



Reconstitution of human atlastin fusion activity reveals autoinhibition by the C-terminus

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August 17, 2021

Re: JCB manuscript #202107070

Dr. Tina H Lee
Carnegie Mellon University
Biological Sciences
4400 5th Avenue
Pittsburgh, PA 15213

Dear Dr. Lee,

Thank you for submitting your manuscript entitled "Reconstitution of human atlastin fusion activity reveals autoinhibition by the C-terminus". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

As you will see, the reviewers are positive about both the advance and technical quality of your work. However, there have provided constructive comments to further validate some of your conclusions. While we agree that examining the role(s) of the twin glutamates (reviewer 2 point 1) could provide interesting mechanistic insight, we do not find that addressing this is required for further consideration at JCB, as elucidating the full mechanism of action of the C-terminus can be the subject of a follow up study. However, all other reviewer comments should be addressed with experimental revisions where requested.

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, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, <https://jcb.rupress.org/site/misc/ifora.xhtml>. All figures in accepted manuscripts will be screened prior to publication.

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Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on 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,

Jodi Nunnari, Ph.D.
Editor-in-Chief

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

Journal of Cell Biology

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

Atlastins are large GTPases that mediate fusion of ER membranes. The work presented here represents the first reconstituted membrane fusion with purified full length vertebrate At1 and At2. At1 purified from HEK cells had fusion activity that was comparable to *Drosophila* At1 (dAt1). The consequences of amino acid substitutions at R77 and R217 was also similar in At1 and dAt1, indicating that At1 likely functions via a similar mechanism that requires intermolecular GTPase domain dimers and crossover of the helical bundles. However, there were also significant differences from dAt1 indicating functional distinctions between the vertebrate and *Drosophila* homologs. First, an amino acid substitution associated with SPG3 (R239C) did not alter dAt1 function but reduced fusion by At1. Second, full length At2 did not support any lipid mixing in the reconstituted system. Authors found that unique extensions at the C-terminus of At1 and At2 possess inhibitory activity and fusion increased by several fold when this region was removed so that deltaC-At1 and deltaC-At2 had similar fusion activity. Domain swap experiments indicate that At2 CTD was a more potent auto-inhibitory domain. Truncations from the C-terminus indicate that most of the region contributes to the inhibitory role, but not equally. The strongest positive effect on fusion was observed upon removal of 30 amino acids. Data from the experiments presented in this manuscript indicate that CTD does not affect GTP binding, but does suppress tethering and GTPase activity. Finally, charge reversals in the CTD indicate that charges proximal to the amphipathic helix are important contributors to the regulatory function of this domain and alternative splicing of At2 alters these charged positions and removes autoinhibitory function of the CTD.

In all, the manuscript presents important data and advances the field through biochemical characterization of At1 and At2. Description of autoinhibition of some vertebrate atlastin isoforms is also an important advance that impacts understanding of the basic mechanism of ER membrane fusion and its regulation in cells. Some additional experiments would help to validate the conclusions drawn.

Concerns:

1. While the CTD of both At1 and At2 had inhibitory activity, At2 was more potent and the sole focus of Figures 4-7. The sequence conservation is limited and therefore, it is not clear that the conclusions drawn for At2-CTD would also hold true for At1 and why authors did not characterize both. A few experiments informed by At2 results could be done with At1. For example, charge reversals or serial truncations or analysis of tethering and GTPase activity of full length and At1-deltaC.
2. For the tethering assay in Figure 5B, proteoliposomes are treated the same as in fusion assays, but absorbance at 405 is measured rather than fluorescence. Is it known whether the tethered proteoliposomes are on-pathway? Do these go on to fuse? Given the importance of this result in the model for autoinhibition, another measure of the intermolecular GTPase-dependent dimer would greatly increase confidence in the conclusions drawn by authors. This could be FRET or SEC-MALS or immunoprecipitation of differentially tagged At2.
3. The rate of fusion for deltaCTD-At2 is 500 fold higher than full length. Yet changes in tethering and GTP hydrolysis are 100 fold less than this. Authors could comment on the discrepancy.
4. When overexpressed in U2OS cells, HA-At2 is localized to ER tubules and in some bright spots throughout the network. In contrast, the EER->KKE CTD charge reversal was primarily localized to collapsed ER. While the difference is striking, it's difficult to infer functional changes due to lack of controls. Is the same phenomenon observed upon overexpression of At2-deltaC or At2-isoform 2?
5. The role of the amphipathic helix in fusion is not tested. The helix was tested in a chimeric protein with *Drosophila* atlastin in Faust et al, where it did not support the same amount of fusion as full-length *drosophila* atlastin. This raises the possibility that it functions differently in the vertebrate protein and given the proximity to the inhibitory CTD, it's important to test this. It could be useful to show that amino acid substitutions that disrupt the hydrophobic properties of the amphipathic helix attenuate fusion in the presence and absence of the CTD.

Minor Comments:

1. The shape of the fusion curve is different for At2 (1-547) and At2 (E555K, E556K, R559E). Authors could comment on this difference in the text.

