The hypervariable region of atlastin-1 is a site for intrinsic and extrinsic regulation

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Re: JCB manuscript #202104128

Prof. Holger Sondermann
Deutsches Elektronen-Synchrotron DESY
Centre for Structural Systems Biology (CSSB)
Notkestr. 85
Hamburg 22607
Germany

Dear Prof. Sondermann,

Thank you for submitting your manuscript entitled "The hypervariable region of atlastin-1 is a site for intrinsic and extrinsic regulation". 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.

You will see that all reviewers are very enthusiastic about your study and feel it provides important new insights into atlastin-mediated membrane tethering. The major experimental requests are to confirm the role of ATL1-HVR in tethering either by EM or fluorescence microscopy and to examine whether mutations in the GTPase domain near the HVR contact site affect tethering. While these are certainly interesting questions we don't believe that these are essential for revision. However, if you have data in hand or can do the experiments to address these points in a reasonable timeframe, we encourage you to do so as it would substantially enhance the paper. We don't believe that additional work on the kinases that phosphorylate ATL1 is necessary. Reviewers also have several other points which we believe can be addressed by text and figure revisions.

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.

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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,

James Hurley, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

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

Atlastin GTPases are dynamin-related mechano-chemical enzymes implicated in the generation and maintenance of 3-way junctions of the ER. Reconstitution experiments indicate that atlastins can tether membranes in trans in a GTPase-dependent reaction. Previous structural studies mostly focusing on the GTPase and middle domains provided a framework how tethering can be achieved via contacts in the G domain. However, the N-terminal hypervariable region (HVR), which is highly conserved amongst isoforms of different species but deviates between the isoform within a species, has so far been neglected in mechanistic and structural studies.

In this manuscript, Kelly et al. report two crystal structures of ATL1 and ATL3, in which the N-terminal HVRs were partly resolved and stabilized by contacts in the crystals. In case of ATL1, these crystal contact may represent a functional interaction during membrane fusion. Indeed, the
authors use in vitro assays to demonstrate a role of the ATL1 HVR, but not the ATL3 HVR, in membrane tethering. Furthermore, they identify in vivo phosphorylation sites in the ATL1 HVR and show that phosphorylation at Ser10 reduces liposome tethering. Some of the phosphomimetic mutants alter ATL1 distribution in the cell. Putative kinases responsible for the phosphorylation were identified in an in vitro kinase screen, a hierarchical phosphorylation order determined and the consequences of phosphorylations discussed in the context of the structural data.

The manuscript is rich in new structural and mechanistic insights. Experiments appear sound, reliable and of high quality. The topic is exciting since the mechanism of ATL-mediated membrane tethering and fusion is still not fully resolved. The described involvement of the HVR adds interesting aspects of the tethering reaction and provides a rationale how ER fusion events may be regulated. Having said this, an important aspect of the study is currently not conclusive and should be still addressed.

Major:
Fig. 1D-E: The observed crystal contacts of the HVR in ATL1 are at the heart of this manuscript since they provide a structural explanation of how the HVR contributes to membrane tethering. However, this contact is not systematically followed up.
(1) The authors should show a detailed figure of the ATL1-HVR contacts with the neighboring ATL molecule.
(2) Key residues in the interface should be mutated, e.g. both in the HVR and in the adjacent G domain. Subsequently, the authors should analyze whether the mutants are still able to tether membranes.

Without successfully demonstrating the importance of this contact, the mechanism of the HVR in atlastin-membrane tethering remains vague.

Minor
Fig. 1: Please show the intermolecular contacts of the ATL3 HVR in an additional SI figure.
Fig. 1E: Do atlastin G domains in the crystals interact via the G-interface? If yes, please mention and show the nucleotide in both G domains.
Fig. 2B: Add in the legend that this experiment was carried out in the presence of 500 µM GTP.
Fig. 2E: What is shown here? A view into a reaction tube? What is one supposed to see?
Fig. 3: These experiments could go to the SI.
Fig. 6/Fig. S4: I did not fully understand these experiments. Is the ATL1 ER distribution altered or the ER morphology in general when the phospho-mimetic mutants are added back? E.g. does 'tubular' and 'punctate' ATL localization correspond to 'tubular ER' and 'fuzzy ER' morphology?
Fig. 8: Kinase reactions with substrate 'ATL1 catalytic core' - Please define the exact construct - is the HVR really part of the 'ATL1 catalytic core'?

Introduction
Another widespread feature is a GTPase effector domain (GED) that functions in intramolecular regulation of GTPase activity (Chappie et al, 2009), however this domain is absent in ATLs.

The GED in dynamin corresponds to the C-terminal sequence stretch of the stalk and bundle signaling element (BSE). Atlastin has also a sequence stretch at this position, which is not included in the crystallized constructs, e.g. a similar role as the GED in dynamin cannot be excluded. Do the authors mean 'BSE' instead of GED? However, whether the middle domain of Atlastin corresponds to the BSE or stalk is also difficult to say.

Discussion
The Discussion is a bit lengthy and could be shortened. For example, there is not much evidence in the manuscript for a role of the HVR in the intrinsic regulation of atlastin's GTPase activity or the prevention of futile GTPase cycles.

