EMBO
reports

Manuscript EMBOR-2013-37108

The myotubularin-amphiphysin 2 complex in membrane tubulation and centronuclear myopathies.

Barbara Royer, Karim Hnia, Christos Gavriilidis, Helene Tronchere, Valerie Tosch and Jocelyn Laporte

Corresponding author: Jocelyn Laporte, IGBMC

Review timeline:	Submission date:	25 January 2013
	Editorial Decision:	01 March 2013
	Revision received:	04 June 2013
	Correspondence:	20 June 2013
	Correspondence:	27 June 2013
	Correspondence:	28 June 2013
	Correspondence:	17 July 2013
	Accepted:	18 July 2013

Editor: Barbara Pauly

Transaction Report:

(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.)

1st Editorial Decision 01 March 2013

Thank you very much for the submission of your research manuscript to our editorial office. Please accept my apologies for the unusual delay in the review process. We have just now received the full set of reviews on your manuscript.

You will see that all reviewers appreciate the interest of your findings and support publication of your study in our journal. However, they also point out aspects of your study that would need to be further strengthened to make the claims fully conclusive. For instance, referee 1 feels that the contribution of the individual domains of BIN1 to MTM1 binding need to be further clarified and referee 3 also comments on this point. Along these lines, referee 2 states that a more detailed analysis of the BIN1/MTM1 interaction and the consequences of this interaction on BIN1-mediated tubulation would strengthen the study. Reviewer 1 points out that the analysis of the requirement for MTM1's catalytic activity on tubulation should be strengthened and referee 2 also makes a statement along these lines. Finally, referee 3 feels that the co-localization data of BIN1 and MTM1 would benefit from further strengthening and all referees also point out some minor technical/experimental issues that would need to be fixed.

© European Molecular Biology Organization

Given these positive evaluations, the reviewers constructive comments and the potential interest of the study, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the reviewers should be addressed. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. If you feel that this period is insufficient for a successful submission of your revised manuscript I can potentially extend this period slightly. Also, the length of the revised manuscript should not exceed roughly 29,000 characters (including spaces and references). If you feel that the additional data requested by the reviewers would make the manuscript too long you may consider including some peripheral data in the form of Supplementary information. However, materials and methods essential for the repetition of the key experiments should be described in the main body of the text and may not be displayed as supplemental information only.

When submitting your revised manuscript, please include:
• a word-formatted version of the manuscript text
• editable, high-resolution TIFF or EPS-formatted or figure files
• a separate single PDF file of all the Supplementary information (in its final format)
• a letter detailing your responses to the referee comments (as word file if possible)
• a short, two-sentence summary of the manuscript

We have also started encouraging authors to submit the raw data of biochemical and/or microscopical images to our editorial office. These data will be published online as part of the supplementary information. This is voluntary at the moment, but if you agree that this would be useful for readers I would like to invite you to supply these files when submitting the revised version of your study.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

We also welcome the submission of cover suggestions or motifs that might be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

With kind regards and best wishes for a nice weekend

REFEREE REPORTS:

Referee #1:

In this manuscript, Royer et al characterize a novel interaction between Myotubularin (MTM1) and amphiphysin 2 (BIN1). They went on to show that MTM1 enhances BIN1-mediated membrane tubulation. Interestingly, MTM1 and BIN1 are two proteins mutated in different forms of centronuclear myopathy (CNM) and BIN1 mutations found in CNM patients alter its binding and

regulation by MTM1.
This is a very intriguing manuscript supporting a common pathological mechanism in different forms of CNM, nevertheless additional experiments are required to set the conclusions on firmer ground.

Major points.

Figure 1B indicates that the BAR or the SH3 domains of BIN1 are sufficient to interact with MTM1. Nevertheless, when the BAR and SH3 domains are together (construct called delta PI in Figure) no interaction is detected. This is a very surprising result that must be explained.

While the IF staining clearly indicates that BIN1 and MTM1 are present on the triad of skeletal muscle, only marginal co-localization is seen on merged images of Figure 1E and Figure S1B-C. This result suggests that only a small fraction of BIN1 and MTM1 interact on the triad. Similarly, a very limited BIN1/MTM1 co-localization is shown in Figure 2. This discrepancy must be discussed.