2. Figure legends indicate that fusion data are the averages of two independent traces. Are these technical replicates or biological replicates? If technical, are similar results obtained with biological replicates?
3. The black text in a red box is difficult to see in Figure 2. Consider changing text to white.
4. The overall ER structure is difficult to see in Figure 6C. It would be informative to stain with an ER marker in addition to the Atlastin signal.
5. Does H258R increase Atl1-mediated fusion by removing the CTD auto-inhibition?

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

This is an elegant, well-controlled study of the mechanism(s) of auto-inhibition in Atlastin (ATL) membrane fusion activity by the extreme C-terminus, which is divergent among ATL paralogs. Despite my best efforts, I really could not find any glaring flaws or omissions in this well-crafted study. However, the mechanism(s) still remain at large with multiple models being proposed in the Discussion. I would appreciate if at least one of these models is tested in the manuscript as detailed below.

Major concern:

1. What exactly are the role(s) of the twin glutamates (Es) in the C-term? Among other scenarios, it is plausible that they could orient and/or regulate the insertion of the upstream amphipathic α -helix (AH) by providing charge-repulsion to negatively charged lipids. Charge-reversal mutants (E to K), on the other hand, could enhance direct membrane interactions/insertion of this C-term segment as part of a contiguous helix with the AH. This should be tested by direct assays of AH or C-term membrane insertion/interactions, and the influence of mutations on it. Have these been done yet? These assays will provide some mechanistic insight into the C-term's mechanism of action.

Minor points:

2. Does the C-term also influence ATL GTPase activity or G-domain dimerization in a minimal construct lacking the 3HB (akin to the dynamin GG construct), and independent of membranes? (this is perhaps a relatively minor concern)
3. The light-scattering assay for liposome tethering must be supported by EM, as light scattering could increase even with protein instability and/or aggregation, especially with the C-term mutants.
4. Folding issue: *E. coli* vs HEK293; The authors indicate that a folding issue (and not phosphorylation) might be the reason why *E. coli*-expressed ATLs have no fusion activity, while mammalian cell-expressed versions do. Where is the evidence for it? Indeed, no such differences have been seen with other dynamin family members (especially for dynamin), and *E. coli* expression has become quite the norm in the field for the last decade. Only phosphorylation as a difference is ruled out here. What other PTMs might be involved? If no evidence exists currently, it is better left out from the Results section, and not speculated upon.

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

Atlastins (ATL1, ATL2, and ATL3) are multidomain GTPases that catalyze homotypic fusion of ER membranes. Understanding the function and regulation of these enzymes is critical from a clinical perspective as atlastin mutations are associated with Hereditary Spastic Paraplegia. Despite extensive characterization using structural and cellular imaging techniques, the regulatory mechanisms controlling different human atlastin isoforms remains poorly defined. In this manuscript, Crosby and Lee successfully reconstitute the membrane fusion activity human ATL1 and ATL2 and measure the kinetics of initial fusion rates for different atlastin constructs and mutants. This alone represents a significant advancement for the field, as to date only the *Drosophila* atlastin has been functionally reconstituted *in vitro*. Surprisingly, the authors find that both ATL1 and ATL2 are autoinhibited and negatively regulated by the C-terminus. Using truncations, chimeras, and charge reversal mutants, the authors map important residues critical to the autoinhibition and show that the C-terminus impairs membrane tethering and GTP hydrolysis *in vitro* without affecting GTP binding. The authors further demonstrate that autoinhibition plays a role in maintaining normal ER network homeostasis *in vivo*. In the absence of structural data, the authors draw from recent studies of GBP1 to present a speculative model for autoinhibition, which details how the C-terminus could modulate atlastin tethering and hydrolysis. While this work is suitable for JCB, a number of minor things should be addressed prior to publication:

- 1) Given the non-native folding states you observe for ATL1 and ATL2 in *E. coli* and the potential for autoinhibition, have you tried to analyze DATL expressed from another source like HEK cells or insect cells to see if there's a different activity? Does that change the fusion activity and/or kinetics of DATL in your assays?

- 2) What happens to fusion and kinetics if you mix a full-length construct on one set of membranes and a truncated form on another set of membranes (e.g. ATL2 and ATL2 Δ C)? This might provide some additional insights as to the mechanism of inhibition by the extended C-terminus.

- 3) In rationalizing the phenotypes for the Δ L549,G550 construct, the authors state: "However, deletion of two residues from the helix was expected to place the identified charged cluster on the opposite, rather than the same, side as the nonpolar face of the amphipathic helix (Fig 6A, DL549, G550, lower helix). Strikingly, simply deleting the two residues increased the fusion rate to

nearly the same extent as the E555K, E556K charge reversal (Fig 6B), indicating that the autoinhibition mechanism likely depends on the charged residues pointing in the same direction as the nonpolar face of the amphipathic helix." This assumes that deletion of these two residues simply induces a register shift in the helix. If removing these residues actually disrupts the structural integrity of the helix, then it's equally possible that the downstream side chains might become disordered, which could have the same net effect of displacing the charged residues and relieving inhibition (much like deletion of the whole segment). Similarly, the authors interpret the P548A,G550A phenotype as "indicating a lack of requirement for a substantial helix disruption or a turn". This statement again makes assumptions about effects of potential changes without any structural validation. I would suggest softening the language and/or presenting alternative plausible interpretations in the absence of supporting structural data.

