS transporters. The fundamental knowledge gathered may prove relevant not only to other researchers in the transport field, but also to those interested in developing biotechnological tools related to the control of protein localization and turnover. Moreover, it paves the way for a broader view on the role of these sequences in transporters, particularly of the

MFS, from other organisms. Plant MFS structural features remain largely unknown, with only a handful of proteins structurally resolved, and a single report tackling the potential role of a sugar transporter C-terminal in the regulation of protein localization (Yamada, Osakabe and Yamaguchi-Shinozaki, PLOS One, 2017). Thus, this study may serve as an important starting point for a more detailed look into understudied features of MFS transporters.

Reviewer 3

Evidence, reproducibility and clarity

In this manuscript, the authors provided insights into the roles of cytosolic termini of a monocarboxylate transporter Jen1 that belongs to the MFS superfamily. Through functional assays, fluorescence imaging and protein quantification, the authors found the N- and C- termini of Jen1 play important roles in trafficking, endocytosis and transport activity. They also identified arrestin proteins that are associated with N- and C- termini. Their data also suggested potential interactions between N- and C- termini of Jen1, which might be linked to the transport activity.

Major comments:

1) The potential interaction between N- and C-termini of Jen1 was proposed based on the results of bimolecular fluorescence (BiFC) assay. Since the split YFP is linked to the N- and C- terminus of the same transporter, YFPn and YFPc are already in close approximate. It is unclear whether the reconstitution of YFP requires the interaction between N- and C- termini in this case. Presumably, the flexibility of N- and C- terminal region of Jen1 might be able to allow YFPn and YFPc to interact. Is it possible to use FRET-based method, which can yield more quantitative information about the distance? Similarly, it is unclear how the conformational switch of the transporter can change the interaction between N- and C- termini. It will be useful to have a quantitative discussion about the distance change based on the known MFS transporter structures in different conformational states. In addition, does the presence of lactic acid affect the protein level of Jen1?

2) The experiments on UapA / Jen1 chimera are informative. It will be interesting to test whether the N- or C- terminal fragment can be placed on the other end. This can tell whether the fragment is truly portable and context independent.

3) Does the N- or C- terminal truncation affect the protein expression (synthesis) level? Such information will be needed to interpret the steady state level of the protein.

Minor comments:

It is unclear why Jen1-delta-NT94 in the triple knockout (rod1/bul1/bul2) is much less than in the rod1 knockout background. Any possible reason?

Significance

Transporters often have unstructured N- and C- termini. However, their functions are not well understood. The results presented in this manuscript provided significant insights into Jen1 transporter, enriched our knowledge about the roles of transporters' N- and C- termini, and contributed to our understanding about transporter regulation.

Author response to reviewers' comments

RESPONSE TO REVIEWERS

We thank the Reviewers for their careful and positive assessment of our manuscript. We have incorporated many of their suggestions (please see below) and we consider that it resulted in a stronger and clearer manuscript.
Please find below our detailed responses to the Reviewers' comments.
We hope that our responses to their comments will satisfactorily address their concerns and that they find the new revised manuscript suitable for publication in eLife.

Reviewer 1

SUMMARY
In this manuscript, Barata-Antunes investigate the effect of truncating wether the N, C or N and C termini of Jen1 on plasma membrane expression levels. They use cell lines that are deficient in certain alpha-arrestins to probe questions regarding what arrestins might be interacting with and with which termini. The authors then use split YFP to demonstrate that the N and C domain come within proximity of each other throughout the transport cycle.

MAJOR COMMENTS
- Deletion of the entire N and C termini resulted in non-functional Jen1. This implies that the entire sequences are important for plasma membrane (PM) localization of Jen1. Can the authors please justify why shorter truncations provide meaningful insight as to how the N and C termini control PM localization?

We created a series of N- and C-terminal truncations to more precisely define regions within the termini involved in transporter biogenesis, activity, and internalization. When truncated versions of genes result in total loss-of-function, it is a standard genetic strategy to construct shorter truncations that might lead to partially affected proteins with measurable function or, in our case, detectable subcellular trafficking. Also, in some cases concerning transporters, shorter truncations might delete degrons, or specific signals, affecting stability, turnover or regulatable endocytosis in response to a specific signal. Our data shows that one of the most important findings via such truncations is that Jen1 can be modified positively, leading to versions of improved substrate affinity or transport capacity.

