Membrane fusion by Drosophila atlastin does not require GTP hydrolysis

Daniel Crosby and Tina Lee

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

RE: Manuscript #E22-05-0164
TITLE: Membrane fusion by Drosophila atlastin does not require GTP hydrolysis

Dear Dr. Lee:

Thank you for submitting this manuscript to MBoC. As you can see below, the manuscript was reviewed by two reviewers who have substantial expertise in membrane fusion and GTPase mechanisms. Although the reviewers agree that the manuscript is interesting and well written, they differ in their opinion of the impact of the work for the field. They also both suggest several additional experiments that may further support your conclusions.
Therefore, I am willing to consider a revised manuscript that addresses their concerns.

Sincerely,

Mary Munson
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Lee,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

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In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

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Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

Dynamin-family GTPases including mitofusin and atlastin mediate membrane fusion, but the mechanisms that couple the GTP binding and hydrolysis cycle to membrane tethering and fusion are contentious. Here, Crosby and Lee present careful and focused biochemical experiments with a recently discovered atlastin mutant (D129N) to explore this question. In a previous paper with a soluble truncation construct, D129N exhibited an almost total inability to hydrolyze GTP while retaining the ability to enter into a "crossover" homodimer. The crossover dimer is hypothesized to correspond to a pre-fusion intermediate that upon fusion becomes a post-fusion cis- dimer. The authors here use full-length atlastins and reconstituted proteoliposomes (RPLs) to test the idea that GTP binding drives formation of the trans-crossover dimer and membrane fusion, while GTP hydrolysis dissociates the cis-crossover dimer, liberating monomers for additional rounds of fusion. Data from kinetic analyses of GTP hydrolysis, fusion, and crossover formation are employed to argue that GTP hydrolysis is needed for multiple, but not single rounds of fusion.

The paper is beautifully written, placing the experiments in the broader context of the GTPase superfamily and logically presenting the experiments, which are carefully controlled and very persuasive. The study is "old school" in the best sense: a concise set of carefully designed experiments are used to rigorously test a focused hypothesis.

Specific comments:

1. In the Introduction, microtubules and the bacterial Z-ring might be mentioned as paradigms for how GTP binding and hydrolysis are coupled to oligomerization and disassembly reactions that, like the dynamins, do mechanical work.

2. Can the authors verify that WT and D129N atlastin are incorporated into RPLs with similar efficiency?

3. In Fig. 1 B low (1 μM) GTP is shown to allow about the same amount of fusion with WT atlastin as with D129N at much higher GTP concentrations. This is a key result, consistent with the authors' interpretation that multiple rounds of fusion require the ongoing presence of GTP, and that at low concentrations the WT hydrolyzes and thereby depletes the GTP in the reaction. However, as the authors mention, the data are also consistent with the possibility that WT and D129N have different Km's for GTP. For this reason, it would be valuable to repeat the fusion assay with WT in the presence of either 1 μM GTP, or in the presence of 1 μM GTP plus a GTP-regenerating system. For example: 1 μM GTP, 5 mM Mg•ATP, and with/without nucleotide diphosphate kinase. The authors' model would predict that the regeneration system should allow sustained, multi-round fusion. If this is not seen, it may indicate that the RPL population is heterogenous and that WT atlastin can drive fusion of some RPLs in the population at 5 μM steady-state GTP, but not 1 μM.

4. In Fig. 1D, the extent of quenching by dithionite (>80%) is surprising. Can the authors suggest an explanation for this? Could it indicate the presence of leaky RPLs, perhaps due to incomplete removal of detergent?

5. In Fig. 3B, it might be useful to repeat the WT FRET experiment with 1 μM rather than 5 μM GTP. The fusion data in Fig. 1 suggest that this might result in trapping of crossover dimers rather than the apparently full disassembly seen at 5 μM.

6. In the Methods, both Triton X-100 and Anapoe X-100 are mentioned. These are marketed as equivalent detergents. Does the nomenclature reflect different brands of similar detergent used for differerent procedures?

7. The methods mention that "Data shown are the average of two individual traces." How many overall replicates were done per experiment, and are the two traces within- or between- experiment replicates? Some indication of experimental variance should also be provided.

