JCB

Human atlastins are sufficient to drive fusion of liposomes with a physiological lipid composition

Eunhong Jang, Yeojin Moon, So Young Yoon, Joyce Anne Diaz, Miriam Lee, Naho Ko, Jongseo Park, Soo Hyun Eom, Changwook Lee, and Youngsoo Jun

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Re: JCB manuscript #202109090

Dr. Youngsoo Jun
Gwangju Institute of Science and Technology
Life Sciences
123 Cheomdangwagi-ro, Buk-gu
Gwangju 61005
Korea, Republic of (South Korea)

Dear Dr. Jun,

Thank you for submitting your manuscript entitled "Human atlastins are sufficient to drive fusion of liposomes with a physiological lipid composition". Your manuscript has been assessed by expert reviewers, whose comments are appended below. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

As you will see, the reviewers appreciate the importance of demonstrating that human atlastins can catalyze membrane fusion in vitro. While we agree with their assessment that this is of interest for the field, we also agree that for the broad readership of JCB further novel mechanistic insight into atlastin-mediated fusion needs to be derived from these fusion assays. The reviewers have provided constructive suggestions on avenues to investigate, therefore for resubmission to JCB you must significantly extend the study in one or more of these directions. In addition, we expect you to address all of the remaining reviewer comments in a revised manuscript.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. Once you have had time to consider their comments, we highly encourage you to send us a revision plan that we may discuss with the reviewers to ensure that your proposed experiments will provide a sufficient mechanistic extension.

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 measures that limit spread of COVID-19 also pose challenges to scientific researchers. Therefore, JCB has waived the revision time limit. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
Text limits: Character count 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: Your manuscript may have up to 10 main text figures. To avoid delays in production, 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.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Your manuscript 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.

Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots

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Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, 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. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

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 (ATL1, ATL2, and ATL3) are multidomain GTPases that play a critical role in the homotypic fusion of ER membranes. The exact function and contribution of each human isoform remains poorly defined and hotly debated as only the Drosophila atlastin has been shown to induce liposome fusion in vitro. In this manuscript, Moon et al. present evidence that all three human atlastins can catalyze fusion when reconstituted into liposomes with a lipid composition mimicking that of the ER and yeast derived microsomes, with ATL1 exhibiting a much lower fusogenic activity under all the conditions tested. The authors show that GFP-tagged ATL2 and ATL3 localize to localize to three-way junctions when expressed in COS7 cells whereas neuronal ATL1 is dispersed along ER tubules. Addition of the neuronal-specific factor M1-spastin increased ATL1-catalyzed liposome fusion in vitro and redistributed GFP-ATL1 localization to three-way junctions when co-expressed in COS7 cells. From this, the authors conclude that neuronal actors are required for the optimal fusogenic activity of ATL1. Using siRNA and mixing experiments, the authors show ATL2 and ATL3 have redundant, overlapping functions in vivo but have the capacity to function together in vitro in a synergistic manner that yields a higher overall fusion activity. Finally, the authors describe a new assay to examine fusion using ER microsomes derived from HEK293T cells and use it to characterize ATL2 fusion functions. Although the finding that human atlastins can catalyze membrane fusion in vitro represents an important step forward for the field, the manuscript provides little in terms of leveraging the fusion assays developed to gain a better mechanistic understanding of atlastin function, the observed differences between ATL1, ATL2, and ATL3, and the potential effects of disease-associated mutations. The work also lacks a number of important controls and fails to consider alternate explanations, which undercut the impact and conclusions.

1) The authors observe that neuronal ATL1 has a lower fusogenic activity and hypothesize that ATL requires additional/neuronal-specific factors to function as effective fusogen. In addition to differential expression, the three ATL isoforms show significant variation at their N- and C-termini. Have the authors considered that this as an alternative cause for the observed phenotypic differences in their fusion assays? Examining how isoform-specific differences in these regions affect fusion activity would greatly increase the impact of the paper.

2) Addition of spastin increases ATL1 fusion activity of in vitro (Fig. 4A). As presented, this data raises a number of questions: Is this effect simply due to the physical interaction between ATL1 and spastin or does it require spastin's ATPase activity? Is it known which part of ATL1 spastin is interaction with and, if so, does altering this by mutagenesis affect the fusion readout in your assay? Does adding spastin alter ATL1's GTPase activity? Is the stimulation of ATL1 fusion activity specific to spastin or can other neuronal-specific factors produce a similar effect in your assays? Does adding similar concentrations of something non-specific like BSA change the fusion activity?

3) The enlarged view of the Figure 4B appears to only show the EGFP signal. It would be beneficial to the reader to show the

enlarged view of the merge well. This would assist in interpreting the localization of spastin and thus its role in modulating the distribution of ATL1. It's difficult to tell how well the two colocalize in how it's currently presented. Does the apparent redistribution of ATL1 require spastin's ATPase activity?

4) The authors show that ATL2 and ATL3 can function together to catalyze fusion and can stimulate the fusogenic activity of the individual proteins (Fig 5B andC). Does substituting ATL1 in these experiments elicit the same effects (i.e. can fusion occur between ATL1 and ATL2 and is the fusion similarly enhanced)?

5) Inclusion of previously characterized hydrolysis-defective and/or nucleotide binding mutants (e.g. R77A or R77E and K80A in ATL1) is an important control needed to confirm that the observed activity in each fusion assay is specific for the reconstituted ATL homolog.

6) Given the numerous structural, biochemical, and genetic analyses of atlastins that exist in the literature, it seems like missed opportunity to not assess how specific disease and mechanistic mutants behave in the different fusion assays presented here. This type of analysis would increase the impact of the work.

7) The data in Supplementary Figure S5 is described as an afterthought in the discussion. It would better serve the reader in the results section, given that this lipid mixing data provides strong support that ATL2 is sufficient to induce full fusion on its own and not simply hemifusion.

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

This is an excellent albeit phenomenological study that unequivocally demonstrates that human atlastins (ATLs) recombinantly expressed in the much maligned, but widely used, E. coli system is fully capable of mediating membrane fusion - when given the right, biomimetic lipid composition. Whereas the phenomenon itself is reconstituted exceptionally well in this study with the appropriate controls, there is very little explanation or mechanistic insight into the differential activities of the three ATL isoforms or their lipid dependencies, which warrants further exploration. Nevertheless, this is an important and informative study that will have a sustained impact on the field.
Major Issues:
1. Very limited mechanistic insight or explanation into the differential activities of the three ATLs: 1) How different are their self-assembly and mechanoenzymatic properties in this reconstituted system, and how are they influenced/regulated by lipid composition? 2) Why is spastin required ONLY for ATL1 activity in membrane fusion? Does it differentially affect ATL1/2/3 GTPase activity? What is its effect on ATL2/3 membrane fusion activity? How does spastin influence ATL self-assembly/cross-bridge formation? What is the basis for ATL2/3 synergy? - Experiments on the protein side of things are lacking.
2. How does ATL membrane fusion activity titrate with the content of conical lipids (with nonbilayer propensities) in membranes? ATL1 fusion activity may very well require a higher content of PE/cholesterol than what is used? - a titration experiment changing both PE and cholesterol content and looking at the membrane fusion activity of all ATL isoforms (with and without spastin) is required for a better explanation of the observed phenomena.
Minor Issue:
1. Fig. 5A. I'm not entirely convinced, just by looking at the images that a combined ablation of ATL2/3 leads to long vs. networked tubules. Can this be quantified (e.g. by measuring length) in some manner?