In the revised version of our manuscript, we also now include a-fold predictions of Jen1, as asked by the Reviewer (see below), which justifies why our larger deletions lead to total loss-of-function. Basically, these truncations delete neighbouring to termini membrane-associated segments of Jen1, whereas shorter truncations delete only cytoplasmic hydrophilic parts of the termini (please see Figure 1 (page 6) and lines 135-142 (page 5)). The originally designed larger deletions were based on homology threading approaches and sequence alignments. This shows the power of a-fold prediction, and we thank the Reviewer for his suggestion.

- Fig. 4: and Lines 290-316: Could the authors please provide more information about how they are extracting crude protein for Western blot analysis, e.g. are total cell proteins isolated or just PM proteins? This is critical for understanding these experiments as internalized proteins will still give off a GFP signal. Along the same line, this reviewer is confused as to why internalized GFP can still be seen in epifluorescence microscopy but not on Western - it would make sense that only PM proteins are being isolated for western however this does not appear to be not detailed in the methods.

We agree with this observation. Often, in studies addressing transporter subcellular localization and turnover, results obtained through epifluorescence microscopy do not match well those from western blot analysis. Epifluorescence microscopy detects PM localization, which westerns cannot distinguish from the fraction of the transporter that remains in internal membranes. On the other hand, westerns detect in a more rigorous quantitative way the turnover of transporters (e.g. Jen1), by estimating the steady state amounts of intact GFP-tagged transporter (e.g. Jen1-GFP). Free GFP levels, estimated by microscopy (vacuolar fluorescent signal) or by westerns (intensity of relevant band), often give different results. The reason of this remains obscure, but might well be related to turnover of transporters during protein purification. In the present work, we tried to interpret our results based on steady state amounts of Jen1- GFP in westerns (quantitative criterion), and the presence of internalized fraction of Jen1-GFP (qualitative criterion).

- Lines 302-305 and 311-316: The authors claim that Jen1 localization and stability is similar between ROD1+ BUL1/2+ and the strain lacking Bul1/2 proteins and conclude that Bul1/2 participate little, if at all, in the endocytosis of the wild-type transporter. This reviewer does not quite see this reflected in the data. Rather, it appears that the deltaCT33 is not internalized by the strain lacking Bul1/2 proteins. This discrepancy should be to be addressed and re-analyzed. This reviewer has an issue with claims regarding the sequential action of rod1 and bul1/2 (e.g. lines 344-346), as these come across as vague and non-quantitative, and do not appear to be substantiated by the western blot analysis.

We agree with the Reviewer, and we have revised our conclusions accordingly in the revised manuscript (pages 12-14, lines 284-343).

This reviewer strongly feels that this manuscript would be improved if a more quantitative approach to data analysis was taken. In the absence of this many of the claims made are hand-wavy and solid conclusions cannot be reliably made.
We agree with the Reviewer and in the revised version we now include quantitative data. Please, see in the Figure 2D (page 9) and in Figure 4E (page 15) quantifications of the ratio of the fluorescence at the plasma membrane over the total fluorescence at the time point Gluc 4 h. Additionally, quantifications of the steady state protein levels and degradation of Jen1 truncations were done (see Figure S4, page 6).

- The authors mention specific lysine residues that are important for Jen1 ubiquitylation. However, no specifics regarding these residues seem to be mentioned, and/nor do the authors investigate the effect of mutating these residues on their system. This reviewer views this as a missed opportunity that would provide substantial insight into the molecular determinants required for Rod1/Bul1&2 ubiquitylation and feels that the paper would benefit from a more in-depth discussion and investigation of these residues.