4) Does overexpression of ATL2 Δ C in U2OS cells also lead to catastrophic defects in ER morphology? One would expect it would recapitulate the same phenotypes observed with the charge reversal mutants since the assumption is that both changes remove the autoinhibitory effect.

5) Conformational coupling and indirect, allosteric modulation of G domain functions by other domains is a common theme that has also emerged from studies of dynamin superfamily proteins and Arf GTPases. For example, membrane binding interactions mediated by dynamin's PH domain via the bundle signaling element or the N-terminal helix of Arf family members directly influence the catalytic activity and structural changes in the GTP binding pocket in a back-to-front manner. The discussion section nicely lays out a potential analogous mechanism based on recent work characterizing GBP1 intramolecular interactions. Including a comparison to other similar examples would broaden the impact of the story and help further delineate distinctions among large, mechanochemical GTPases.

6) Based on comparisons with GBP1, it's tantalizing to suggest that there are residues in the upper portion of the 3HB, the linker, and/or the back side of the G domain that directly interact with the C terminus. Have you looked further at the available atlastin structures to see if this might be true (i.e. are there clusters of oppositely charged residues that could associate with the region containing residues 554-560)? If so, mutating those side chains and analyzing their effects in your assays may further your mechanistic description and could strengthen the model presented in Figure 8C.

7) I appreciate the authors' attempt to present a model that best describes the current data and their honest assessment of what information is still missing and/or has yet to be tested. Given our current understanding, I would suggest adding labels to the upper panels of Figure 8C to denote that the depicted AH-3HB and C-term-G domain interactions/conformations are predicted and modeled and have yet to be validated. This would ensure the readers know this is still speculation rather than dogma.

Minor comments:

1) Page 7, line 140: "...a truncation removing the entire ATL1 tail harboring the required amphipathic helix"
It would be helpful to the reader to clarify the boundaries of this truncation with residue numbers and/or label it specifically as "tail" in domain diagram such as shown in Figure 2A to distinguish it from just the C-terminal segment.

Similarly, it would be helpful to number the domain boundaries in Figure 2C to explicitly orient the reader as to what segments are present in each of the constructs analyzed in Figure 2D.

2) Page 9, line 188: remove the hyphen in "G-domain"

3) Page 13, line 264-265: "we focused on the residues closest to the amphipathic helix, within AA554-565."
I would suggest changing the numbering to "AA548-565" as the mutations discussed here and show in Figure 6 span this entire region and it would be more correct as far as describing this segment as "closest to the amphipathic helix".

4) Page 13, line 265-266: "...secondary structure prediction algorithms predict..."
The figure legend mentions JPRED. Were other algorithms used? How well do the results compare across different algorithms and what are the confidence levels in the different outputs? Please clarify as this all helical model is presented without any supporting structural data.

5) Figure 6A: It would be beneficial to label the position of 565 in the domain diagram as this is referenced in the text and it would help orient the reader with respect to the helical cartoon below. The authors might also consider realigning the "547" label which is currently hovering over part of the C-terminal extension but in fact denotes the end of the AH. Additionally, it would be useful to label residues 457, 554, and 560 along with 527 in the alignment to again orient the reader with respect to domain boundaries and specific mutations tested.

6) Figure 6 legend: A reference for JPRED should be added.

7) Figure 8A: The images of the GBP1 and G domain structures appear to be low resolution. Ray tracing these prior to publication would avoid unintended pixilation, assuming they were generated with Pymol.

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

Atlastins are large GTPases that mediate fusion of ER membranes. The work presented here represents the first reconstituted membrane fusion with purified full length vertebrate Atl1 and Atl2. Atl1 purified from HEK cells had fusion activity that was comparable to Drosophila Atl (dAtl). The consequences of amino acid substitutions at R77 and R217 was also similar in Atl1 and dAtl, indicating that Atl1 likely functions via a similar mechanism that requires intermolecular GTPase domain dimers and crossover of the helical bundles. However, there were also significant differences from dAtl indicating functional distinctions between the vertebrate and Drosophila homologs. First, an amino acid substitution associated with SPG3 (R239C) did not alter dAtl function but reduced fusion by Atl1. Second, full length Atl2 did not support any lipid mixing in the reconstituted system. Authors found that unique extensions at the C-terminus of Atl1 and Atl2 possess inhibitory activity and fusion increased by several fold when this region was removed so that deltaC-Atl1 and deltaC-Atl2 had similar fusion activity. Domain swap experiments indicate that Atl2 CTD was a more potent auto-inhibitory domain. Truncations from the C-terminus indicate that most of the region contributes to the inhibitory role, but not equally. The strongest positive effect on fusion was observed upon removal of 30 amino acids. Data from the experiments presented in this manuscript indicate that CTD does not affect GTP binding, but does suppress tethering and GTPase activity. Finally, charge reversals in the CTD indicate that charges proximal to the amphipathic helix are important contributors to the regulatory function of this domain and alternative splicing of Atl2 alters these charged positions and removes autoinhibitory function of the CTD.

In all, the manuscript presents important data and advances the field through biochemical characterization of Atl1 and Atl2. Description of autoinhibition of some vertebrate atlastin isoforms is also an important advance that impacts understanding of the basic mechanism of ER membrane fusion and its regulation in cells. Some additional experiments would help to validate the conclusions drawn.