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

Atlastins are large GTPases that mediate fusion of ER membranes. Drosophila Atlastin has been shown to be sufficient for membrane fusion in reconstituted liposome mixing assays; however, none of the three human atlastins isoforms were sufficient in the same assay. The work presented here demonstrates that recombinantly purified human atlastin paralogs possess fusion activity in reconstituted liposome mixing assays if the lipid composition mimics that of the ER. In particular, PE and cholesterol are required. Notably, liposome fusion by ATL1 is significantly lower than either ATL2 or ATL3 in the context of reconstituted liposomes and yeast microsomes. Data presented here demonstrate that membrane fusion by the neural-specific ATL1 is augmented by recombinantly purified spastin in the reconstituted assay, indicating that atlastins can be regulated by additional proteins. This was confirmed in cells where co-expression of spastin converted ATL1 from diffuse on the ER membrane to a pattern more similar to ATL2 and ATL3 with enrichment at three-way junctions. The redundancy of ATL2 and ATL3 is tested in the proteoliposome fusion assay, which revealed that fusion is higher if both isoforms are present in the same proteoliposomes. Finally, a microsome fusion assay is developed and data support the conclusion that ATL2 mediates membrane fusion in cells. Overall, the manuscript represents an advance in understanding the function of human atlastin isoforms. A few points should be addressed prior to publication.

Specific Comments:
1. For liposome fusion experiments with ATL1 and spastin, dependence on cholesterol and PE should also be tested.

2. For siRNA experiments in Cos7 cells, images should be quantified by a blinded experimenter in at least 3 biological replicates and data expressed as proportion of cells counted.
3. Proteoliposome fusion assays with both ATL2 and ALT3 indicate that when both isoforms are on the same membrane, fusion is more efficient than either isoform alone. In contrast, when ATL2 and ATL3 are present on opposing membranes, or in trans, fusion is comparable to homotypic controls. Authors should speculate on how ATL2 & ATL3 might cooperate in cis.
4. Additional details must be added for the HEK microsome fusion assay in the Materials and Methods or within the text. What does 100% represent in Figure 6B? What is the efficiency of microsome fusion (how many microsomes in the population fuse)? Does fusion increase if ATL3 expression is increased in the HEK parent cells?

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October 21th, 2022

Dr. Jodi Nunnari
Editor-in-Chief
Journal of Cell Biology
Rockefeller University Press
950 Third Ave., 2nd Floor
New York, NY 10022 USA

Dear Dr. Nunnari,

Please find enclosed our revised manuscript (20219090R) and point-by-point responses to the three thorough reviews. Each comment of each reviewer has been carefully addressed by making the changes to the text or providing the additional data that were requested. We have worked very hard to address each of the reviewers' comments by incorporating substantial additional experiments and data, and thank the reviewers for strengthening this paper. While our manuscript was under review, Lee and colleagues reported similar results to ours (human ATL1 and ATL2-2 are sufficient to induce liposome fusion) in *Journal of Cell Biology (Crosby et al., (2022) 221:e2021107070).* In addition, they found that ATL2-1, the major splice isoform of ATL2 predominantly expressed in non-neuronal cells, is auto-inhibited by its C-terminal helix (CH) and that deletion of the CH restores the fusion activity of ATL2-1. However, they did not explain how the CH inhibits the fusion activity of ATL2-1 or how the inhibition is relieved to support fusion *in vivo*. In this revised manuscript, we have addressed these important issues and included several pieces of data providing clues of how the CH inhibits the fusion activity of ATL2-1 and how this inhibition is relieved to support ER fusion *in vivo*. We are very grateful for the careful editing and expert review, and hope that our paper is now acceptable for publication in *Journal of Cell Biology*.

Yours sincerely,

Youngsoo Jun

Youngsoo Jun, Ph.D.
Professor
Gwangju Institute of Science and Technology, Gwangju, Korea

Point-by-point responses

Reviewer Comments:

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

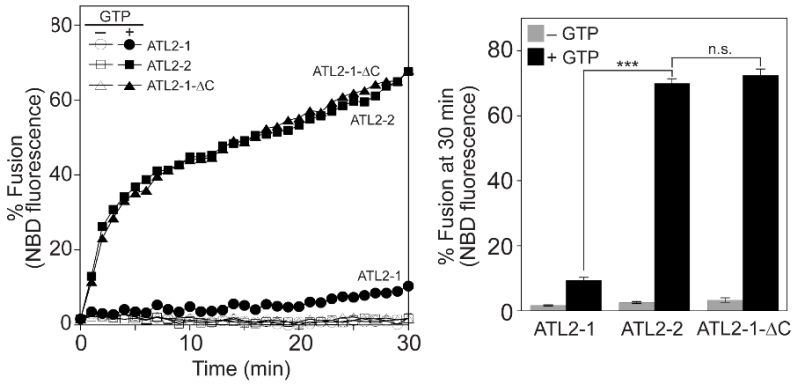
Atlastins (ATL1, ATL2, and ATL3) are multidomain GTPases that play a critical role in the homotypic fusion of ER membranes. The exact function and contribution of each human isoform remains poorly defined and hotly debated as only the Drosophila atlastin has been shown to induce liposome fusion in vitro. In this manuscript, Moon et al. present evidence that all three human atlastins can catalyze fusion when reconstituted into liposomes with a lipid composition mimicking that of the ER and yeast derived microsomes, with ATL1 exhibiting a much lower fusogenic activity under all the conditions tested. The authors show that GFP-tagged ATL2 and ATL3 localize to localize to three-way junctions when expressed in COS7 cells whereas neuronal ATL1 is dispersed along ER tubules. Addition of the neuronal-specific factor M1-spastin increased ATL1-catalyzed liposome fusion in vitro and redistributed GFP-ATL1 localization to three-way junctions when co-expressed in COS7 cells. From this, the authors conclude that neuronal actors are required for the optimal fusogenic activity of ATL1. Using siRNA and mixing experiments, the authors show ATL2 and ATL3 have redundant, overlapping functions in vivo but have the capacity to function together in vitro in a synergistic manner that yields a higher overall fusion activity. Finally, the authors describe a new assay to examine fusion using ER microsomes derived from HEK293T cells and use it to characterize ATL2 fusion functions. Although the finding that human atlastins can catalyze membrane fusion in vitro represents an important step forward for the field, the manuscript provides little in terms of leveraging the fusion assays developed to gain a better mechanistic understanding of atlastin function, the observed differences between ATL1,

ATL2, and ATL3, and the potential effects of disease-associated mutations. The work also lacks a number of important controls and fails to consider alternate explanations, which undercut the impact and conclusions.

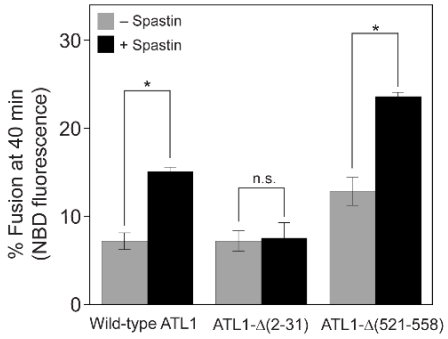
➔ *We greatly appreciate the reviewer's kind and constructive comments, which greatly helped improve our manuscript.*

1) The authors observe that neuronal ATL1 has a lower fusogenic activity and hypothesize that ATL requires additional/neuronal-specific factors to function as effective fusogen. In addition to differential expression, the three ATL isoforms show significant variation at their N- and C-termini. Have the authors considered that this as an alternative cause for the observed phenotypic differences in their fusion assays? Examining how isoform-specific differences in these regions affect fusion activity would greatly increase the impact of the paper.