-Alex Merz

Reviewer #2 (Remarks to the Author):

Dynamin superfamily proteins are mechanochemical enzymes that remodel cellular membranes. The mechanistic basis of membrane fusion by DSPs is not well defined. Atlastin is a DSP localized to the endoplasmic reticulum that mediates homotypic membrane fusion. Early models posited that GTP hydrolysis would provide the energy required to overcome the energetic barrier of membrane fusion. Consistent with this, variants of Atlastin that cannot hydrolyse GTP fail to fuse membranes and non-hydrolysable GTP analogs block fusion in reconstituted systems. Work from several labs revealed that a key aspect of the atlastin mechanism was a large conformational change in the context of an intermolecular dimer where the helical bundle domains of each molecule crossover relative to their GTPase domain. Kinetic analysis of purified, truncated Drosophila atlastin protein (dATL) suggested that formation of the crossover dimer occurs prior to GTP hydrolysis, raising the question of whether membrane fusion occurs following formation of the crossover dimer or following GTP hydrolysis. Winsor et al. initially tested this model using a novel atlastin variant that can generate the crossover dimer in solution but has very slow hydrolysis activity (dATL D129N). This published work revealed limited fusion by the variant and extremely delayed disassembly or persistence of the crossover dimer, both consistent with a model where GTP hydrolysis is required for disassembly of the crossover dimer and therefore additional rounds of membrane fusion by atlastin.

In this manuscript, Crosby and Lee take a similar approach to further support this model for the role of GTP hydrolysis in disassembly of the crossover dimer. By limiting the concentration of GTP to concentrations that are predicted to be sufficient for only one round of hydrolysis by wildtype dATL, they observe proteoliposome fusion that is similar to dATL-D129N with excess GTP. To support the conclusion that this is complete fusion and not lipid mixing resulting from a hemifusion intermediate, they quench NBD-fluorescence on the outer leaflet. Kinetic analysis of fusion activity and GTP hydrolysis are consistent with the conclusion that GTP hydrolysis occurs after the first round of lipid mixing. The paper is well-written and the data broadly support the conclusions made. It is not entirely clear that this is more than a very incremental advance from the Winsor et al paper published in 2018 as it employs the same variant, the same assays and makes the same conclusion as that paper.

Specific comments:
1. Authors should consider explaining in detail why 1 micromolar GTP is sufficient for one round of atlastin-mediated fusion and not two in the specific context of their fusion reactions. This is key to their interpretation of the dATL D129N fusion data. To further support this conclusion, additional GTP could be spiked into the reaction at 200 seconds with the expectation that wild type dATL proteoliposomes would fuse again while dATL D129N proteoliposomes would not.

2. The text suggests that the increase in proteoliposome fusion observed with dATL D129N with 5 micromolar GTP over 1 micromolar GTP is due to decreased affinity for GTP. This should be measured experimentally.

3. The quenching of NBD fluorescence in the context of proteoliposomes reduced total fluorescence from 800 units to 200 units. One would predict a reduction by half if the NBD were evenly distributed between the leaflets. Did authors consider that proteoliposomes lack membrane integrity, possibly due to residual detergent?

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The paper is beautifully written, placing the experiments in the broader context of the GTPase superfamily and logically presenting the experiments, which are carefully controlled and very persuasive. The study is "old school" in the best sense: a concise set of carefully designed experiments are used to rigorously test a focused hypothesis.

Thank you for your review. Your positive comments are much appreciated.

Specific comments:

1. In the Introduction, microtubules and the bacterial Z-ring might be mentioned as paradigms for how GTP binding and hydrolysis are coupled to oligomerization and disassembly reactions that, like the dynamins, do mechanical work.

Thank you for the suggestion; we have included these GTPases in the introduction (line 110-115).

2. Can the authors verify that WT and D129N atlastin are incorporated into RPLs with similar efficiency?

Verification that the proteins are incorporated with similar efficiency has been added as Figure S1.

3. In Fig. 1 B low (1 μM) GTP is shown to allow about the same amount of fusion with WT atlastin as with D129N at much higher GTP concentrations. This is a key result, consistent with the authors' interpretation that multiple rounds of fusion require the ongoing presence of GTP, and that at low concentrations the WT hydrolyzes and thereby depletes the GTP in the reaction. However, as the authors mention, the data are also consistent with the possibility that WT and D129N have different Km's for GTP. For this reason, it would be valuable to repeat the fusion assay with WT in the presence of either 1 μM GTP, or in the presence of 1 μM GTP plus a GTP-regenerating system. For example: 1 μM GTP, 5 mM Mg•ATP, and with/without nucleotide diphosphate kinase. The authors' model would predict that the regeneration system should allow sustained, multi-round fusion. If this is not seen, it may indicate that the RPL population is heterogenous and that WT atlastin can drive fusion of some RPLs in the population at 5 μM steady-state GTP, but not 1 μM.