Response: We thank the reviewer for their appreciation of the advances we have made and have done our best to address their concerns as detailed below.

Concerns:

1. While the CTD of both Atl1 and Atl2 had inhibitory activity, Atl2 was more potent and the sole focus of Figures 4-7. The sequence conservation is limited and therefore, it is not clear that the conclusions drawn for Atl2-CTD would also hold true for Atl1 and why authors did not characterize both. A few experiments informed by Atl2 results could be done with Atl1. For example, charge reversals or serial truncations or analysis of tethering and GTPase activity of full length and Atl1-deltaC.

Response: As suggested by the reviewer, we have furthered our analysis of ATL1 autoinhibition by testing serial truncations of its C-term. Interestingly, while ATL1 and ATL2 share no sequence similarity in the C-term, the inhibition of ATL1 also maps to residues very near the amphipathic helix, with the inhibitory element being relatively

localized. This new data has been added as the latter part (C, D) of the new Figure 3 (previously Figure 4). The fact that ATL1 has only one relatively tightly localized inhibitory element whereas ATL2 has multiple inhibitory elements that span the C-term length could explain why inhibition by the ATL1 C-term is less potent. However, more in depth analysis of both ATL1 and ATL2 C-terms and how they influence tethering and fusion will be required to assess whether the autoinhibition mechanisms are the same or different.

2. For the tethering assay in Figure 5B, proteoliposomes are treated the same as in fusion assays, but absorbance at 405 is measured rather than fluorescence. Is it known whether the tethered proteoliposomes are on-pathway? Do these go on to fuse? Given the importance of this result in the model for autoinhibition, another measure of the intermolecular GTPase-dependent dimer would greatly increase confidence in the conclusions drawn by authors. This could be FRET or SEC-MALS or immunoprecipitation of differentially tagged AtI2.

Response: We agree that FRET, SEC-MALS or Co-IP would be interesting approaches to try. However, these techniques may not distinguish between weaker and stronger trans dimers nor distinguish between trans dimers that are on pathway and those that are not. On the other hand, one indication that the greater apparent tethering seen in the ATL2 Δ C liposome sample is on path would be demonstration of an increase in full fusion. Therefore, we have added 3 new pieces of data demonstrating full fusion by ATL2 Δ C: 1) Inner leaflet mixing (new Figure 5A); 2) an irreversible (EDTA insensitive) substantial shift in liposome size by dynamic light scattering (new Figure 5B); 3) cryo-EM tomography showing much larger vesicles (new Figure 6A). Additionally, the lack of any increase in 405 nm absorbance by the K107A (GTP binding defective) ATL2 Δ C liposomes (new Figure 4B) strongly suggests that our absorbance assay is a valid measure of GTP binding dependent trans dimer mediated tethering. (See also our response to reviewer 2 #3).

3. The rate of fusion for deltaCTD-AtI2 is 500 fold higher than full length. Yet changes in tethering and GTP hydrolysis are 100 fold less than this. Authors could comment on the discrepancy.

Response: This is a very good point that we should have commented on earlier. Indeed, our calculations show that each approximate doubling of tethering and GTP hydrolysis rates leads to a ~10-fold increase in fusion rate. We currently do not have an explanation for this phenomenon, but one possibility is that the C-term also regulates the transition from the tethered to the fused state. Whether such an effect would occur as a downstream consequence of a primary effect on tethering, or whether there is a separate effect on fusion per se remains to be determined. Nonetheless, we have added mention of this intriguing discrepancy in the text (line 269).

4. When overexpressed in U2OS cells, HA-AtI2 is localized to ER tubules and in some bright spots throughout the network. In contrast, the EER->KKE CTD charge reversal was primarily localized to collapsed ER. While the difference is striking, it's difficult to

infer functional changes due to lack of controls. Is the same phenomenon observed upon overexpression of AtI2-deltaC or AtI2-isoform 2?

Response: These controls have been done and added as part of the new Figure 8A, B. For technical reasons, we have switched to COS-7 cells, which show the same distinctive collapsed ER phenotype for EER->KKE seen in U2OS. As anticipated, ATL2ΔC also causes ER collapse when overexpressed, though the morphology of the collapsed structure appears different. Perhaps somewhat surprisingly, ATL2 iso 2 also causes ER collapse when overexpressed in COS-7 cells. On the one hand this is not surprising given how fusion active it is in vitro; but on the other hand, it is surprising given that this isoform is normally expressed in certain neuronal cell types. Presumably, neurons that express ATL2-2 must either express levels sufficiently low that it does not collapse the ER or negatively regulate its activity in some way.

5. The role of the amphipathic helix in fusion is not tested. The helix was tested in a chimeric protein with Drosophila atlastin in Faust et al, where it did not support the same amount of fusion as full-length drosophila atlastin. This raises the possibility that it functions differently in the vertebrate protein and given the proximity to the inhibitory CTD, it's important to test this. It could be useful to show that amino acid substitutions that disrupt the hydrophobic properties of the amphipathic helix attenuate fusion in the presence and absence of the CTD.