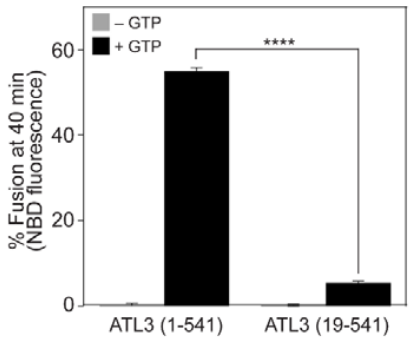
➔ *We greatly appreciate the reviewer pointing out these critical issues. A recent paper published in Journal of Cell Biology reported that although the only difference between the two isoforms of ATL2 (ATL2-1 and ATL-2) is confined to their C-terminal amino acids, ATL2-1 only supports negligible liposome fusion while ATL2-2 has strong fusion activity (Crosby et al., 2022). It further showed that this is due to the inhibitory effect of the C-terminal 38 amino acids (hereafter termed the C-terminal helix or CH) of ATL2-1 because its deletion made the mutant fuse comparably to ATL2-2. We were able to reproduce these results (Fig. 6A or below).*



➔ *As shown in Figure 5A (or see gray bars below), while deletion of the N-terminal part of ATL1 did not affect its fusion activity, deletion of the C-terminal region markedly increased ATL1-mediated liposome fusion, consistent with the findings of Crosby et al. (Crosby et al., 2022).*



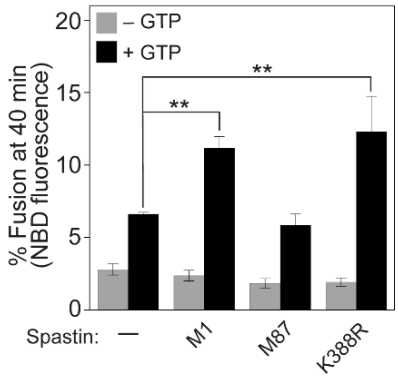
➔ *Finally, there are two major isoforms of ATL3. ATL3-1 contains an additional 18 amino acids at its N-terminus compared with ATL3-2. Surprisingly, deletion of the additional 18 amino acids from ATL3-1, which converts ATL3-1 to ATL3-2, markedly impaired the fusion activity of ATL3-1 (Fig. 10E or below).*



2) Addition of spastin increases ATL1 fusion activity of in vitro (Fig. 4A). As presented, this data raises a number of questions:

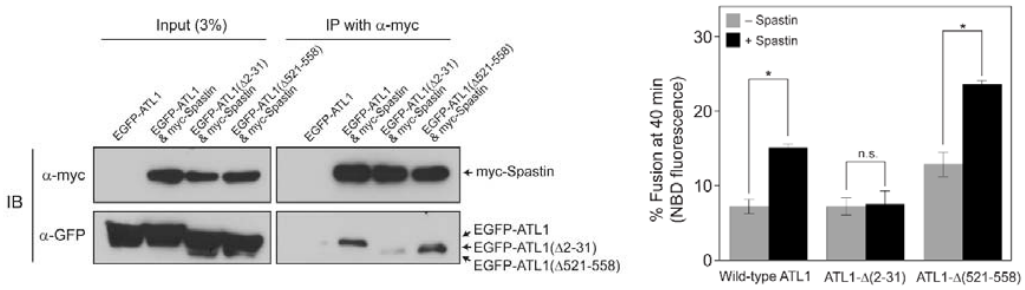
① Is this effect simply due to the physical interaction between ATL1 and spastin or does it require spastin's ATPase activity?

➔ *As shown in Figure 4C (or below), the spastin mutant (K388R) lacking ATPase activity enhanced ATL1-mediated fusion comparably to wild-type spastin. However, the M87 isoform of spastin, which retains ATPase activity but cannot interact with ATL1, failed to stimulate ATL1-mediated fusion, suggesting that the stimulatory effect of spastin on ATL1-mediated fusion requires the physical interaction between ATL1 and spastin, but not the ATPase activity of spastin.*



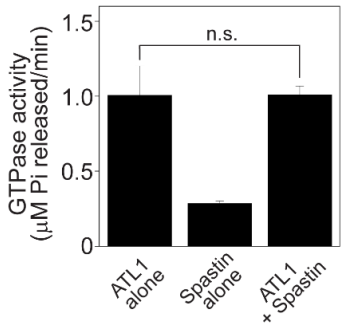
② Is it known which part of ATL1 spastin is interaction with and, if so, does altering this by mutagenesis affect the fusion readout in your assay?

➔ *The C-terminal tail of ATL1 was proposed to interact with spastin (Evans et al., 2006; Rismanchi et al., 2008). By contrast, Sanderson et al. suggested that the N-terminal region of ATL1 associates with spastin (Sanderson et al., 2006). Our co-immunoprecipitation experiment suggests that spastin preferentially interacts with the N-terminal region of ATL1 because deletion of the N-terminal region (2–31 amino acids) largely abolished the interaction (Fig. 5B or the blot shown below). Consistently, spastin stimulated fusion mediated by C-terminally deleted ATL1 [ATL1-Δ(521–558)], but not that mediated by mutant ATL1 lacking the N-terminal region (2–31 amino acids) (Fig. 5A or the bar graph shown below).*



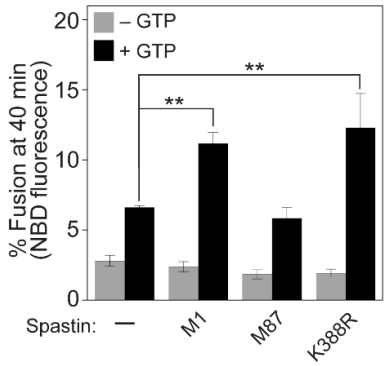
③ Does adding spastin alter ATL1's GTPase activity?

➔ *As shown in Figure 4D (or below), addition of spastin did not affect the GTPase activity of ATL1.*



④ Is the stimulation of ATL1 fusion activity specific to spastin or can other neuronal-specific factors produce a similar effect in your assays? Does adding similar concentrations of something non-specific like BSA change the fusion activity?