Specific lysine residues have been shown to play a role in Jen1 endocytic turnover in previous reports (1–3). *Importantly, one of these studies revealed that only the replacement of all cytosolic lysine (K) residues of the transporter by arginine blocks Jen1 internalization* (2). *This construct was functional, while some of the lysine residues proved critical, but not essential, for transport activity. We believe that investigating these lysine residues individually would not bring new light into the role of Jen1 termini, which was the main focus of this work. We now shortened the introduction, as requested by one of the Reviewers, removing details regarding lysines, and only mentioning Ub target sites, in general.*

References:

1. *Fujita, S., Sato, D., Kasai, H., Ohashi, M., Tsukue, S., Takekoshi, Y., Gomi, K., and Shintani, T. (2018) The C-terminal region of the yeast monocarboxylate transporter Jen1 acts as a glucose signal-responding degron recognized by the α-arrestin Rod1. J. Biol. Chem.* ***293****, 10926-10936*
2. *Becuwe, M., and Léon, S. (2014) Integrated control of transporter endocytosis and recycling by the arrestin-related protein Rod1 and the ubiquitin ligase Rsp5. Elife.* ***3****, 1- 23*
3. *Paiva, S., Vieira, N., Nondier, I., Haguenauer-Tsapis, R., Casal, M., and Urban-Grimal, D. (2009) Glucose-induced ubiquitylation and endocytosis of the yeast Jen1 transporter: role of lysine 63-linked ubiquitin chains. J. Biol. Chem.* ***284****, 19228-36*

- Time points for YFPn-Jen1-YFPc should also be provided in the absence of lactic acid as a control.

This is now included in the manuscript (Figure 8B, middle panel, page 22).

- The major aims and conclusions of this paper are never clearly defined. Saying a region of a protein has a "cryptic role" in modifying transporter function doesn't, in the Reviewer's opinion, quite suffice. The authors should use quantitative measures and focus on more specific regions or amino acids within these terminal regions in order to reach meaningful conclusions regarding which alpha-arrestins interact with what regions of the termini.

We do not agree with this comment, however, in the revised version we did our best to make our conclusions more explicit. As 'cryptic' seems to create confusion, we replace it with 'complex'. Basically, the main aim of this work was to see what the Jen1 termini do. Do they affect function? Which function? How? Do they interact with each other and the rest of the transporter body? In most cases, to define specific sequence motifs is complex. Their role proves to be context-dependent, which strongly suggest that termini motifs affect interactions with other parts of the protein. We regret that some of the most original findings described were not emphasized in the original version of the manuscript. In brief, the novel findings are:

1. *Termini interact with each other dynamically to control function. To our knowledge, this has been formally detected only in FurE, which belongs to the NCS1/APC superfamily, distinct structurally and functionally distinct from Jen1/MFS.*
2. *We defined a segment that in a context-independent manner elicits glucose triggered endocytosis. To our knowledge, this is the first time such a finding is revealed.*
3. *We used rigorous functional assays to characterize chimeras of Jen1 with heterologous glucose-insensitive transporter (UapA) which, to our opinion, is an elegant genetic approach to investigate the issue of context-dependence. The aforementioned points are now explicitly stated in the revised version of the manuscript and quantitative data was also added as mentioned above.*

We consider that we have now clearly highlighted the novelty of our work.

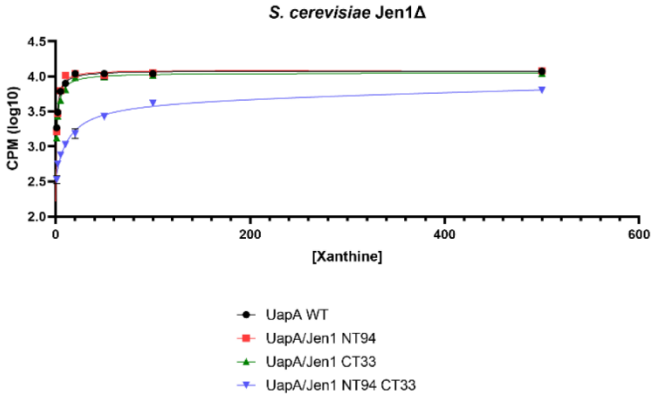
MINOR COMMENTS

- The introduction provides an overwhelming amount of information regarding the different types of alpha-arrestins in *Saccharomyces cerevisiae*, *Aspergillus nidulans*, and mammals (lines 69-19). Most of these are rarely mentioned again throughout the paper. This reviewer questions the relevance of these broad summaries of different types of arrestins in different organisms and finds it of little objective utility. Lines 125-136 (currently in the results section) would be a more suitable and relevant introduction, and this reviewer suggests cutting back on the current introduction's discussion of different alpha-arrestins to only include those that are directly relevant to understanding the results presented in this manuscript, and instead incorporating lines 125-136 into the introduction.
We agree with the Reviewer. The introduction has now been shortened as requested by the reviewer (pages 3-4).