Response: As suggested, we have tested ATL1 with a point mutation I507D analogous to the nonpolar to charge point mutation on the hydrophobic face of the *DATL* amphipathic helix that blocks fusion (Liu et al, PNAS 2012). This mutation essentially blocked fusion by full length ATL1. The new data has been added to the new Figure 1E.

Minor Comments:

1. The shape of the fusion curve is different for AtI2 (1-547) and AtI2 (E555K, E556K, R559E). Authors could comment on this difference in the text.

Response: The initial fusion rate is 3-fold lower for the charge reversal than for the truncation, while the final extent of fusion is the same. The 3-fold lower rate could be due to incomplete relief of autoinhibition by the charge reversal. This point has been added to the text (line 317).

2. Figure legends indicate that fusion data are the averages of two independent traces. Are these technical replicates or biological replicates? If technical, are similar results obtained with biological replicates?

Response: Most fusion data are technical replicates from a single protein prep. In a few instances, for key constructs, we have repeated the assay from an entirely different protein prep also using independently prepared liposomes, and the deviation in fusion rates ranges only 5-10%. This is now indicated in the methods section (line 540). In our experience, independent preps have not (so far) ever resulted in a qualitative difference in fusion activity.

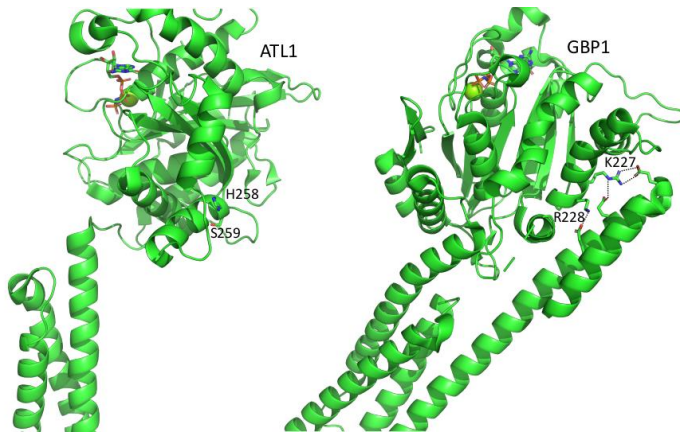
3. The black text in a red box is difficult to see in Figure 2. Consider changing text to white.

Response: This has been done; thank you for the suggestion.

4. The overall ER structure is difficult to see in Figure 6C. It would be informative to stain with an ER marker in addition to the Atlastin signal.

Response: This has been done and we now include co-staining with the ER marker calnexin (new Figure 8A).

5. Does H258R increase Atl1-mediated fusion by removing the CTD auto-inhibition?



Response: We wondered the same thing because H258/S259 in ATL1 aligns with R227/K228 in GBP1 (primary sequence) and the latter are the very residues in the GBP1 G domain that mediate critical salt bridges to the C-term helical extension for autoinhibition (PDB 1F5N). However, although both sets of residues are on the same helix of the G domain, the ATL1 α -4' helix is shorter than and

oriented differently from the equivalent helix in GBP1, with H258/S259 not as surface exposed as R227/K228. Also, the α -4' helix is unstructured in the extended conformation (crossover conformation is shown here) so it may undergo significant conformational change during the reaction cycle. Still, we did test the effect of an alanine substitution of the equivalent H285/N286 residues in ATL2 and sadly this did not activate ATL2. At this point, we think that the best way to address the possible role of ATL1 H258 and other residues in the autoinhibition mechanism is to obtain structural data on full length ATL1 and ATL2.

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

This is an elegant, well-controlled study of the mechanism(s) of auto-inhibition in Atlastin (ATL) membrane fusion activity by the extreme C-terminus, which is divergent among ATL paralogs. Despite my best efforts, I really could not find any glaring flaws or omissions in this well-crafted study. However, the mechanism(s) still remain at large with multiple models being proposed in the Discussion. I would appreciate if at least one of these models is tested in the manuscript as detailed below.

Response: We thank the reviewer for their appreciation of the advances we have made. We did make substantial efforts to gain further mechanistic insight, but not yet fruitful. A full understanding of the inhibition mechanism will likely require extensive further work. Our responses to this and other concerns are detailed below.

Major concern:

1. What exactly are the role(s) of the twin glutamates (Es) in the C-term? Among other scenarios, it is plausible that they could orient and/or regulate the insertion of the upstream amphipathic α -helix (AH) by providing charge-repulsion to negatively charged lipids. Charge-reversal mutants (E to K), on the other hand, could enhance direct membrane interactions/insertion of this C-term segment as part of a contiguous helix with the AH. This should be tested by direct assays of AH or C-term membrane insertion/interactions, and the influence of mutations on it. Have these been done yet? These assays will provide some mechanistic insight into the C-term's mechanism of action.