➔ *The lack of stimulation by the M87 isoform of spastin (shown in Fig. 4C or below) indicates that stimulation of ATL1-mediated fusion by the M1 isoform of spastin is specific. In addition, the observation that C-terminally deleted ATL1, but not N-terminally deleted ATL1, was stimulated by spastin further supports the specificity of spastin-mediated stimulation (Fig. 5A).*



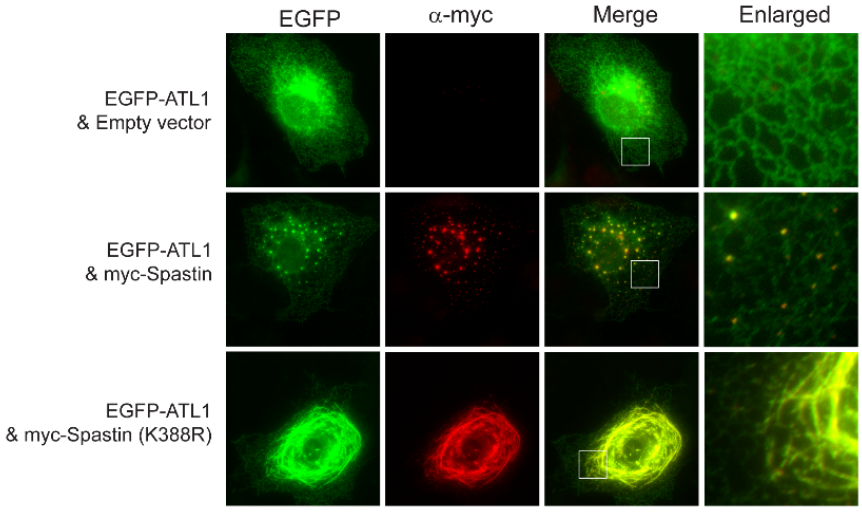
3) The enlarged view of the Figure 4B appears to only show the EGFP signal. It would be beneficial to the reader to show the enlarged view of the merge well. This would assist in

interpreting the localization of spastin and thus its role in modulating the distribution of ATL1. It's difficult to tell how well the two colocalize in how it's currently presented.

➔ *This has been done. The previous Figure 4B is Figure 4A in the revised manuscript.*

Does the apparent redistribution of ATL1 require spastin's ATPase activity?

➔ *To address this question, we co-expressed the ATPase-defective mutant M1-spastin (K388R) with EGFP-ATL1 and compared these cells with those expressing wild-type M1-spastin and EGFP-ATL1 (see below). Although the co-localization of EGFP-ATL1 and M1-spastin was unaffected by expression of M1-spastin (K388R), three-way junctions were largely lost. M1-spastin (K388R) lacks ATPase activity and thus is unable to sever microtubules; therefore, its expression results in excessive filamentation of microtubules and an unbranched ER morphology (Lumb et al., 2012). To avoid confusion, we decided not to include these data in the revised manuscript.*

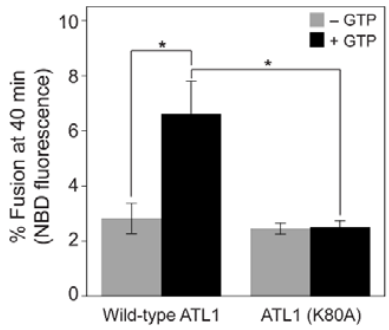


4) The authors show that ATL2 and ATL3 can function together to catalyze fusion and can stimulate the fusogenic activity of the individual proteins (Fig 5B and C). Does substituting ATL1 in these experiments elicit the same effects (i.e. can fusion occur between ATL1 and ATL2 and is the fusion similarly enhanced)?

➔ *The experiments shown in previous Figure 5 were performed based on the premise that non-neuronal cells predominantly express ATL2 and ATL3. In particular, the HEK293T cell line used in this study predominantly expresses ATL2 and ATL3, although ATL2 (ATL2-1 isoform) expression is 50-fold higher than ATL3 expression. Thus, we respectfully think that the experiment that the reviewer requested provides little biologically meaningful insight. More importantly, we have removed the previous Figure 5 because the revised manuscript places more emphasis on the molecular mechanisms by which ATL2-1, a fusion-incompetent isoform of ATL2, can possibly support ER fusion in HEK293T cells, which predominantly express ATL2-1 and ATL3.*

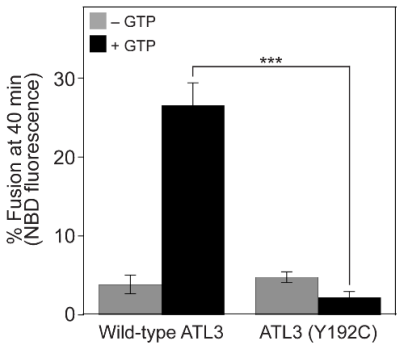
5) Inclusion of previously characterized hydrolysis-defective and/or nucleotide binding mutants (e.g. R77A or R77E and K80A in ATL1) is an important control needed to confirm that the observed activity in each fusion assay is specific for the reconstituted ATL homolog.

➔ *As shown in Figure 3A or below, the ATL1 mutant defective for nucleotide binding (K80A) did not support liposome fusion. Thus, the fusion signal that we saw with wild-type ATL1 is indeed mediated by ATL1.*



6) Given the numerous structural, biochemical, and genetic analyses of atlastins that exist in the literature, it seems like missed opportunity to not assess how specific disease and mechanistic mutants behave in the different fusion assays presented here. This type of analysis would increase the impact of the work.

➔ *As the reviewer suggested, we examined a well-known ATL3 mutant (Y192C) that causes sensory neuropathy (Blackstone, 2012; Krols et al., 2018). As shown in Figure 10F or below, the ATL3 mutant (Y192C), which exhibited extremely low GTPase activity (Supplementary Fig. S9), did not support liposome fusion, consistent with the previous finding that this mutation decreases the density of three-way junctions (Behrendt et al., 2019), which are formed by homotypic fusion between ER tubules.*



7) The data in Supplementary Figure S5 is described as an afterthought in the discussion. It would better serve the reader in the results section, given that this lipid mixing data provides strong support that ATL2 is sufficient to induce full fusion on its own and not simply hemifusion.

➔ *This has been done. The previous Supplementary Figure S5 is Figure 9 in the revised manuscript.*

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➔ *We greatly appreciate the reviewer's kind and constructive comments, which greatly helped improve our manuscript.*

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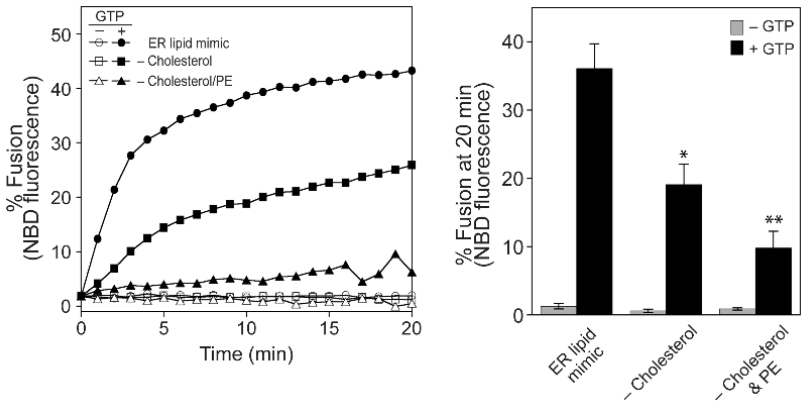
1. Very limited mechanistic insight or explanation into the differential activities of the three ATLs:

1) How different are their self-assembly and mechanoenzymatic properties in this reconstituted system, and how are they influenced/regulated by lipid composition?