- Line 126 - a "model cargo" doesn't make much sense to this reviewer as cargo is typically the substrate of the transporter, not the transporter itself.
The word model is used in a different sense. The Jen1 protein itself is a membrane trafficking "cargo" protein. This is a standard terminology in studies addressing protein trafficking. So, in this work, we use Jen1 as model cargo protein to study membrane trafficking.

- Line 152-158: did the reviewers use alpha-fold to assist in construct design? This reviewer feels it would be a very useful tool and it could even be used for visualization. *We thank the reviewer for this suggestion. We have now included the alpha-fold prediction and its analysis was invaluable to interpret some of our data (please see Figure 1 (page 6) and lines 135-142 (page 5)).*

- Fig. 3 and lines 262-265: Is expression accounted for in substrate transport assays? The authors should be cautious when describing kinetic properties of the truncated proteins as substrate binding and transport cannot be clearly distinguished in this type of assay. Additionally, a log10 scale on the x-axis would provide a much clearer visualization of the data distribution.
Jen1 expression was accounted, not by westerns, but by microscopy. Notice also, that we mostly discuss K_m values, which are not affected by expression levels. A log10 scale does not provide a clearer visualization of the data (see the example below), therefore, we decided to keep the graphic without the log10 scale.



- Fig. 5A: Concentration response curves should be provided, preferably with a log10 scale on the x-axis - as is, the claim that they reached saturable xanthine import is not substantiated.

We agree with the Reviewer. Concentration response curves are now included in the manuscript (please, see Figure 5B, page 17).

In this reviewers opinion the significance of these results is not as high as it could be should the authors perform more directed and quantitative experiments. The authors make claims that this study is informative for other transporters however aside from saying that the N and C termini are important (which is unsurprising) the study cannot really be extended to other proteins. The conclusions made in this paper need to be more concrete and specific in order for true significance to be assessed. Readers of the paper in its current state would have to be in the same field to find the conclusions informative.

As already stated above, in the revised manuscript, we tried to make our conclusions and novel findings more explicit. In conclusion, by using genetic approaches, functional assays and BiFC, we provided compelling evidence that Jen1 termini interact functionally. This is the first report of functional termini interactions in a MFS transporter. Importantly, termini modifications were shown to lead to hypermorphic Jen1 mutants with increased substrate affinity and increased stability. This finding opens the way for creating modified or improved MFS transporters.

Reviewer 2

In the present work, Barata-Antunes et al. investigate the potential role of the N- and C-terminal regions of the *Saccharomyces cerevisiae* Jen1 transporter in the control of the protein endocytosis. For this, they generated truncated versions of Jen1 lacking either or both cytosolic N- and C-terminal fragments, and characterized the corresponding proteins. As confirmed by transport experiments, three out of the five originally obtained Jen1 truncated versions (called ΔNT94, ΔCT33 and ΔNT94- ΔCT33) were functional, exhibiting even higher substrate affinity to lactate than the wild type counterpart. Interestingly, the stability of these truncated proteins in the plasma membrane (PM) was contrasting: Whereas ΔNT94 underwent very rapid internalization and degradation, ΔCT33 and ΔNT94-ΔCT33 stably localized at the PM even under conditions known to trigger Jen1 endocytosis, with the latter being even more resistant to internalization than the former. To investigate the potential binding sites of the α-arrestins Rod1 and Bul1/2, known to mediate Jen1 degradation, the authors then studied the dynamics of the functional Jen1 truncated versions in the presence or absence of either or both Rod1 and Bul1/2. The results suggested that Rod1 and Bul1/2 interact with the C- and the N-termini of Jen1, respectively, to induce the transporter degradation, and that these α-arrestins act sequentially. The preponderance of Rod1 action via its interaction with the C-terminus of Jen1 was confirmed by studying the dynamics of protein chimeras in which the N- and C-terminal fragments corresponding to the functional Jen1 truncations were added to the *Aspergillus nidulans* UapA transporter, insensitive to endocytosis when expressed in *S. cerevisiae*. An interesting observation concerns the role of cER-PM contact points in protein retention in the ER, as their abolishment led to an enhanced protein chimera sorting to the PM. Finally, Barata-Antunes et al. explored the possibility of Jen1 protein turnover being regulated by the direct interaction of its N- and C-termini. BiFC experiments suggest that the two Jen1 tails do interact in cis, and that this interaction is dependent on the conformational changes owed to the protein transport activity, triggering protein endocytosis upon substrate translocation.