Response: We had also thought of the possibility that the negative charges on E555, E556 could cause charge repulsion with the 15% PS/85% PC membranes in our in vitro assay. But on the other hand, charge reversal of R559 also stimulated in vitro fusion (new Figure 7A, B), which is at odds with the idea. Moreover, though membrane insertion of the amphipathic helix is absolutely required for in vitro fusion, it is dispensable for tethering (Saini et al, MBoC 2014; Liu et al, PNAS 2015), and C-term deletion stimulated tethering even with a mutation designed to block crossover and fusion (previous Figure 5B, new Figure 4B). As mentioned above (response to reviewer #1 comment 3), one possibility is that the C-term regulates multiple steps in the reaction cycle, first tethering and then fusion, in which case charge repulsion between the C-term and anionic head groups may come into play for the latter (though we would still need to explain why the R559E mutation also stimulates fusion). In all, the experiments suggested by the reviewer are clearly important but putting the results into a context in which they can be clearly interpreted is arguably beyond the scope of this study. In the meantime, we feel that the body of work presented in our current study lays a solid foundation to pursue the detailed mechanism in future work.

Minor points:

2. Does the C-term also influence ATL GTPase activity or G-domain dimerization in a minimal construct lacking the 3HB (akin to the dynamin GG construct), and independent of membranes? (this is perhaps a relatively minor concern)

Response: Our initial attempt at making a minimal construct for testing without membranes was a deletion of just the ATL2 TM domain (Δ TM), leaving the 3HB intact because it is required for proper GTP loading (Byrnes et al, EMBOJ 2013). This Δ TM construct had a GTPase rate lower than ATL2 Δ C, but not as low as full length ATL2 in membranes, indicating that the autoinhibition was not fully reconstituted. However, we are reluctant to make a firm conclusion based on this single construct because it is possible that the TM truncation may hinder the C-term's ability to make contacts with the G/3HB. Therefore, our plan is to add in a linker between the 3HB and amphipathic

helix to increase flexibility. Given that this is a relatively minor concern, we have chosen not to pursue it for the current paper.

3. The light-scattering assay for liposome tethering must be supported by EM, as light scattering could increase even with protein instability and/or aggregation, especially with the C-term mutants.

Response: We have added 2 new pieces of data to address this concern. First, we show that the increase in light scattering in the most active C-term truncation (ATL2 1-547) is reversed by the addition of EDTA, which has been used frequently to dissociate ATL tethers because the tethers require Mg-GTP binding to be maintained (Orso et al, Nature 2009; Liu et al, PNAS 2015). Reversibility would not be expected for protein denaturation or nonspecific protein aggregation. This has been added as the new Figure 4C. Also, as indicated above, we have added new cryo-EM data, which shows not only larger liposomes with ATL2 (1-547) that indicate that fusion is occurring, but also many examples of what appear to be tethered liposomes. This has been added as the new Figure 6.

4. Folding issue: E. coli vs HEK293; The authors indicate that a folding issue (and not phosphorylation) might be the reason why E.coli-expressed ATLs have no fusion activity, while mammalian cell-expressed versions do. Where is the evidence for it? Indeed, no such differences have been seen with other dynamin family members (especially for dynamin), and E. coli expression has become quite the norm in the field for the last decade. Only phosphorylation as a difference is ruled out here. What other PTMs might be involved? If no evidence exists currently, it is better left out from the Results section, and not speculated upon.

Response: The reviewer is right that we have not ruled out other PTMs. The statement regarding a possible folding defect in bacterially produced ATL1 has accordingly been removed from the text.

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

Atlastins (ATL1, ATL2, and ATL3) are multidomain GTPases that catalyze homotypic fusion of ER membranes. Understanding the function and regulation of these enzymes is critical from a clinical perspective as atlastin mutations are associated with Hereditary Spastic Paraplegia. Despite extensive characterization using structural and cellular imaging techniques, the regulatory mechanisms controlling different human atlastin isoforms remains poorly defined. In this manuscript, Crosby and Lee successfully reconstitute the membrane fusion activity human ATL1 and ATL2 and measure the kinetics of initial fusion rates for different atlastin constructs and mutants. This alone represents a significant advancement for the field, as to date only the *Drosophila* atlastin has been functionally reconstituted in vitro. Surprisingly, the authors find that both ATL1 and ATL2 are autoinhibited and negatively regulated by the C-terminus. Using truncations, chimeras, and charge reversal mutants, the authors map important residues critical to the autoinhibition and show that the C-terminus impairs membrane

tethering and GTP hydrolysis in vitro without affecting GTP binding. The authors further demonstrate that autoinhibition plays a role in maintaining normal ER network homeostasis in vivo. In the absence of structural data, the authors draw from recent studies of GBP1 to present a speculative model for autoinhibition, which details how the C-terminus could modulate atlastin tethering and hydrolysis. While this work is suitable for JCB, a number of minor things should be addressed prior to publication:

Response: We thank the reviewer for their careful reading of our manuscript and many helpful suggestions for improving it. Our response to each point is detailed below.

1) Given the non-native folding states you observe for ATL1 and ATL2 in *E. coli* and the potential for autoinhibition, have you tried to analyze DATL expressed from another source like HEK cells or insect cells to see if there's a different activity? Does that change the fusion activity and/or kinetics of DATL in your assays?

*Response: As requested, we tested DATL expressed and purified from HEK cells following the same procedure as for ATL1 and ATL2. Indeed, even DATL shows a fusion rate substantially higher than the same protein produced from *E. coli*. We have added this data to the new Figure S1C.*

2) What happens to fusion and kinetics if you mix a full-length construct on one set of membranes and a truncated form on another set of membranes (e.g. ATL2 and ATL2 Δ C)? This might provide some additional insights as to the mechanism of inhibition by the extended C-terminus.