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

The Manuscript entitled "The hypervariable region of atlastin-1 is a site for intrinsic and extrinsic regulation" by Kelly et al demonstrates the role of the Hypervariable Region (HVR) of Atlastin-1 (ATL1) in the enzyme's tethering efficiency and potentially Endoplasmic Reticulum (ER) membrane fusion. Towards understanding the mechanism of ATL, authors crystallized the ATL1 and ATL3. They highlight the novel N-terminal HVR motif of ATL which was absent in the previously determined crystal structures of ATL. Sequence comparison between the 3 isoforms is highly divergent, hence termed as hypervariable region (HVR). The biochemical experiments performed on ATL1 suggests that the ATL1 HVR has a role in membrane tethering and possibly membrane fusion, but no significant function in GTPase activity or oligomerization of the protein. Using proteomic predictions and immunoblotting experiments, authors identified 3 different Serine positions (S10, S22 and S23) within the HVR which can be phosphorylated and furthermore found potential kinase candidates for S10 and S22/S23. They found AurA kinase as the top candidate for phosphorylating S10, a possible link between mitotic progression and previously observed changes in ER morphology. Other potential S10 kinase candidate is PKA, a common kinase for other dynamin family members. For S22/23, the CaMKII kinases are top candidates and also have been shown to modify Drp1. Overall, the present work defines a role for the HVR domain of ATL1 in tethering and the subsequent effects of phosphorylation in this region.

Comments:
Comment on the G-domain dimer interfaces observed in the crystal structure of ATL1. The crystal packing of ATL1 appears to have the canonical G domain dimer interface seen in other dynamins and shown to stimulate GTPase hydrolysis. (It is stated that the ATL3 HVR conformation does not represent a physiologically relevant state and therefore may not have the canonical G domain interface.)

The tethering assay (fig 2B) needs clarification. A description of what the graph represents over the 45 min time frame would be helpful. It is not clear what part of the graph is indicating tethering - the high signal at early timepoints (0-10 mins)? Also, why does the signal decrease and rise again? Is there constant stirring during the 45 min assay to prevent sedimentation of large complexes? What cause the increase in signal for ATL1-delta-HRV and ATL3? Indicate the time points that correspond to the following comments "tethering rate constants as a
function of protein concentration indicate a robust tethering enhancement of wild-type ATL1" and "later time-points, wild-type ATL1 samples produced macroscopic, tethered clusters that were visually discernable and occurred when tether formation began to plateau causing high signal variation"

Considering alterations in tethering is the main observation in this manuscript, it is important to show in vitro tethering directly through electron or fluorescent microscopy (idealy cryoEM).

The S10A mutant had no effect on tethering or GTPase activity but did result in more puncta in the cell. This is briefly addressed in the discussion on line 549 but needs more clarification.

Minor comments:

The main result of the paper is an effect on tethering with delta-HRV and phosphomimetic mutants and thus tethering should be mentioned in the title.

Figure 1C: add G domain is colored orange.

Figure 2B: mention GTP is present in the figure legend.

Figure 2C: change in legend, (C) As in (C), correct it to as in (B).

Figure 2B and 2C: shapes in graphs do not match legend icons. In 2B legend, the shape of ATL1∆HVR is shown as square in light blue, but in graph it is a light blue triangle.

Line 534: Clarify what is meant by " and S22 and S23 existing only in the same phosphorylation state"?

Explain why the tethering traces vary significantly between experiments, compare figure 2B to S3C.

Figure 3 can be moved to supplemental, or reduced to one panel and the rest shown in supplemental.

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

In this work, new structures of soluble parts of ATL1 and ATL3 capture secondary structure from sequence immediately upstream of the respective ATL GTPase domains. These sequences have been noted previously as they are highly conserved within ATL isoforms from different species but vary considerably between isoforms. In previous structures these were disordered. In the case of ATL1, the so-called HyperVariable Region (HVR) preceding the G domain forms a beta hairpin, which is of interest as the sequence in and around the hairpin contains serines that are shown in this work to be phosphorylated. In the case of the structure of the isolated ATL3 GTPase domain, the sequence is ordered in one of the 2 copies in the ASU, where it forms an extended helix.

Of greater interest regarding possible insight into the mechanisms of regulation of tethering and/or fusion activity, is that the ATL1 HVR hairpin makes a small-surface contact with its adjacent protomers that may explain array formation in the context of the ER membrane. This may help
poise or prepare the ATL1 G domain monomers for trans dimer formation, required for tethering and/or fusion. This is an attractive idea as fusion efficiency does depend on density of atlastin on the opposed membranes and this may be a molecular mechanism to facilitate this.

Much of the current manuscript sets out to explore the function of this HVR. A significant body of existing work has already extensively characterized the hydrolysis kinetics and light scattering properties of the soluble parts of the Atlastins (some of this work on ATL1 already included the HVR - e.g. Byrnes, 2011, 2013). Hydrolysis turnover numbers for constructs bearing and lacking the HVR were similar, molar mass determinations by light scattering in the presence of various nucleotides or nucleotide analogs were not different either. The SAXS data are exquisite: and the pair-distance distribution plots indicated similar profiles, estimated maximum dimensions and the Kratky plots indicated no differences in folding in constructs with or without the HVR. Overall, the data show that, in solution at least, the presence of the HVR makes no difference.

A difference was detected when the catalytic core proteins were tagged with a histidine tag and assessed in a liposome tethering assay, using liposomes doped with DGS-NTA(Ni). The presence of the ATL1 HVR resulted in increased rates of change in solution turbidity, and this could be detected macroscopically.

ATL1 HVR and the region around it harbors multiple phosphorylation sites. LC-MS/MS was used to demonstrate S10, which lies upstream of the hairpin, was essentially constitutively phosphorylated (though in vivo data suggests phosphate cycling at this site) while S22/S23 was partially phosphorylated. In exhaustive mutant screening, the only difference observed in catalytic activity and tethering assays using phosphomimetic or S to A mutations at these sites was that S10E mutants were impaired in tethering.