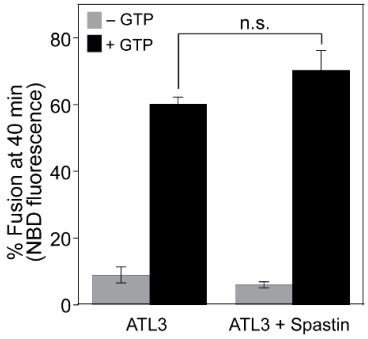
➔ *We sincerely appreciate the reviewer's critical comments and constructive suggestions. Since Drosophila atlastin (dATL) was discovered as a fusogen for ER membrane fusion in 2008, it took almost 15 years to successfully reconstitute human atlastin-mediated liposome fusion. Thus, we respectively think that even reconstitution of human atlastin-mediated fusion in vitro per se is a huge step toward a better understanding of how these proteins work to regulate the ER structure. We finally have a facile tool to investigate the important questions that the reviewer raised (such as those concerning the mechano-enzymatic properties of human atlastins) and hope to address these critical questions more extensively in future studies. The lipid composition plays a crucial role in various membrane fusion events, such as SNARE-mediated fusion and viral fusion. In the current study, we showed that omission of both cholesterol and PE (neutral lipids with small head groups that tend to form non-bilayer structures) markedly reduced ATL2-mediated fusion (Supplementary Fig. S1 or below), consistent with the findings of studies of other membrane fusion events. Membrane fusion mediated by Sey1p, the yeast atlastin, requires regulatory lipids (sterol, PE, DAG, and PI) for optimal fusion (Lee et al., 2019; Sugiura and Mima, 2016).*

Because we have successfully reconstituted human atlastin-mediated liposome fusion in vitro, it is feasible to examine the effect of various lipid compositions on human atlastin-mediated membrane fusion in future studies. The effect of a few different concentrations of cholesterol and PE on ATL-mediated fusion was assessed in this study (Supplementary Fig. S2).



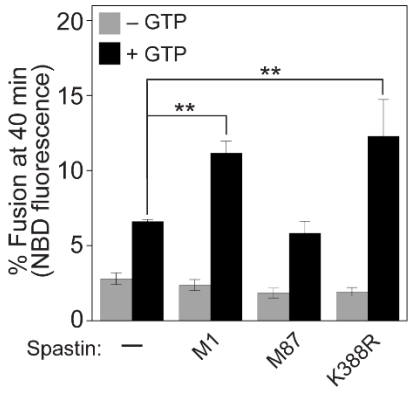
2) Why is spastin required ONLY for ATL1 activity in membrane fusion?

➔ *We cannot exclude the possibility that spastin stimulates atlastins other than ATL1. However, because ATL1 is the major atlastin expressed in neuronal cells, and spastin is also preferentially expressed in neuronal cells, ATL1 is probably the major target of spastin. More importantly, it was reported that spastin specifically interacts with ATL1, but not with ATL2 or ATL3 (Rismanchi et al., 2008). Furthermore, our data indicate that stimulation of ATL1-mediated fusion by spastin requires the physical interaction between ATL1 and spastin (Fig. 5). Consistently, as shown below, spastin did not significantly stimulate ATL3-mediated fusion.*



What is its effect on ATL2/3 membrane fusion activity?

➔ *As shown above, spastin did not significantly stimulate ATL3-mediated fusion. As shown in Figure 4C and below, spastin-mediated stimulation requires the physical interaction of spastin with ATL, and it was reported that spastin specifically interacts with ATL1, but not with ATL2 or ATL3 (Rismanchi et al., 2008). Thus, spastin-mediated fusion stimulation is specific to ATL1.*



How does spastin influence ATL self-assembly/cross-bridge formation?

➔ *We greatly appreciate the reviewer's critical comments. Unfortunately, however, it is not possible for us to analyze the effect of spastin on self-assembly/cross-bridge formation of atlastins at the moment. We hope to address these issues in future studies. Based on the data shown in Figure 4A, a simple idea is that spastin clusters ATL1, which facilitates the formation of multimeric complexes of ATL1, at three-way junctions where fusion actively occurs. Accumulation or clustering of SNAREs at fusion sites markedly enhances SNARE-mediated membrane fusion (Li et al., 2020; Wang et al., 2002).*

What is the basis for ATL2/3 synergy?

➔ *In the revised manuscript, we removed the previous Figure 5, which described ATL2/3 synergy. While this paper was under revision, Lee and colleagues reported that ATL2-1, the*

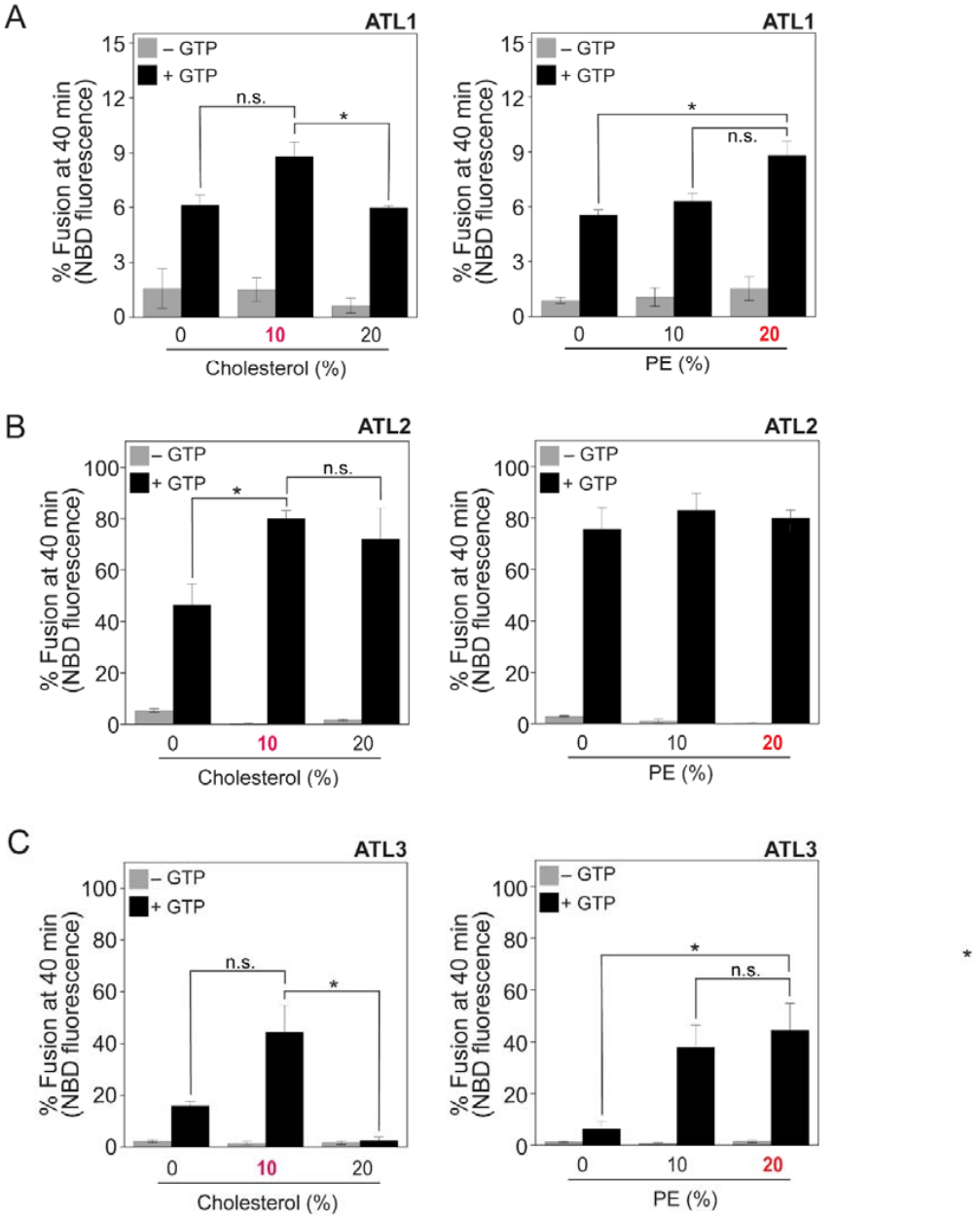
major isoform of ATL2 predominantly expressed in non-neuronal cells, is auto-inhibited by its C-terminal helix (CH) and thereby supports little fusion (Crosby et al., 2022). However, they did not provide any data to explain how the auto-inhibition is relieved and regulated during ER fusion in vivo. Therefore, in the revised manuscript, we focused on elucidating the molecular mechanism underlying auto-inhibition by the CH and relief of this auto-inhibition. We found that ATL2-1, the fusion-incompetent isoform, and ATL3 were predominantly expressed in HEK293T cells and that ATL2-1 was essential for ER microsome fusion in vitro. Our data also suggest that ATL3 plays a critical role in relieving the auto-inhibition of ATL2-1 and allows ATL2-1 to mediate ER fusion (Fig. 9). Although the basis of stimulation of ATL2-1 by ATL3 remains largely elusive, one possibility is that ATL2-1 oligomerization, which generates fusion-incompetent oligomers, is blocked by ATL3, resulting in formation of ATL2-1/ATL3 hetero-oligomers, which support fusion (Fig. 9C). In addition, our data indicate that a cytosolic factor may relieve the auto-inhibition of ATL2-1 (Fig. 9E).