Thank you for the suggestion. We performed this experiment and observed multi-round fusion upon the addition of nucleoside diphosphate kinase (NDK), 5mM ATP, and 1uM GTP. Addition of 5mM ATP and 1uM GTP without NDK did not cause a sustained increase. This data has been added as Figure 1D.

We also performed an additional experiment (in response to reviewer 2 comment 1) by spiking with 1μM GTP after each fusion plateau and observed the predicted stepwise increases predicted. This data has been added as Figure 1C.

4. In Fig. 1D, the extent of quenching by dithionite (>80%) is surprising. Can the authors suggest an explanation for this? Could it indicate the presence of leaky RPLs, perhaps due to incomplete removal of detergent?

As rightly noted by the reviewer, this >50% quenching is larger than expected if NBD lipids were distributed equally between outer and inner leaflets. It is not seen with protein free liposomes (the expected 50% quenching of protein free liposomes is added as Figure S2). Therefore, it is somehow due to the process of inserting atlastin or the presence of atlastin in the proteo-liposomes. We believe that it is unlikely due to leaky liposomes for two reasons. First, we tested for leakage by performing two further sequential additions of sodium dithionite (four total) to both protein-free and ATL-containing liposomes (Figure S2) and observed that a similar and relatively flat baseline is achieved after the first two dithionite additions for both types of liposomes. What NBD fluorescence remains after the first two dithionite additions is retained similarly in both.

Second, we perform 4-1hr detergent removal steps with Bio-Beads SM2 during our incorporation protocol. Each step has sufficient bead mass to remove the amount of detergent present. Therefore, it is highly unlikely that any residual detergent remains.

Finally, this larger than expected decrease in NBD fluorescence by dithionite for ATL containing liposomes has also been seen previously (Orso et al, 2009, Figure S13).

5. In Fig. 3B, it might be useful to repeat the WT FRET experiment with 1 μM rather than 5 μM GTP. The fusion data in Fig. 1 suggest that this might result in trapping of crossover dimers rather than the apparently full disassembly seen at 5 μM.

The NDK experiment suggested by the reviewer and performed in Figure 1D, as well as the sequential GTP addition experiment in Figure 1C, is at odds with the suggestion of the trapping of the wildtype crossover dimer.

6. In the Methods, both Triton X-100 and Anapoe X-100 are mentioned. These are marketed as equivalent detergents. Does the nomenclature reflect different brands of similar detergent used for different procedures?

Anapoe X-100 is simply a higher quality version of Triton X-100 that contains less oxidized Triton. We elute our protein into the higher quality reagent and use it in all subsequent assays. This has been noted in the methods (line 360).

7. The methods mention that "Data shown are the average of two individual traces." How many overall replicates were done per experiment, and are the two traces within- or between- experiment replicates? Some indication of experimental variance should also be provided.

Thank you for the comment. Most traces are the average of 2 technical replicates and have been repeated with similar results from 2 biological replicates (2 independent protein preps and incorporations). Technical replicates are typically very similar and only a 5-10% deviation in initial fusion rates is typically seen from 2 independent protein preps. All of this has been added into the figure legends and methods.

-Alex Merz

Reviewer #2 (Remarks to the Author):

Dynamin superfamily proteins are mechanochemical enzymes that remodel cellular membranes. The mechanistic basis of membrane fusion by DSPs is not well defined. Atlastin is a DSP localized to the endoplasmic reticulum that mediates homotypic membrane fusion. Early models posited that GTP hydrolysis would provide the energy required to overcome the energetic barrier of membrane fusion. Consistent with this, variants of Atlastin that cannot hydrolyse GTP fail to fuse membranes and non-hydrolysable GTP analogs block fusion in reconstituted systems. Work from several labs revealed that a key aspect of the atlastin mechanism was a large conformational change in the context of an intermolecular dimer where the helical bundle domains of each molecule crossover relative to their GTPase domain. Kinetic analysis of purified, truncated Drosophila atlastin protein (dATL) suggested that formation of the crossover dimer occurs prior to GTP hydrolysis, raising the question of whether membrane fusion occurs following formation of the crossover dimer or following GTP hydrolysis. Winsor et al. initially tested this model using a novel atlastin variant that can generate the crossover dimer in solution but has very slow hydrolysis activity (dATL D129N). This published work revealed limited fusion by the variant and extremely delayed disassembly or persistence of the crossover dimer, both consistent with a model where GTP hydrolysis is required for disassembly of the crossover dimer and therefore additional rounds of membrane fusion by atlastin.