In vivo consequences of ATL1 HVR phosho-site mutations were assessed by reintroduction of ATL1 and various mutants into NIH-3T3 cells where the 3 ATL isoforms were deleted. Some modest effects are seen in localization and in ER morphology but mutation of S10 to either E or A results in an increased "punctate" localization.

Finally, a kinase screen using the kinase domains from 58 ser/thr kinases showed several can phosphorylate the HVR, with some specificity as to which kinases phosphorylate which region

Overall, the crystallography, the small angle X-ray scattering, the tethering assays, and the supporting in vitro characterization are beautiful. By contrast, the in vivo and kinase work seem relatively incomplete and preliminary in comparison. This latter part dampens my current enthusiasm for supporting publication in the current form.

Comments:
A confirmation of the role of the HVR in tethering would be a duplication of the tethered structures (perhaps with DGS-NTA(Ni)-bearing lipids) seen by EM by Siani, 2014, and its predicted loss in the absence of the HVR.

Do mutations in and around the groove on the back of the GTPase domain (around E202 in PDB 3Q5E), where the HVR is predicted to interact, have any effects in vitro or in the in vivo assays?

The authors suggest the concentration on a membrane template may be required to see the effects of the presence or absence of ATL1 HVR. This, and given the small contact area, means the lack of effects on catalytic core kinetics in solution are not a surprise. What about on
liposomes? Does the presence of the HVR have any effects on protein tethered to liposomes using the DGS-NTA(Ni) approach?

The in vitro kinase assays show that several kinase domains can in principle phosphorylate the HVR in solution. In vitro tethering assays show that the S10E mutants have strong effect. The effects of the kinase mutants in vivo are, by contrast, conflicting and surprisingly mild. Might these mutants be more involved in isoform segregation in vivo?

Minor:
S2A. This gel is denaturing PAGE. What is the relatively abundant band at @70 kDa? And is this present in all the solution characterizations?
Dear Dr. Hurley,

We would like to thank you and the reviewers for the constructive and thoughtful feedback on our manuscript entitled “The hypervariable region of atlastin-1 is a site for intrinsic and extrinsic regulation”. We are also grateful for the opportunity to submit a revised manuscript. We hope you agree with us that we have addressed satisfactorily in the response below and our amended submission all points that were raised during the previous review. Thank you for considering this revised manuscript for publication in the Journal of Cell Biology. We are looking forward to hearing from you.

You will see that all reviewers are very enthusiastic about your study and feel it provides important new insights into atlastin-mediated membrane tethering. The major experimental requests are to confirm the role of ATL1-HVR in tethering either by EM or fluorescence microscopy and to examine whether mutations in the GTPase domain near the HVR contact site affect tethering. While these are certainly interesting questions we don't believe that these are essential for revision. However, if you have data in hand or can do the experiments to address these points in a reasonable timeframe, we encourage you to do so as it would substantially enhance the paper. We don't believe that additional work on the kinases that phosphorylate ATL1 is necessary. Reviewers also have several other points which we believe can be addressed by text and figure revisions.

Thank you for sharing your enthusiasm regarding our study with us. We agree with the reviewers that the novel insight into the regulation of ATL warrants an in-depth characterization of the newly described protein-interaction interface and further studies into the role of this interaction for membrane tethering. We are planning to conduct the suggested experiments in the future. Our main goal with the present study was establishing a role of the HVR for ATL function, which led to the discovery of intrinsic and extrinsic regulatory mechanisms that can be clearly tied to the HVR and its posttranslational modification, respectively. Given the scope and extent of our current submission, we feel the suggested studies deserve a separate, in-depth characterization, allowing us to test several hypotheses and models that we mention in our Discussion. Hence, we thank you for providing us with the choice to conduct the suggested experiments in the context of future studies.
Reviewer #1:
...The manuscript is rich in new structural and mechanistic insights. Experiments appear sound, reliable and of high quality. The topic is exciting since the mechanism of ATL-mediated membrane tethering and fusion is still not fully resolved. The described involvement of the HVR adds interesting aspects of the tethering reaction and provides a rationale how ER fusion events may be regulated. Having said this, an important aspect of the study is currently not conclusive and should be still addressed.

We thank this Reviewer for acknowledging the novel aspects of our study and for the constructive feedback.

Major:
Fig. 1D-E: The observed crystal contacts of the HVR in ATL1 are at the heart of this manuscript since they provide a structural explanation of how the HVR contributes to membrane tethering. However, this contact is not systematically followed up.

In the current manuscript, we investigated the functional implications of the N-terminal HVR in ATLs on a broad scale, providing the first evidence for their role in membrane tethering. These studies were inspired by the crystallographic data that provides a model for how the HVR could impact ATL1-mediated membrane tethering. We agree with the reviewers that the structural models and associated hypotheses require further validation and testing. We consider these as the logical next steps for future mechanistic studies. Our analysis and main findings presented in the manuscript do not hinge on the validation of the crystallographic interface, while the crystal packing provides the basis for some of the discussed models awaiting assessment.

(1) The authors should show a detailed figure of the ATL1-HVR contacts with the neighboring ATL molecule.

We included such a figure and a brief description of the protein interface in the revised manuscript (Figure S1 and figure legend).

(2) Key residues in the interface should be mutated, e.g. both in the HVR and in the adjacent G domain. Subsequently, the authors should analyze whether the mutants are still able to tether membranes.