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1. In lines 312-314, the authors say that "the role of Bul1/2 in glucose-triggered endocytosis was found to be more complex, as it seemed to depend on the presence or absence of Rod1". In fact, the western blots in Figure 4E-H are consistent with a negligible role for Bul1/2, as Jen1 levels decrease in a similar fashion in strains with a functional Rod1 (wild type and bul1Δbul2Δ), whereas the protein amount is relatively constant in the strains lacking this arrestin (rod1Δ and rod1Δbul1Δbul2Δ), irrespective of Bul1/2 presence. However, the images in Figure 4A-D convincingly suggest that Bul1/2 do participate in Jen1 endocytosis. The authors should address this apparent contradiction and propose potential explanations for these results.
This observation is in line with another comment by Reviewer 1 (above). We agree with the Reviewers, and we have revised our conclusions in the updated manuscript (pages 12-14, lines 284-343).

2. The arrows pointing towards some cell structures in the images in Figure 2C are not described in the figure legend.
This is now included in the legend of Figure 2 (pages 9-10, line 236).

3. In line 389, the reference for the value of native Km of UapA measured in *A. nidulans* is missing, as, if I understood correctly, the first value shown in Figure 5A corresponds to UapA expressed in *S. cerevisiae*.
We confirm that the first value in the table of the Figure 5A (now Figure 5B, page 17) correspond to the K_m value of UapA expressed in S. cerevisiae. However, we also add in the main text the value of the native K_m of UapA measured in *A. nidulans* (described in the literature), as suggested by the Reviewer (page 18, line 396).

4. For consistency, the position of Jen1ΔCT33 and Jen1ΔNT94 images in panel A of Figure S3 should be swapped.
The position of the images is now swapped (please see Figure S3 in supplementary material, page 5).

The results of this work advance our understanding on: i) the importance of the N- and C- cytosolic tails of transporters of the MFS superfamily for protein localization, stability and function in yeast, and ii) the central role that the physical interactions between these structural elements play for the regulation of MFS transporters. The fundamental knowledge gathered may prove relevant not only to other researchers in the transport field, but also to those interested in developing biotechnological tools related to the control of protein localization and turnover. Moreover, it paves the way for a broader view on the role of these sequences in transporters, particularly of the MFS, from other organisms. Plant MFS structural features remain largely unknown, with only a handful of proteins structurally resolved, and a single report tackling the potential role of a sugar transporter C-terminal in the regulation of protein localization (Yamada, Osakabe and Yamaguchi-Shinozaki, PLOS One, 2017). Thus, this study may serve as an important starting point for a more detailed look into understudied features of MFS transporters.

Reviewer 3
In this manuscript, the authors provided insights into the roles of cytosolic termini of a monocarboxylate transporter Jen1 that belongs to the MFS superfamily. Through functional assays, fluorescence imaging and protein quantification, the authors found the N- and C- termini of Jen1 play important roles in trafficking, endocytosis and transport activity. They also identified arrestin proteins that are associated with N- and C- termini. Their data also suggested potential interactions between N- and C- termini of Jen1, which might be linked to the transport activity.