Response: Interestingly, mixing results in intermediate fusion rate, suggesting that activation of just one of the two partners is sufficient to stimulate fusion by ATL2. This has been added as the new Figure 2E.

3) In rationalizing the phenotypes for the Δ L549,G550 construct, the authors state: "However, deletion of two residues from the helix was expected to place the identified charged cluster on the opposite, rather than the same, side as the nonpolar face of the amphipathic helix (Fig 6A, DL549, G550, lower helix). Strikingly, simply deleting the two residues increased the fusion rate to nearly the same extent as the E555K, E556K charge reversal (Fig 6B), indicating that the autoinhibition mechanism likely depends on the charged residues pointing in the same direction as the nonpolar face of the amphipathic helix." This assumes that deletion of these two residues simply induces a register shift in the helix. If removing these residues actually disrupts the structural integrity of the helix, then it's equally possible that the downstream side chains might become disordered, which could have the same net effect of displacing the charged residues and relieving inhibition (much like deletion of the whole segment). Similarly, the authors interpret the P548A,G550A phenotype as "indicating a lack of requirement for a substantial helix disruption or a turn". This statement again makes assumptions about effects of potential changes without any structural validation. I would suggest softening the language and/or presenting alternative plausible interpretations in the absence of supporting structural data.

Response: These are all excellent points. Of relevance, we became aware, since our original submission, of the AlphaFold structure prediction tool and server (Jumper et al, Nature, 2021) <https://alphafold.ebi.ac.uk/>, whose structure prediction of the ATL2 tail shows, with a 70-90% confidence score, that the amphipathic helix residues (AA528-547) and the inhibitory residues (AA548-567) indeed are likely part of a continuous alpha helix (predicted structure now shown in the new Figure 7A). In this prediction, the charged residues E555, R559 are shown *not* to emanate from the nonpolar face as we had previously assumed (though E556 may still be). Considering all this, we have now 1) removed speculation that all 3 charged residues are on the nonpolar face; 2) removed the Δ L549,G550 test for orientation in light of the very valid caveats raised by the reviewer; 3) redrawn the autoinhibited molecule in our model (Figure 10C) to show the nonpolar face of the amphipathic helix now inserted in the membrane as more commonly accepted; and 4) deleted discussion of the possibility that the amphipathic helix might not be membrane inserted prior to fusion. We have, however, retained the P548A, G550A variant fusion data because the AlphaFold prediction does show an intriguing slight kink in the otherwise continuous helix and this mutant variant does attempt to address the importance of the residues that may contribute to the kink. All that said, we wholly acknowledge that any firm conclusions about how the inhibition is working must await structure determination (even the AlphaFold prediction could be wrong) and have accordingly attempted to soften our language throughout.

4) Does overexpression of ATL2 Δ C in U2OS cells also lead to catastrophic defects in ER morphology? One would expect it would recapitulate the same phenotypes observed with the charge reversal mutants since the assumption is that both changes remove the autoinhibitory effect.

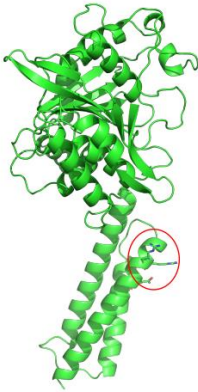
Response: Yes, as predicted, overexpression of ATL2 Δ C does lead to ER collapse. This data has been added to the new Figure 8A, B.

5) Conformational coupling and indirect, allosteric modulation of G domain functions by other domains is a common theme that has also emerged from studies of dynamin superfamily proteins and Arf GTPases. For example, membrane binding interactions mediated by dynamin's PH domain via the bundle signaling element or the N-terminal helix of Arf family members directly influence the catalytic activity and structural changes in the GTP binding pocket in a back-to-front manner. The discussion section nicely lays out a potential analogous mechanism based on recent work characterizing GBP1 intramolecular interactions. Including a comparison to other similar examples would broaden the impact of the story and help further delineate distinctions among large, mechanochemical GTPases.

Response: We thank the reviewer for this suggestion. A paragraph discussing the potential broader impact of our findings has been added to the end of the discussion (line 455).

6) Based on comparisons with GBP1, it's tantalizing to suggest that there are residues

in the upper portion of the 3HB, the linker, and/or the back side of the G domain that directly interact with the C terminus. Have you looked further at the available atlastin structures to see if this might be true (i.e. are there clusters of oppositely charged residues that could associate with the region containing residues 554-560)? If so, mutating those side chains and analyzing their effects in your assays may further your mechanistic description and could strengthen the model presented in Figure 8C.



Response: Indeed, we have made several guesses, but unfortunately without success. An example is shown here, where we identified a charge cluster on the ATL2 3HB and made a triple charge reversal R442E, R443E, D446K, which failed to activate the full-length protein. As mentioned above, we feel at this point that the best way forward is to obtain a structure of the full-length protein and let it reveal what the C-term is interacting with.