We are planning to conduct an in-depth mechanistic study in the future, including a mutational analysis of the crystallographic interface. For the current study, we feel that the HVR deletion mutant and the regulation by phosphorylation clearly establish a functional role of the HVR in membrane tethering – the main focus of our manuscript.

Without successfully demonstrating the importance of this contact, the mechanism of the HVR in atlastin-membrane tethering remains vague.

A detailed mechanistic follow-up should definitively include a mutational analysis of the HVR-G domain interface of a putative ATL1 oligomer, as it could validate the involvement of the specific interface in the tethering reaction.

Minor:
**Fig. 1:** Please show the intermolecular contacts of the ATL3 HVR in an additional SI figure.

We included the figure requested in the revised manuscript (Figure S1B).

**Fig. 1E:** Do atlastin G domains in the crystals interact via the G-interface? If yes, please mention and show the nucleotide in both G domains.

The G-domain dimer recognized in earlier structures (Byrnes and Sondermann, 2011, DOI: 10.1073/pnas.1012792108; Bian et al., 2011, DOI: 10.1073/pnas.1101643108) is present also in the structure of ATL1 presented here. This aspect is illustrated in Figure 1E (and now mentioned in the main text). We also included a Figure panel showing the nucleotide density in Figure S1 of the amended manuscript.

**Fig. 2B:** Add in the legend that this experiment was carried out in the presence of 500 µM GTP.

We made the requested change.

**Fig. 2E:** What is shown here? A view into a reaction tube? What is one supposed to see?

We added clarification to the figure legends.

**Fig. 3:** These experiments could go to the SI.

To comply with JCB’s number of allowed supplementary figures, we combined the SEC-MALS data with the SAXS data to be included in the main text.

**Fig. 6/Fig. S4:** I did not fully understand these experiments. Is the ATL1 ER distribution altered or the ER morphology in general when the phospho-mimetic mutants are added back? E.g. does ‘tubular’ and ‘punctate’ ATL localization correspond to ‘tubular ER’ and ‘fuzzy ER’ morphology?

We purposefully referred to ATL1 ER distribution since this is what we mainly looked at experimentally in Figure 6. However, since ATL1 is associated with the ER membrane, it can be indicative of ER morphology. It likely correlates with overall ER morphology, as is indicated in the experiments shown in Figure S4, which were conducted with U2OS cells expressing endogenous ATL isoforms. ‘Tubular’ or ‘fuzzy’ ATL localization correlates with ‘tubular’ and ‘fuzzy’ ER, but ‘punctate ATL1 localization is distinct from the former two. We clarified this in the text.

**Fig. 8:** Kinase reactions with substrate ‘ATL1 catalytic core’ - Please define the exact construct - is the HVR really part of the ‘ATL1 catalytic core’?

We clarified this point in the text. Thus far, we have referred to the catalytic core as constructs that start with the first residue of the respective isoforms. This nomenclature was introduced before we knew about the functional role of the HVR. Basic catalytic activity of full-length protein, catalytic core with or without HVR are comparable, not changing our argument for the nomenclature, which is largely based on catalytic activity.

**Line 58** - ‘Another widespread feature is a GTPase effector domain (GED) that functions in intramolecular regulation of GTPase activity (Chappie et al, 2009), however this domain is
absent in ATLs.’ The GED in dynamin corresponds to the C-terminal sequence stretch of the stalk and bundle signaling element (BSE). Atlastin has also a sequence stretch at this position, which is not included in the crystallized constructs, e.g. a similar role as the GED in dynamin cannot be excluded. Do the authors mean ‘BSE’ instead of GED? However, whether the middle domain of Atlastin corresponds to the BSE or stalk is also difficult to say.

We amended this section in the Introduction by removing the statement as it does not directly relate to the current study and to avoid confusion.

The Discussion is a bit lengthy and could be shortened. For example, there is not much evidence in the manuscript for a role of the HVR in the intrinsic regulation of atlastin’s GTPase activity or the prevention of futile GTPase cycles.

As suggested, we shortened the discussion.

Reviewer #2:

Major:

Comment on the G-domain dimer interfaces observed in the crystal structure of ATL1. The crystal packing of ATL1 appears to have the canonical G domain dimer interface seen in other dynamins and shown to stimulate GTPase hydrolysis. (It is stated that the ATL3 HVR conformation does not represent a physiologically relevant state and therefore may not have the canonical G domain interface.)

The structural observations in this comment are correct and echo our descriptions in the revised manuscript. While aspects of the conformation and oligomerization may have functional implications, we established earlier that this particular G domain interface may not be physiological relevant, at least not in the GDP-bound state that was crystallized (ATLs do not dimerize with GDP). Also, unlike dynamins, under the conditions tested we don’t observe robust stimulation of GTPase activity due to dimerization, which we documented previously. Finally, ATL3 used for crystallization lacks the middle domain, which could impact G domain conformation and dimerization.

The tethering assay (fig 2B) needs clarification. A description of what the graph represents over the 45 min time frame would be helpful. It is not clear what part of the graph is indicating tethering - the high signal at early timepoints (0-10 mins)? Also, why does the signal decrease and rise again?

We added more clarifying text to the figure legend.

Is there constant stirring during the 45 min assay to prevent sedimentation of large complexes?

There is no stirring as this could interfere with tethering itself. The initial rise is due to microscopic tethering. At longer time points we observe macroscopic tethering (as you indicate large complexes) in the wells, which causes the apparent erratic absorbance/scattering traces.