Major comments:

1) The potential interaction between N- and C-termini of Jen1 was proposed based on the results of bimolecular fluorescence (BiFC) assay. Since the split YFP is linked to the N- and C- terminus of the same transporter, YFPn and YFPc are already in close approximate (proximity). It is unclear whether the reconstitution of YFP requires the interaction between N- and C- termini in this case. Presumably, the flexibility of N- and C- terminal region of Jen1 might be able to allow YFPn and YFPc to interact. Is it possible to use FRET-based method, which can yield more quantitative information about the distance?

We believe that FRET will not provide more accurate or detailed spatial resolution, as the long termini can undergo flexible and dynamic interactions. Moreover, both methods (BiFC and FRET) rely on close proximity (at the nanometer scale) for signal generation. FRET assays may be better suited to monitor temporal dynamics (and lifetime fluorescence), but they will not provide additional information on distances between the N- and C-termini. While we appreciate the reviewer's suggestion, this would require sophisticated spin-coupling experiments between cysteine residues engineered throughout the termini or NMR studies using the purified, reconstituted Jen1 protein that are beyond the scope of the current study.

Similarly, it is unclear how the conformational switch of the transporter can change the interaction between N- and C- termini. It will be useful to have a quantitative discussion about the distance change based on the known MFS transporter structures in different conformational states.

We do not think we can comment on molecular distances based on alpha-fold prediction of regions that are intrinsically flexible and unstructured.

In addition, does the presence of lactic acid affect the protein level of Jen1?

The presence of lactic acid only affects the protein level of Jen1 after a prolonged growth under this carbon source. Our previous data (4) *(please, see Figure 2B of this reference) showed that Jen1 transporter is localized at the plasma membrane even after 8 h in the presence of lactic acid, being internalized and targeted to the vacuoles only after 24 h. In line with this, the steady-state levels of JEN1-GFP are also stabilized after 8 h, in the presence of lactate, as observed by the western blot in the same figure. However, as a control and to exclude the hypothesis of lactate-induced internalization of Jen1, we also analyzed the localization of the JEN1-GFP by fluorescent microscopy under the growth conditions and time-points used for the BiFC assay. These results are now added in the manuscript (Figure 8B, left panel), confirming that 50 min in lactic acid does not trigger endocytosis of Jen1.*

References:

4. *Talaia, G., Gournas, C., Saliba, E., Barata-Antunes, C., Casal, M., André, B., Diallinas, G., and Paiva, S. (2017) The a-Arrestin Bul1p Mediates Lactate Transporter Endocytosis in Response to Alkalinization and Distinct Physiological Signals. J. Mol. Biol.* ***429****, 3678-3695*

2) The experiments on UapA / Jen1 chimera are informative. It will be interesting to test whether the N- or C- terminal fragment can be placed on the other end. This can tell whether the fragment is truly portable and context independent.

As suggested by the Reviewer, a new chimera was designed, in which the CT33 portion of the Jen1 was fused to the N-terminus of the UapA transporter (Jen1CT33/UapA, please see Figure below). This chimera is mostly ER-retained, and its steady state levels and localization are little affected by the presence of glucose (please see below Figure B-C).