2. How does ATL membrane fusion activity titrate with the content of conical lipids (with nonbilayer propensities) in membranes? ATL1 fusion activity may very well require a higher content of PE/cholesterol than what is used? - a titration experiment changing both PE and cholesterol content and looking at the membrane fusion activity of all ATL isoforms (with and without spastin) is required for a better explanation of the observed phenomena.

➔ *While this paper was under revision, Lee and colleagues reported that human ATL1 and ATL2-2, an alternative spliced isoform of ATL2, prepared from HEK293T cells are sufficient to induce fusion of PC/PS liposomes (Crosby et al., 2022), indicating that the physiological lipid composition per se is not essential to reconstitute human atlastin-mediated fusion. Although neutral lipids with small head groups that tend to form non-bilayer structures, such as*

phosphatidylethanolamine (PE) and cholesterol, generally enhanced human atlastin-mediated fusion, they differentially affected the fusion activity of different atlastin paralogs (Supplementary Fig. S2 or below).

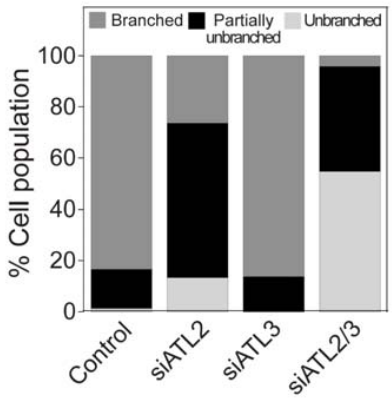
Supplementary Figure S2



Minor Issue:

1. Fig. 5A. I'm not entirely convinced, just by looking at the images that a combined ablation of ATL2/3 leads to long vs. networked tubules. Can this be quantified (e.g. by measuring length) in some manner?

➔ *Although we have not included the previous Figure 5 in the revised manuscript, we quantified the long vs. branched tubules as shown below.*



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

Atlastins are large GTPases that mediate fusion of ER membranes. Drosophila Atlastin has been shown to be sufficient for membrane fusion in reconstituted liposome mixing assays; however, none of the three human atlastins isoforms were sufficient in the same assay. The work presented here demonstrates that recombinantly purified human atlastin paralogs possess fusion activity in reconstituted liposome mixing assays if the lipid composition mimics that of the ER. In particular, PE and cholesterol are required. Notably, liposome fusion

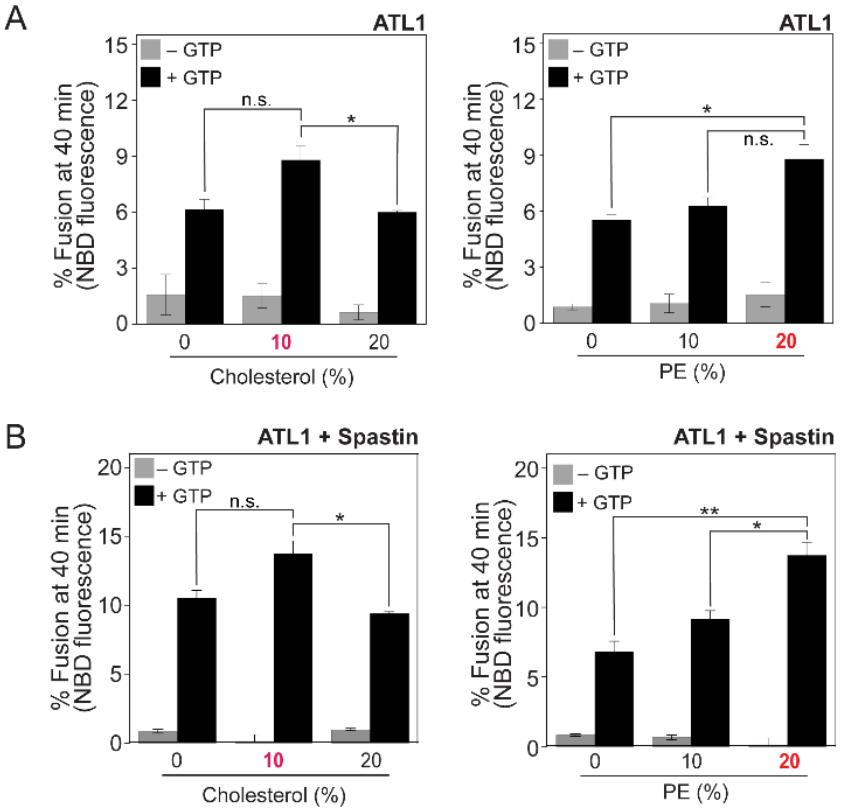
by ATL1 is significantly lower than either ATL2 or ATL3 in the context of reconstituted liposomes and yeast microsomes. Data presented here demonstrate that membrane fusion by the neural-specific ATL1 is augmented by recombinantly purified spastin in the reconstituted assay, indicating that atlastins can be regulated by additional proteins. This was confirmed in cells where co-expression of spastin converted ATL1 from diffuse on the ER membrane to a pattern more similar to ATL2 and ATL3 with enrichment at three-way junctions. The redundancy of ATL2 and ATL3 is tested in the proteoliposome fusion assay, which revealed that fusion is higher if both isoforms are present in the same proteoliposomes. Finally, a microsome fusion assay is developed and data support the conclusion that ATL2 mediates membrane fusion in cells. Overall, the manuscript represents an advance in understanding the function of human atlastin isoforms. A few points should be addressed prior to publication.

➔ *We greatly appreciate the reviewer's kind and constructive comments, which greatly helped improve our manuscript.*

Specific Comments:

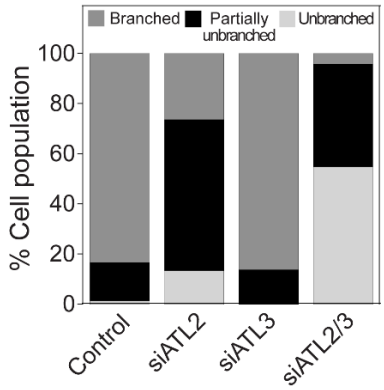
1. For liposome fusion experiments with ATL1 and spastin, dependence on cholesterol and PE should also be tested.