What cause the increase in signal for ATL1-delta-HRV and ATL3?
Based on this assay, we interpret the increase to signal still as tethering events, but with delayed kinetics/potency compared to the ATL1 protein with HVR. This interpretation is supported by the fact that ATL1-delta-HVR still dimerizes in a GTPase-dependent manner and that the reactions are reversible by EDTA addition (removing ATL from the vesicles).

*Indicate the time points that correspond to the following comments “tethering rate constants as a function of protein concentration indicate a robust tethering enhancement of wild-type ATL1” and “later time-points, wild-type ATL1 samples produced macroscopic, tethered clusters that were visually discernable and occurred when tether formation began to plateau causing high signal variation”.

The data that was included for the determination of apparent tethering rate constant is described in the Material and Methods section. As requested, we indicated the onset of macroscopic tethering in the amended figure.

*Considering alterations in tethering is the main observation in this manuscript, it is important to show in vitro tethering directly through electron or fluorescent microscopy (ideally cryoEM).

The turbidity assay is well accepted in the field (see, for example, reference Liu et al. 2015 in the manuscript) and is a direct assay for tethering. Important controls (i.e., reversibility by removing protein from the liposomes, dependence on GTP hydrolysis) were included in our study (see Figure S2B), indicating an ATL-specific function. While we see the suggested approaches as complementary to the one chosen here and agree they could potentially yield additional information, we feel these studies go beyond the scope of the current study.

*The S10A mutant had no effect on tethering or GTPase activity but did result in more puncta in the cell. This is briefly addressed in the discussion on line 549 but needs more clarification.

Unfortunately, at this point, we do not have a mechanistic explanation beyond the possible explanation provided in the discussion, that the dynamic modification of S10 is important for its function.

*Minor:
**The main result of the paper is an effect on tethering with delta-HRV and phosphomimetic mutants and thus tethering should be mentioned in the title.**

We feel the chosen title best reflects the scope of our study. Tethering is one of the functional read-outs we report on but may not be the only one affected by HVR deletion or phosphorylation of the HVR. Hence, we prefer to keep the more general title for this study.

*Figure 1C: add G domain is colored orange.*

This is already mentioned in the figure legend.

*Figure 2B: mention GTP is present in the figure legend.*

Done.

*Figure 2C: change in legend, (C) As in (C), correct it to as in (B).*

Done.
**Figure 2B and 2C:** shapes in graphs do not match legend icons. In 2B legend, the shape of ATL1ΔHVR is shown as square in light blue, but in graph it is a light blue triangle.

We addressed this in the revised manuscript.

**Line 534: Clarify what is meant by "and S22 and S23 existing only in the same phosphorylation state"?**

We clarified this statement in the revised manuscript.

**Explain why the tethering traces vary significantly between experiments, compare figure 2B to S3C.**

The variability could stem from small differences in the vesicle preparations, which include extrusion steps. Difference in extrusion efficiency and/or NiNTA-lipid presentation could affect the onset of the HVR-dependent component of the vesicle tethering reaction. Importantly, all experimental sets are internally controlled, each including background and wild-type controls, making a comparison possible.

**Figure 3 can be moved to supplemental, or reduced to one panel and the rest shown in supplemental.**

Given guidelines regarding figure and supplemental figure items allowed, we combined the SEC-MALS and SAXS in Figure 2 of the amended manuscript.

**Reviewer #3:**

**Major:**

A confirmation of the role of the HVR in tethering would be a duplication of the tethered structures (perhaps with DGS-NTA(Ni)-bearing lipids) seen by EM by Siani, 2014, and its predicted loss in the absence of the HVR.

We agree that a careful analysis of the tethering structures on liposomes could provide additional support for an effect of the HVR on liposome tethering. Considering that we still observe tethering but detect a change in kinetics and apparent vesicle-cluster size could be a technical challenge for this approach. The purpose of our current study was the elucidation of a functional role of the HVR. We envision a more detailed study testing several of the hypotheses we mention in the discussion in the future.

Do mutations in and around the groove on the back of the GTPase domain (around E202 in PDB 3Q5E), where the HVR is predicted to interact, have any effects in vitro or in the in vivo assays?

At this time, we do not know if mutations at the G-domain of the G:HVR interface affect ATL1 function similar to HVR mutations. We are planning to incorporate those experiments for a future study. In the meantime, we made sure to not overstate the potential relevance of the crystallographic interface and rather see it as a plausible model for higher-order organization.
The authors suggest the concentration on a membrane template may be required to see the effects of the presence or absence of ATL1 HVR. This, and given the small contact area, means the lack of effects on catalytic core kinetics in solution are not a surprise. What about on liposomes? Does the presence of the HVR have any effects on protein tethered to liposomes using the DGS-NTA(Ni) approach?

We tried to measure phosphate release kinetics of ATL1 on liposomes. Unfortunately, ATL1/GTPase-dependent liposome tethering interferes substantially with the readout of the enzymatic assay. We also attempted an end-point assay using malachite green, but the results were unconvincing so far.

The in vitro kinase assays show that several kinase domains can in principle phosphorylate the HVR in solution. In vitro tethering assays show that the S10E mutants have strong effect. The effects of the kinase mutants in vivo are, by contrast, conflicting and surprisingly mild. Might these mutants be more involved in isoform segregation in vivo?

Involvement of the HVR and its regulation by phosphorylation in isoform segregation is one of the models we discuss in the manuscript. It is a particularly appealing hypothesis we plan to test in the future.