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Thank you for your review. We agree to a certain extent that this study is 'incremental' in the sense that the assays employed are conceptually very similar to those in our previous study (Winsor et al, 2018). However, we respectfully disagree that the advance is incremental. The key kinetic assays in the previous paper were done using the truncated soluble domain of atlastin, thus in the absence of the TM domain, amphipathic helix and tail, and in the absence of any membranes, and with atlastin concentrations very

different from those used in our fusion assay. Thus, our earlier study left open the possibility – however remote - that GTP hydrolysis could still be required. Perhaps because of the lack of conclusiveness of our prior paper, several papers/reviews published since 2018 have continued to maintain or question that fusion is triggered by hydrolysis. For instance, a recent paper from the Sondermann group (Kelly, Byrnes, Neela, Sondermann and O'Donnell, JCB 2021) states that "Upon initiation of hydrolysis, G domains are released followed by dimerization in a crossover formation, bringing membranes in close proximity for fusion." In another example, this time from a review on the dynamin superfamily published soon after our 2018 paper (Ford and Chappie, Traffic 2019), "Different biophysical studies and experimental conditions have yielded alternative models for the timing of these conformational changes relative to atlastin's GTP hydrolysis cycle. Although each is consistent with the respective assays employed, a definitive consensus has yet to be reached in the field."

For all these reasons, it was imperative that the nucleotide hydrolysis and crossover dimerization assays be repeated under conditions as close as possible to the fusion assay conditions. And we have done that in this study. We have measured GTP hydrolysis by full-length atlastins under conditions nearly identical to the fusion assay, which has not been done before. As a result, we can conclude, for the first time, that the atlastin GTPase can definitely mediate fusion without the need for hydrolyzing GTP and that like for most other G proteins, the hydrolysis of nucleotide provides a means for destabilizing and resetting the biologically active, functional version of the protein.

Specific comments:

1. Authors should consider explaining in detail why 1 micromolar GTP is sufficient for one round of atlastin-mediated fusion and not two in the specific context of their fusion reactions. This is key to their interpretation of the dATL D129N fusion data. To further support this conclusion, additional GTP could be spiked into the reaction at 200 seconds with the expectation that wild type dATL proteoliposomes would fuse again while dATL D129N proteoliposomes would not.

We thank the reviewer for the comment and the suggested experiment. We added text to indicate that 1 µM GTP is greater than one but less than 2 molar equivalents of atlastin in the fusion assay (line 181). Additionally, we performed the spiking experiment and observed further fusion as expected by the reviewer. This new data has been added as Figure 1C. Additionally, we performed a similar experiment (response to reviewer 1) utilizing 1uM GTP and a GTP regeneration system which yielded similar results. This data has been added as Figure 1D.

2. The text suggests that the increase in proteoliposome fusion observed with dATL D129N with 5 micromolar GTP over 1 micromolar GTP is due to decreased affinity for GTP. This should be measured experimentally.

Thank you for the comment. Looking back at our previously published intrinsic tryptophan fluorescence data (Winsor et al, 2018) comparing wildtype to D127N atlastin (albeit the truncated soluble domain), we realized that we already had sufficient data to suggest that the GTP binding affinity between wildtype and mutant is unlikely to be different. On the other hand, there appears to be slight difference in the amplitude of the curves, suggesting that the GTP binding induced G domain conformational change (prior to dimerization) might be slightly different for the mutant. Based on this, we speculate that the G domain dimer affinity might be slightly lower for the D127N mutant and hence more sensitive to nucleotide concentration. We have modified our comment on this difference accordingly on line 206.

3. The quenching of NBD fluorescence in the context of proteoliposomes reduced total fluorescence from 800 units to 200 units. One would predict a reduction by half if the NBD were evenly distributed between the leaflets. Did authors consider that proteoliposomes lack membrane integrity, possibly due to residual detergent?

Please see the response to comment 4 from reviewer 1.

4. The number of technical replicates and biological replicates needs to be specified for all experiments.

Thank you for the comment; as indicated in response to reviewer 1 comment 7, this information has been added to the figure legends and methods.

RE: Manuscript #E22-05-0164R
TITLE: "Membrane fusion by Drosophila atlastin does not require GTP hydrolysis"

Dear Dr. Lee:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Sincerely,
Mary Munson
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Lee:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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We are pleased that you chose to publish your work in MBoC.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

The authors have done a really thorough job of addressing the referees' comments.

Reviewer #2 (Remarks to the Author):

Authors have addressed all experimental concerns.