➔ *This has been done. The effect of cholesterol and PE on ATL1-mediated fusion was similar regardless of whether spastin was absent or present as shown below.*



2. For siRNA experiments in Cos7 cells, images should be quantified by a blinded experimenter in at least 3 biological replicates and data expressed as proportion of cells counted.

➔ *Although we have not included the previous Figure 5 in the revised manuscript, we quantified long vs. branched tubules as shown below.*

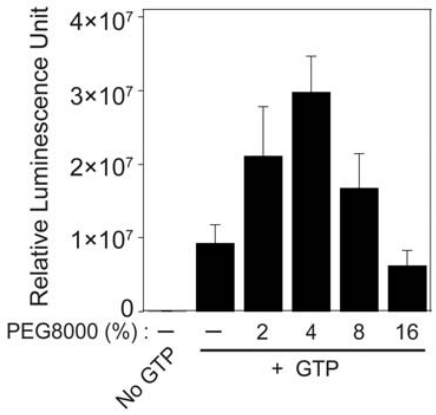


3. Proteoliposome fusion assays with both ATL2 and ALT3 indicate that when both isoforms are on the same membrane, fusion is more efficient than either isoform alone. In contrast, when ATL2 and ATL3 are present on opposing membranes, or in trans, fusion is comparable to homotypic controls. Authors should speculate on how ATL2 & ATL3 might cooperate in cis.

➔ *In the revised manuscript, we removed the previous Figure 5, which described ATL2/3 synergy. While this paper was under revision, Lee and colleagues reported that ATL2-1, the major isoform of ATL2 predominantly expressed in non-neuronal cells, is auto-inhibited by its C-terminal helix (CH) and thereby supports little fusion (Crosby et al., 2022). However, they did not provide any data to explain how the auto-inhibition is relieved and regulated during ER fusion in vivo. Therefore, in the revised manuscript, we focused on elucidating the molecular mechanism underlying auto-inhibition by the CH and relief of this auto-inhibition. We found that ATL2-1, the fusion-incompetent isoform, and ATL3 were predominantly expressed in HEK293T cells and that ATL2-1 was essential for ER microsome fusion in vitro. Our data also suggest that ATL3 plays a critical role in relieving the auto-inhibition of ATL2-1 and allows ATL2-1 to mediate ER fusion (Fig. 9). Although the basis of stimulation of ATL2-1 by ATL3 remains largely elusive, one possibility is that ATL2-1 oligomerization, which generates fusion-incompetent oligomers, is blocked by ATL3, resulting in formation of ATL2-1/ATL3 hetero-oligomers, which support fusion (Fig. 9C). In addition, our data indicate that a cytosolic factor may relieve the auto-inhibition of ATL2-1 (Fig. 9E).*

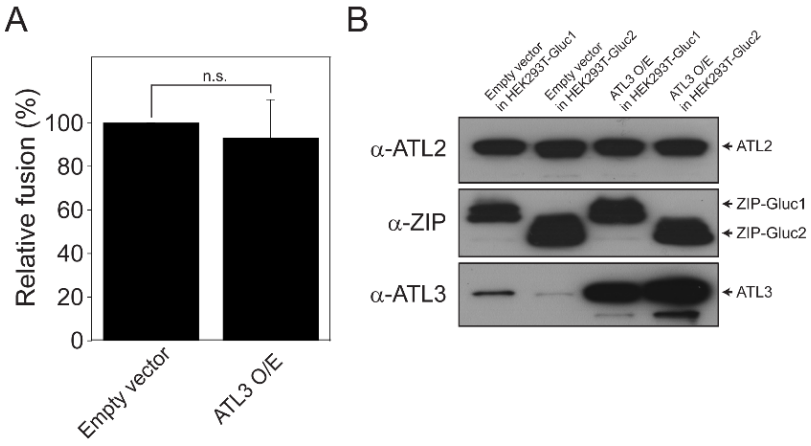
4. Additional details must be added for the HEK microsome fusion assay in the Materials and Methods or within the text. What does 100% represent in Figure 6B? What is the efficiency of microsome fusion (how many microsomes in the population fuse)?

➔ *We greatly appreciate the reviewer raising this critical issue. We initially attempted to perform microsome fusion reactions in the presence of mild detergents, such as Triton X-100, from the beginning of the reactions, but were unable to observe any signal generated by luciferase reconstitution. Thus, the protein fragment complementation assay using Gaussia princeps luciferase may not work properly in the presence of detergents. We tested a variety of detergents, including β-OG, CHAPS, and NP-40, but none worked. Therefore, instead of measuring extra-microsomal reconstitution of luciferases in the presence of detergents, we decided to take advantage of polyethylene glycol (PEG), which promotes liposome tethering and fusion through dehydration and protein association. At high levels, PEG directly triggers fusion even in the absence of fusion proteins (Burgess et al., 1992; Lentz, 2007). Thus, in the presence of high concentrations of PEG, it is possible to measure the maximal fusion signal between ER microsomes that are added to fusion reactions. ER microsome fusion reactions were performed in the presence of increasing concentrations of PEG (Supplementary Fig. S6). In the presence of 4% PEG, the maximal signal was obtained, and our fusion signal, which depends on GTP and ATL2/3, was about 25% of the maximal fusion signal. Thus, the relative fusion (100%) in our data indicates about 25% of the maximal fusion signal.*



Does fusion increase if ATL3 expression is increased in the HEK parent cells?

➔ *As shown below, ATL3 overexpression (>10-fold overexpression compared with the endogenous level) did not increase fusion between ER microsomes isolated from HEK293T cells. Although ATL3, in particular the ATL3-1 isoform, is sufficient to induce liposome fusion, the physiological role of ATL3 remains mysterious. Previous studies suggest that the main role of ATL3 is not as an ER fusogen. ATL3 functions as a receptor for ER-phagy by interacting with GABARAP (Chen et al., 2019a; Chen et al., 2019b). In our siRNA experiments, ER microsomes isolated from cells treated with siRNA targeting ATL3 almost fully support fusion. These data are consistent with the idea that ATL3 may not directly mediate fusion. However, they do not explain how ATL3 contributes to relief of ATL2-1 auto-inhibition. The finding that ATL2 expression increased upon ATL3 knockdown indicates that ATL2-2, the fusion-competent isoform of ATL2, is increased upon ATL3 knockdown and thus helps to relieve auto-inhibition of ATL2-1 (Fig. 9B). Alternatively, an unknown cytosolic factor may be mainly responsible for relief of the auto-inhibition of ATL2-1 (Fig. 9E).*



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December 13, 2022

Re: JCB manuscript #202109090R

Dr. Youngsoo Jun
Gwangju Institute of Science and Technology
Life Sciences
123 Cheomdangwagi-ro, Buk-gu
Gwangju 61005
Korea, Republic of (South Korea)

Dear Dr. Jun,

Thank you for submitting your revised manuscript entitled "Human atlastins are sufficient to drive fusion of liposomes with a physiological lipid composition". The manuscript has been seen by the original reviewers whose full comments are appended below. While the reviewers continue to be overall positive about the work in terms of its suitability for JCB, some important issues remain.