Minor:

S2A. This gel is denaturing PAGE. What is the relatively abundant band at @70 kDa? And is this present in all the solution characterizations?

We propose that the abundant band at 70 kDa is an artifact of electrophoresis, though we cannot conclusively determine the source. The bands are not present in the input sample preparations and are oddly present in both ATL1 and ATL3 samples. This evidence suggests the Nycodenz density gradient may interfere with electrophoresis.
August 23, 2021

RE: JCB Manuscript #202104128R

Prof. Holger Sondermann  
Deutsches Elektronen-Synchrotron DESY  
Centre for Structural Systems Biology (CSSB)  
Notkestr. 85  
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Dear Prof. Sondermann,

Thank you for submitting your revised manuscript entitled "The hypervariable region of atlastin-1 is a site for intrinsic and extrinsic regulation." We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

In addition, we ask that you please further revise the legend of Figure 2 to address the referees' requests in full. Reviewers #1&2 requested to state the GTP concentration for panel 2B, this is still missing. There is also still not enough information about the meaning of these experiment as requested by Reviewer #2. Please expand the description to clearly state what the graphs represent, what part indicates tethering, and why the signal decreases and then increases again.

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.**

1) Text limits: Character count for Articles 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 may have up to 10 main text figures.

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. Please add MW marker to top blot in Fig.4B and scale bars for insets in Figures 5 & S4.

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 (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.). 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."

5) Abstract and title: The summary 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. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

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. 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.

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 reconstructions, 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 and 10 videos. 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. Please include one brief sentence per item.

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. It should begin with "First author name(s) et al..." to match our preferred style.
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) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

14) 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.

B. FINAL FILES:

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).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

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

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

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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,

James Hurley, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology
Dear Dr. Hurley,

We would like to thank you and the reviewers for the constructive and thoughtful feedback on our manuscript entitled “The hypervariable region of atlastin-1 is a site for intrinsic and extrinsic regulation”. We are also grateful for the opportunity to submit a revised manuscript. We hope you agree with us that we have addressed satisfactorily in the response below and our amended submission all points that were raised during the previous review. Thank you for considering this revised manuscript for publication in the Journal of Cell Biology. We are looking forward to hearing from you.

You will see that all reviewers are very enthusiastic about your study and feel it provides important new insights into atlastin-mediated membrane tethering. The major experimental requests are to confirm the role of ATL1-HVR in tethering either by EM or fluorescence microscopy and to examine whether mutations in the GTPase domain near the HVR contact site affect tethering. While these are certainly interesting questions we don’t believe that these are essential for revision. However, if you have data in hand or can do the experiments to address these points in a reasonable timeframe, we encourage you to do so as it would substantially enhance the paper. We don’t believe that additional work on the kinases that phosphorylate ATL1 is necessary. Reviewers also have several other points which we believe can be addressed by text and figure revisions.

Thank you for sharing your enthusiasm regarding our study with us. We agree with the reviewers that the novel insight into the regulation of ATL warrants an in-depth characterization of the newly described protein-interaction interface and further studies into the role of this interaction for membrane tethering. We are planning to conduct the suggested experiments in the future. Our main goal with the present study was establishing a role of the HVR for ATL function, which led to the discovery of intrinsic and extrinsic regulatory mechanisms that can be clearly tied to the HVR and its posttranslational modification, respectively. Given the scope and extent of our current submission, we feel the suggested studies deserve a separate, in-depth characterization, allowing us to test several hypotheses and models that we mention in our Discussion. Hence, we thank you for providing us with the choice to conduct the suggested experiments in the context of future studies.
Reviewer #1:

...The manuscript is rich in new structural and mechanistic insights. Experiments appear sound, reliable and of high quality. The topic is exciting since the mechanism of ATL-mediated membrane tethering and fusion is still not fully resolved. The described involvement of the HVR adds interesting aspects of the tethering reaction and provides a rationale how ER fusion events may be regulated. Having said this, an important aspect of the study is currently not conclusive and should be still addressed.

We thank this Reviewer for acknowledging the novel aspects of our study and for the constructive feedback.

Major:

Fig. 1D-E: The observed crystal contacts of the HVR in ATL1 are at the heart of this manuscript since they provide a structural explanation of how the HVR contributes to membrane tethering. However, this contact is not systematically followed up.

In the current manuscript, we investigated the functional implications of the N-terminal HVR in ATLs on a broad scale, providing the first evidence for their role in membrane tethering. These studies were inspired by the crystallographic data that provides a model for how the HVR could impact ATL1-mediated membrane tethering. We agree with the reviewers that the structural models and associated hypotheses require further validation and testing. We consider these as the logical next steps for future mechanistic studies. Our analysis and main findings presented in the manuscript do not hinge on the validation of the crystallographic interface, while the crystal packing provides the basis for some of the discussed models awaiting assessment.

(1) The authors should show a detailed figure of the ATL1-HVR contacts with the neighboring ATL molecule.

We included such a figure and a brief description of the protein interface in the revised manuscript (Figure S1 and figure legend).

(2) Key residues in the interface should be mutated, e.g. both in the HVR and in the adjacent G domain. Subsequently, the authors should analyze whether the mutants are still able to tether membranes.

We are planning to conduct an in-depth mechanistic study in the future, including a mutational analysis of the crystallographic interface. For the current study, we feel that the HVR deletion mutant and the regulation by phosphorylation clearly establish a functional role of the HVR in membrane tethering – the main focus of our manuscript.