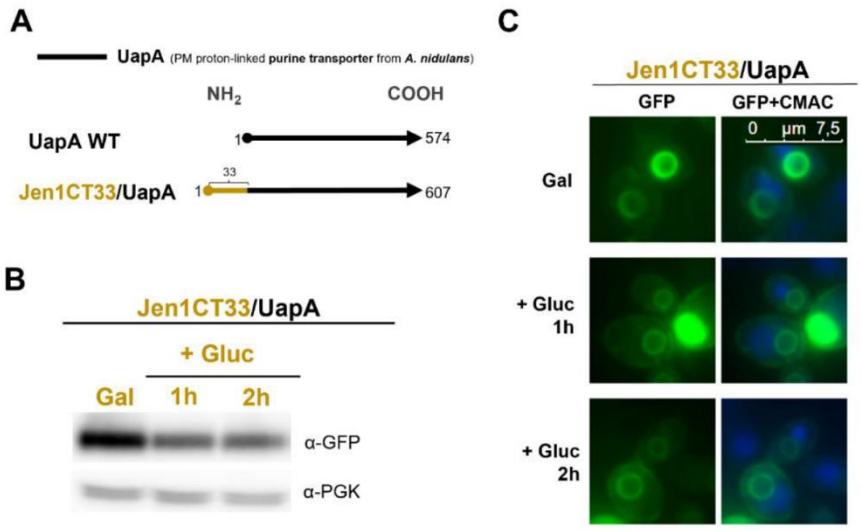

Figure - The CT33 segment of Jen1 fused at the N-terminus of UapA is retained in the ER. Graphical representation of the Jen1CT33/UapA chimera. The CT33 segment of Jen1 (amino acid residues from 584 to 616) is coloured in light brown and was fused to the N-terminal region of the purine transporter UapA from A. nidulans, represented in black (A). Cells of S. cerevisiae ROD1+ BUL1/2+ strain, expressing Jen1CT33-UapA tagged with GFP and expressed under a GAL promoter, were analysed by Western blot (B) and by epifluorescence microscopy (C). Cells were grown overnight in YNB galactose (2 %, w/v) + glucose (0.1 %, w/v) medium until mid-exponential phase and glucose was added when indicated (Gal + Gluc 1 h and Gal + Gluc 2 h). At these time points, protein extracts were prepared for western immunoblotting with an anti-GFP antibody or anti-phosphoglycerate kinase (PGK) antibody (loading control) (B), or cells were visualized by fluorescence microscopy (C). The CMAC dye was used as a vacuolar marker (blue).

3) Does the N- or C- terminal truncation affect the protein expression (synthesis) level? Such information will be needed to interpret the steady state level of the protein.
The N- and C- terminal truncations affect the protein expression as demonstrated by western blots in Figure S4A (page 6). Deletion of the C-terminal portion (Jen1ΔCT33) leads to an increase of the steady state protein levels comparing to the wild type Jen1. Even more apparent in the doubly truncated version Jen1ΔNT94ΔCT33. This is also evident by the fluorescent images shown in Figures 2C (page 9). For instance, Jen1ΔCT33 and Jen1ΔNT94ΔCT33 showed increased expression (increased GFP signal at the PM) when compared to the wild-type Jen1, under lactic acid-grown conditions (Lac 4 h).

Minor comments:
It is unclear why Jen1-delta-NT94 in the triple knockout (rod1/bul1/bul2) is much less than in the rod1 knockout background. Any possible reason?
Indeed, the expression of Jen1ΔNT94 is much lower in cells lacking the bul1/2 arrestin (bul1Δbul2Δ and rod1Δbul1Δbul2Δ), being undetectable by western blot analysis. This was not the first case in which removing specific arrestins resulted in a lower level of expression of PM transporters. In the work of Hovsepian J. and colleagues (5)*, deletion of the crs2/art8 arrestin, resulted in a much lower expression of the Hxt6-GFP and Hxt7-GFP transporters. Importantly, this phenotype was not due to an unconventional role of Crs2 in transporter expression, once it was not suppressed by expressing a plasmid containing Crs2* (5)*. In our work, only the truncated Jen1ΔNT94- GFP, and not the wild type Jen1-GFP, showed a strong decrease in its steady-state protein levels when expressed in bul1/2Δ strain. Therefore, we consider that Bul1/2 does not affect the expression levels of Jen1 transporter, however, we did not test for this hypothesis. Considering that Jen1ΔNT94 is an unstable version of Jen1, it could be more vulnerable to degradation under our conditions of total protein extraction or that a significant part of membrane associated Jen1 is lost. In fact, the detection of Jen1ΔNT94 by western blot was weak even in the wild type background.*

References:

*5. Hovsepian, J., Defenouillère, Q., Albanèse, V., Váchová, L., Garcia, C., Palková, Z., and Léon, S. (2017) Multilevel regulation of an a-arrestin by glucose depletion controls hexose transporter endocytosis. J. Cell Biol. **216**, 1811-1831*

Transporters often have unstructured N- and C- termini. However, their functions are not well understood. The results presented in this manuscript provided significant insights into Jen1 transporter, enriched our knowledge about the roles of transporters' N- and C- termini, and contributed to our understanding about transporter regulation.

Original submission

First decision letter

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