You will see that reviewer #3 raises several points re: the new data you included to respond to the Lee study, which we find are valid and will need to be addressed in an additional revision.

Our general policy is that papers are considered through only one revision cycle; however, given that the suggested changes are relatively minor we are open to one additional short round of revision. Please note that I will expect to make a final decision without additional reviewer input upon resubmission.

Please submit the final revision within one month, along with a cover letter that includes a point by point response to the remaining reviewer comments.

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

Sincerely,

Jodi Nunnari, Ph.D.
Editor-in-Chief
The Journal of Cell Biology

Lucia Morgado-Palacin, PhD
Scientific Editor
Journal of Cell Biology

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

The manuscript is much improved, and authors have done well to address my concerns and the comments of the other reviewers. The additional data and text added to the manuscript provide new insights and further validation of the findings of Crosby et al. published earlier this year, making this work an important contribution to the field. I support publication in JCB.

Minor comment:
The authors make mechanistic inferences about the flexibility of Atlastin and putative domain associations using an AlphaFold model (Fig. 7C). While comments about these potential interactions are valid in the context of this work, it would be beneficial to include the LDDT plot (or a version of the structure with confidence coloring) and the predicted aligned error plot in the supplemental materials to help the reader assess the model's overall confidence and the general flexibility of different regions as determined by the algorithm.

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

This is an extensively revised manuscript on the intrinsic capacity of human atlastins (heterologously expressed in E. coli!) to mediate membrane fusion when provided with a physiologically relevant lipid composition, specifically containing the nonbilayer-prone lipid, PE. Even though this study contrasts, to some extent, with the arguments (pertaining to mammalian vs E. coli expression) made in a recently published study by Tina Lee and colleagues, it also beautifully complements and completes the overall study of the atlastins and their regulation by membrane properties. It appears that the mammalian cell-expressed protein (potentially carrying PTMs other than phosphorylation?) used by Lee and colleagues is more conformationally dynamic than the E.coli-expressed protein (devoid of PTMs) used by the authors, which now requires a more "supple/malleable/ductile" membrane bilayer, facilitated by the presence of curvature-inducing nonbilayer-prone lipids, specifically PE, to catalyze membrane fusion. In essence, both PTMs (apart from phosphorylation) and lipid composition likely cooperatively aid atlastin-mediated membrane fusion. The authors may consider introducing a sentence to this effect in the discussion (only if they agree with this assessment). Otherwise, the authors have adequately addressed my queries from the previous round of reviews. I have no further concerns. This study nicely rounds up the study of Lee and colleagues and will be a valuable addition to our overall understanding of atlastins and membrane fusion.

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

While the authors have satisfactorily addressed the concerned outlined after the initial review, they have now included new experiments to test the model put forth in Crosby and Lee, 2022. In particular, they seek to further define why the fusion activity of ATL2-1 is autoinhibited by the C-terminal domain while ATL2-2 is fusion competent. The results of these experiments are over-interpreted and required additional controls to support the conclusions/model now presented.

1. A dose response analysis of ATL2-1 peptide would be useful to show that the inhibition of fusion is saturable.

2. While the ATL2-2 CTD peptide did not inhibit liposome fusion by ATL2-1deltaCH, it did have moderate binding activity in their co-immunoprecipitation reaction. This raises some concerns about their model. These could be mitigated by also showing that the ATL2-2 CTD peptide does not alter GTPase activity or by providing a dose-response curve for this peptide.

3. It is not clear why the ATL2-1 peptide did not inhibit liposome fusion by ATL2-2 as the proposed binding site on the GTPase domain is the same in these isoforms and the model is that binding of peptide to this site is sufficient for inhibition. If the CTD of ATL2-2 does not bind the GTPase domain, this site should be open for ATL2-1 CTD peptide to bind. This suggests that the model is not as simple as presented. Authors could test binding of the ATL2-1 peptide to ATL2-2 full length and deltaCTD or quantify GTPase activity in the presence and absence of the peptide.

4. The fact that ATL2-2 or ATL3 alleviate ATL2-1 autoinhibition is intriguing. To solidify the conclusion that fusion is mediated by ATL2-1, it would be useful to include control experiments with a variant of ATL2-1 with a mutation that abolishes GTPase activity.

Minor concerns:
1. Authors do not discuss the left panel in the lower part of Figure 6B (initial rate of fusion).

January 9, 2023

RE: JCB Manuscript #202109090RR

Dr. Youngsoo Jun
Gwangju Institute of Science and Technology
Life Sciences
123 Cheomdangwagi-ro, Buk-gu
Gwangju 61005
Korea, Republic of (South Korea)

Dear Dr. Jun:

Thank you for submitting your revised manuscript entitled "Human atlastins are sufficient to drive fusion of liposomes with a physiological lipid composition". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully. Please go through all the formatting points paying special attention to those marked with asterisks.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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

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

2) Figures limits: Articles and Tools may have up to 10 main text figures.

Please note that main text figures should be provided as individual, editable files.

3) Figure formatting:
*** Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please add MW markers to Figs 2B, 3B, 5B, and 8E.

Scale bars must be present on all microscopy images, including inset magnifications.

Also, please avoid pairing red and green for images and graphs to ensure legibility for color-blind readers. If red and green are paired for images, please ensure that the particular red and green hues used in micrographs are distinctive with any of the colorblind types. If not, please modify colors accordingly or provide separate images of the individual channels.

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. We can see that you have indicated the number of independent data points -please, also indicate whether 'n' refers to technical or biological replicates (i.e. number of analyzed cells, samples or animals, number of independent experiments).

If independent experiments with multiple biological replicates have been performed, we recommend using distribution-reproducibility SuperPlots (please, see Lord et al., JCB 2020) to better display the distribution of the entire dataset, and report statistics (such as means, error bars, and P values) that address the reproducibility of the findings.

*** Statistical methods should be explained in full in the materials and methods in a separate section. Please, provide more detail on the statistical methods used in the study.

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 (both in the figure legend itself and 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.).

*** As you used parametric tests in your study (i.e. t-tests), you should have first determined whether the data was normally distributed before selecting that test. In the stats section of the methods, please indicate how you tested for normality. If you did not test for normality, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title:
The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience.

The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

6) Materials and methods:
Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. The text should not refer to methods "...as previously described."

Also, the materials and methods should be included with the main manuscript text and not in the supplementary materials.

7) For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features, even if described in other published work or gifted to you by other investigators (and provide references where appropriate).

Please be sure to provide the sequences for all of your oligos: primers, si/shRNA, RNAi, gRNAs, etc. in the materials and methods.

*** You must also indicate in the methods the source, species, and catalog numbers/vendor identifiers (where appropriate) for all of your antibodies, including secondary. If antibodies are not commercial, please add a reference citation if possible. Please, indicate the species and catalog numbers for all of your antibodies.

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/Tools may have up to 5 supplemental figures. There is no limit for supplemental tables. Currently, you have 11 supplemental figures. We can give you a bit more space, but we would need you to try to reduce the number of supplementary figures (up to 7-8 if possible) by consolidating data from two figures into one or moving supplemental data to one of the main figures. Please be sure to correct the callouts in the text to reflect this change.

*** Please note that supplemental figures and tables should be provided as individual, editable files.

*** A summary of all supplemental material should appear at the end of the Materials and Methods section (please see any recent JCB paper for an example of this summary).

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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.

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