Without successfully demonstrating the importance of this contact, the mechanism of the HVR in atlastin-membrane tethering remains vague.

A detailed mechanistic follow-up should definitively include a mutational analysis of the HVR-G domain interface of a putative ATL1 oligomer, as it could validate the involvement of the specific interface in the tethering reaction.

Minor:
**Fig. 1:** Please show the intermolecular contacts of the ATL3 HVR in an additional SI figure.

We included the figure requested in the revised manuscript (Figure S1B).

**Fig. 1E:** Do atlastin G domains in the crystals interact via the G-interface? If yes, please mention and show the nucleotide in both G domains.

The G-domain dimer recognized in earlier structures (Byrnes and Sondermann, 2011, DOI: 10.1073/pnas.1012792108; Bian et al., 2011, DOI: 10.1073/pnas.1101643108) is present also in the structure of ATL1 presented here. This aspect is illustrated in Figure 1E (and now mentioned in the main text). We also included a Figure panel showing the nucleotide density in Figure S1 of the amended manuscript.

**Fig. 2B:** Add in the legend that this experiment was carried out in the presence of 500 µM GTP.

We made the requested change.

**Fig. 2E:** What is shown here? A view into a reaction tube? What is one supposed to see?

We added clarification to the figure legends.

**Fig. 3:** These experiments could go to the SI.

To comply with JCB’s number of allowed supplementary figures, we combined the SEC-MALS data with the SAXS data to be included in the main text.

**Fig. 6/Fig. S4:** I did not fully understand these experiments. Is the ATL1 ER distribution altered or the ER morphology in general when the phospho-mimetic mutants are added back? E.g. does ‘tubular’ and ‘punctate’ ATL localization correspond to ‘tubular ER’ and ‘fuzzy ER’ morphology?

We purposefully referred to ATL1 ER distribution since this is what we mainly looked at experimentally in Figure 6. However, since ATL1 is associated with the ER membrane, it can be indicative of ER morphology. It likely correlates with overall ER morphology, as is indicated in the experiments shown in Figure S4, which were conducted with U2OS cells expressing endogenous ATL isoforms. ‘Tubular’ or ‘fuzzy’ ATL localization correlates with ‘tubular’ and ‘fuzzy’ ER, but ‘punctate ATL1 localization is distinct from the former two. We clarified this in the text.

**Fig. 8:** Kinase reactions with substrate ‘ATL1 catalytic core’ - Please define the exact construct - is the HVR really part of the ‘ATL1 catalytic core’?

We clarified this point in the text. Thus far, we have referred to the catalytic core as constructs that start with the first residue of the respective isoforms. This nomenclature was introduced before we knew about the functional role of the HVR. Basic catalytic activity of full-length protein, catalytic core with or without HVR are comparable, not changing our argument for the nomenclature, which is largely based on catalytic activity.

**Line 58 - ‘Another widespread feature is a GTPase effector domain (GED) that functions in intramolecular regulation of GTPase activity (Chappie et al, 2009), however this domain is**
absent in ATLs.' The GED in dynamin corresponds to the C-terminal sequence stretch of the stalk and bundle signaling element (BSE). Atlastin has also a sequence stretch at this position, which is not included in the crystallized constructs, e.g. a similar role as the GED in dynamin cannot be excluded. Do the authors mean 'BSE' instead of GED? However, whether the middle domain of Atlastin corresponds to the BSE or stalk is also difficult to say.

We amended this section in the Introduction by removing the statement as it does not directly relate to the current study and to avoid confusion.

The Discussion is a bit lengthy and could be shortened. For example, there is not much evidence in the manuscript for a role of the HVR in the intrinsic regulation of atlastin’s GTPase activity or the prevention of futile GTPase cycles.

As suggested, we shortened the discussion.

Reviewer #2:
Major:
Comment on the G-domain dimer interfaces observed in the crystal structure of ATL1. The crystal packing of ATL1 appears to have the canonical G domain dimer interface seen in other dynamins and shown to stimulate GTPase hydrolysis. (It is stated that the ATL3 HVR conformation does not represent a physiologically relevant state and therefore may not have the canonical G domain interface.)

The structural observations in this comment are correct and echo our descriptions in the revised manuscript. While aspects of the conformation and oligomerization may have functional implications, we established earlier that this particular G domain interface may not be physiological relevant, at least not in the GDP-bound state that was crystallized (ATLs do not dimerize with GDP). Also, unlike dynamins, under the conditions tested we don’t observe robust stimulation of GTPase activity due to dimerization, which we documented previously. Finally, ATL3 used for crystallization lacks the middle domain, which could impact G domain conformation and dimerization.

The tethering assay (fig 2B) needs clarification. A description of what the graph represents over the 45 min time frame would be helpful. It is not clear what part of the graph is indicating tethering - the high signal at early timepoints (0-10 mins)? Also, why does the signal decrease and rise again?

We added more clarifying text to the figure legend.

Is there constant stirring during the 45 min assay to prevent sedimentation of large complexes?

There is no stirring as this could interfere with tethering itself. The initial rise is due to microscopic tethering. At longer time points we observe macroscopic tethering (as you indicate large complexes) in the wells, which causes the apparent erratic absorbance/scattering traces.

What cause the increase in signal for ATL1-delta-HRV and ATL3?
Based on this assay, we interpret the increase to signal still as tethering events, but with delayed kinetics/potency compared to the ATL1 protein with HVR. This interpretation is supported by the fact that ATL1-delta-HVR still dimerizes in a GTPase-dependent manner and that the reactions are reversible by EDTA addition (removing ATL from the vesicles).