7) I appreciate the authors' attempt to present a model that best describes the current data and their honest assessment of what information is still missing and/or has yet to be tested. Given our current understanding, I would suggest adding labels to the upper panels of Figure 8C to denote that the depicted AH-3HB and C-term-G domain interactions/conformations are predicted and modeled and have yet to be validated. This would ensure the readers know this is still speculation rather than dogma.

Response: This is a good point. As suggested, we have labeled the depictions of the interactions in the new Figure 10C as speculative.

Minor comments:

1) Page 7, line 140: "...a truncation removing the entire ATL1 tail harboring the required amphipathic helix"

It would be helpful to the reader to clarify the boundaries of this truncation with residue numbers and/or label it specifically as "tail" in domain diagram such as shown in Figure 2A to distinguish it from just the C-terminal segment.

Similarly, it would be helpful to number the domain boundaries in Figure 2C to explicitly orient the reader as to what segments are present in each of the constructs analyzed in Figure 2D.

Response: These are now all properly labeled.

2) Page 9, line 188: remove the hyphen in "G-domain"

Response: Thank you; this has been done.

3) Page 13, line 264-265: "we focused on the residues closest to the amphipathic helix, within AA554-565."

I would suggest changing the numbering to "AA548-565" as the mutations discussed

here and show in Figure 6 span this entire region and it would be more correct as far as describing this segment as "closest to the amphipathic helix".

Response: This has been changed.

4) Page 13, line 265-266: "...secondary structure prediction algorithms predict..."
The figure legend mentions JPRED. Were other algorithms used? How well do the results compare across different algorithms and what are the confidence levels in the different outputs? Please clarify as this all helical model is presented without any supporting structural data.

Response: As mentioned above, we now cite the AlphaFold tool and server (Jumper et al, Nature, 2021) <https://alphafold.ebi.ac.uk/>, whose structure prediction of the ATL2 tail is now used in our paper and rendered in PyMOL in the new Figure 7A. We also make a note of the confidence score for the helical prediction, which is 70-90% for the region analyzed in our paper (line 304).

5) Figure 6A: It would be beneficial to label the position of 565 in the domain diagram as this is referenced in the text and it would help orient the reader with respect to the helical cartoon below. The authors might also consider realigning the "547" label which is currently hovering over part of the C-terminal extension but in fact denotes the end of the AH. Additionally, it would be useful to label residues 457, 554, and 560 along with 527 in the alignment to again orient the reader with respect to domain boundaries and specific mutations tested.

Response: We apologize for not having had residues clearly labeled; labels have been added as suggested. Please note that this is now the new Figure 7A.

6) Figure 6 legend: A reference for JPRED should be added.

Response: This has been done, though we have largely replaced the prediction from JPred used for Figure 6A with that from AlphaFold. References have been added accordingly. Please note that this is now the new Figure 7.

7) Figure 8A: The images of the GBP1 and G domain structures appear to be low resolution. Ray tracing these prior to publication would avoid unintended pixilation, assuming they were generated with Pymol.

Response: Thank you very much for the suggestion; this has been done. Please note that this is now the new Figure 10A.

November 10, 2021

RE: JCB Manuscript #202107070R

Dr. Tina H Lee
Carnegie Mellon University
Biological Sciences
4400 5th Avenue
Pittsburgh, PA 15213

Dear Dr. Lee:

Thank you for submitting your revised manuscript entitled "Reconstitution of human atlastin fusion activity reveals autoinhibition by the C-terminus". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below). Please also carefully address the text issues of reviewer #3 in your final revision.

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.****

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- 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.
- 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."
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- 6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.
- 7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.
- 8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
 - a. Make and model of microscope
 - b. Type, magnification, and numerical aperture of the objective lenses
 - c. Temperature
 - d. Imaging medium
 - e. Fluorochromes
 - f. Camera make and model
 - g. Acquisition software

h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

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

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

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

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

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

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

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Please 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).

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

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

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

Sincerely,

Jodi Nunnari, Ph.D.
Editor-in-Chief

Andrea L. Marat, Ph.D.

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

Authors have sufficiently addressed all comments. It's a very nice paper.

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

The authors have responded appropriately to my queries. I have no further concerns.

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

The authors have sufficiently addressed my concerns and expanded the manuscript with additional supporting data and experiments in response to critiques raised by other reviewers. I fully support publication of this revised work in JCB.

A few minor changes should be implemented prior to final publication (these do not affect the conclusions or change the findings in any significant way and can be addressed at the stage of proofs if necessary):

1) Hyphenate the following:

Page 5, Line 96: "...pre-steady-state rates of GTP-dependent crossover"

Page 11, Line 239: "...bind one another in an ATL- and GTP-dependent manner..."

Page 18, Lines 394: "...If tight regulation of ATL-mediated ER fusion is cell type-dependent..."

Page 21, Line 455: "...GTPase is rendered assembly-dependent by conformational changes..."

2) Text appears to be missing a closing bracket in the parenthetical phrase on Page 10, lines 215-216, which can replace the semi colon: "... (compare ATL2(1-568) to ATL2(1-573); and ATL2(1-553) to ATL2(1-565)) whereas removal of..."

3) Please switch the places of panels C and D in Figure 4

4) I would suggest relabeling the bottom images in Figure 8A as "HA-ATL2-2" rather than "Iso 2" to be consistent with the description in the text and nomenclature adopted throughout the manuscript.