**Indicate the time points that correspond to the following comments** "tethering rate constants as a function of protein concentration indicate a robust tethering enhancement of wild-type ATL1" and "later time-points, wild-type ATL1 samples produced macroscopic, tethered clusters that were visually discernable and occurred when tether formation began to plateau causing high signal variation".

The data that was included for the determination of apparent tethering rate constant is described in the Material and Methods section. As requested, we indicated the onset of macroscopic tethering in the amended figure.

**Considering alterations in tethering is the main observation in this manuscript, it is important to show in vitro tethering directly through electron or fluorescent microscopy (idealy cryoEM).**

The turbidity assay is well accepted in the field (see, for example, reference Liu et al. 2015 in the manuscript) and is a direct assay for tethering. Important controls (i.e., reversibility by removing protein from the liposomes, dependence on GTP hydrolysis) were included in our study (see Figure S2B), indicating an ATL-specific function. While we see the suggested approaches as complementary to the one chosen here and agree they could potentially yield additional information, we feel these studies go beyond the scope of the current study.

**The S10A mutant had no effect on tethering or GTPase activity but did result in more puncta in the cell. This is briefly addressed in the discussion on line 549 but needs more clarification.**

Unfortunately, at this point, we do not have a mechanistic explanation beyond the possible explanation provided in the discussion, that the dynamic modification of S10 is important for its function.

**Minor:**

**The main result of the paper is an effect on tethering with delta-HRV and phosphomimetic mutants and thus tethering should be mentioned in the title.**

We feel the chosen title best reflects the scope of our study. Tethering is one of the functional read-outs we report on but may not be the only one affected by HVR deletion or phosphorylation of the HVR. Hence, we prefer to keep the more general title for this study.

**Figure 1C: add G domain is colored orange.**

This is already mentioned in the figure legend.

**Figure 2B: mention GTP is present in the figure legend.**

Done.

**Figure 2C: change in legend, (C) As in (C), correct it to as in (B).**

Done.
Figure 2B and 2C: shapes in graphs do not match legend icons. In 2B legend, the shape of ATL1∆HVR is shown as square in light blue, but in graph it is a light blue triangle.
We addressed this in the revised manuscript.

Line 534: Clarify what is meant by "and S22 and S23 existing only in the same phosphorylation state"?
We clarified this statement in the revised manuscript.

Explain why the tethering traces vary significantly between experiments, compare figure 2B to S3C.
The variability could stem from small differences in the vesicle preparations, which include extrusion steps. Difference in extrusion efficiency and/or NiNTA-lipid presentation could affect the onset of the HVR-dependent component of the vesicle tethering reaction. Importantly, all experimental sets are internally controlled, each including background and wild-type controls, making a comparison possible.

Figure 3 can be moved to supplemental, or reduced to one panel and the rest shown in supplemental.
Given guidelines regarding figure and supplemental figure items allowed, we combined the SEC-MALS and SAXS in Figure 2 of the amended manuscript.

Reviewer #3:
Major:
A confirmation of the role of the HVR in tethering would be a duplication of the tethered structures (perhaps with DGS-NTA(Ni)-bearing lipids) seen by EM by Siani, 2014, and its predicted loss in the absence of the HVR.
We agree that a careful analysis of the tethering structures on liposomes could provide additional support for an effect of the HVR on liposome tethering. Considering that we still observe tethering but detect a change in kinetics and apparent vesicle-cluster size could be a technical challenge for this approach. The purpose of our current study was the elucidation of a functional role of the HVR. We envision a more detailed study testing several of the hypotheses we mention in the discussion in the future.

Do mutations in and around the groove on the back of the GTPase domain (around E202 in PDB 3Q5E), where the HVR is predicted to interact, have any effects in vitro or in the in vivo assays?
At this time, we do not know if mutations at the G-domain of the G:HVR interface affect ATL1 function similar to HVR mutations. We are planning to incorporate those experiments for a future study. In the meantime, we made sure to not overstate the potential relevance of the crystallographic interface and rather see it as a plausible model for higher-order organization.
The authors suggest the concentration on a membrane template may be required to see the effects of the presence or absence of ATL1 HVR. This, and given the small contact area, means the lack of effects on catalytic core kinetics in solution are not a surprise. What about on liposomes? Does the presence of the HVR have any effects on protein tethered to liposomes using the DGS-NTA(Ni) approach?

We tried to measure phosphate release kinetics of ATL1 on liposomes. Unfortunately, ATL1/GTPase-dependent liposome tethering interferes substantially with the readout of the enzymatic assay. We also attempted an end-point assay using malachite green, but the results were unconclusive so far.

The in vitro kinase assays show that several kinase domains can in principle phosphorylate the HVR in solution. In vitro tethering assays show that the S10E mutants have strong effect. The effects of the kinase mutants in vivo are, by contrast, conflicting and surprisingly mild. Might these mutants be more involved in isoform segregation in vivo?

Involvement of the HVR and its regulation by phosphorylation in isoform segregation is one of the models we discuss in the manuscript. It is a particularly appealing hypothesis we plan to test in the future.

**Minor:**

*S2A. This gel is denaturing PAGE. What is the relatively abundant band at @70 kDa? And is this present in all the solution characterizations?*

We propose that the abundant band at 70 kDa is an artifact of electrophoresis, though we cannot conclusively determine the source. The bands are not present in the input sample preparations and are oddly present in both ATL1 and ATL3 samples. This evidence suggests the Nycodenz density gradient may interfere with electrophoresis.