I'm not convinced that the results of Figures 2 and S2 indicate that the catalytic activity of MTM1 is required to regulate BIN1-mediated membrane tubulation. For example, authors cannot exclude the possibility that the MTM1 mutations impair MTM1 ability to interact with BIN1. This important control must be performed.

Input for GST proteins must be shown in Figure 5B-C.

Minor points.

Legend to Figure 1, panels E and F has been swapped.

For non-experts, it would be useful to include a description of the different fractions used in subcellular fractionation of Figure 1 (how have the fractions be obtained? what do they contain? what the letter indicating them stands for?).

Referee #2:

Here Laporte and colleagues continue their examination of the regulation and function of myotubularins. The manuscript specifically defines the interaction between MTM1 and amphiphysin 2 (BIN1) in the biology and pathology of centronuclear myopathy. This is clearly an interesting study. However, further supporting data is required to validate some of the stated conclusions. Without this detailed analysis the study remains mostly observational.

Major points:
Through out the manuscript there is a need to be more clear with the details of the analysis that was performed to define the impact of MTM1 on BIN1 trigger tubulation. For example, number of tubules analysed, in what number of cells etc. In legend to Fig. 2 what precisely is 'tubules were considered long when they were more that twice longer than large".

The interaction between BIN1 and MTM1 needs to be mapped in more detail. Through the subsequent generation of interacting deficient mutants the importance of the interaction for tubulation will be more definitively defined. How important is the localised activity of MTM1 for the effect on BIN1 tubulation. If a PI3P/PI3,5P2 phosphatase that does not associate with BIN1 is overexpressed does this affect tubulation? The idea of a localised microdomain mediated regulation of the phosphoinositide environment is an important aspects of the manuscript.

The enzymology of the lack of effect of BIN1 on MTM1 phosphatase activity requires more robust quantification. Similarly, do the experiments in Fig. 5 allow one to conclude that the affinity of any interaction is not perturbed? This needs to be more robustly quantified.

Minor points:
Material and Methods: co-ip assay "300Mm" ?

Referee #3:

The manuscript from Royer et al. presents an thorough molecular analysis of Bin1, a relatively little understood skeletal muscle protein that has been implicated in an complex human disease, central nuclear myopathy. Bin1 is very modular with a BAR domain, which mediates membrane interactions and membrane curvatures and a structurally unique SH3 domain, which is not as well defined (in terms of protein-protein interactions and biologic function). The manuscript focuses on the unique activity imparted by the SH3 domain and the data strongly suggests that there is a relevant functional interaction between MTM1 and Bin1. Collectively the experiments form a convincing argument in establishing this protein interaction, which has bearing on the molecular pathology that derives from central nuclear myopathy.

Despite the general enthusiasm for the manuscript, there is are a number of concerns that need to be addressed prior to acceptance for publication.

1. The data in figure 1 shows that the Bin1 SH3 domain can interact with MTM1, yet the interaction is noticeably reduced compared to full length Bin1. This raises the issue as to the significance of the SH3 interaction in the absence of the BAR domain. What is puzzling in discussing prominent SH3 interactions for Bin1, there is no mention of the reported interactions between the Bin1 SH3 domain and actin/myosin (as reported in Fernando et al. 2009). To be fair, this paper makes use of SH3 over-expression models, which may be difficult to interpret in a physiologic context, yet the authors of this manuscript present compelling biochemical data to demonstrate that the Bin1 SH3 domain mediates a functional interaction with actin and myosin. Moreover, a transgenic over-expression of the SH3 domain leads to measurable disruptions in sarcomeric structure. As such, the author should discuss their observations in light of this prior paper.
2. Regarding the issue raised above, Figure 1E needs further clarification. The authors imply that this immuno-localization of Bin1 and MTM1 has a Triad/t-tuble like distribution. However, the low magnification images indicate a sarcomeric distribution as well. Confocal imaging should be utilized to limit confusion and/or misinterpretation here. Perhaps the authors should think of co-staining with other markers to confirm the exact sub-cellular location of MTM1 (and what happens to MTM1 localization when the mutant Bin1 SH3 domain protein (from Figure 5) is over-expressed).
3. The images in Figure 3 confirm the concerns noted above. For example, Bin1 distribution in the Wt scenario has a very sarcomeric orientation, yet the authors claim a Triad exclusive distribution. Again, these observations need to be discussed in light of Fernando et al. 2009.

1st Revision - authors' response 04 June 2013

Manuscript EMBOR-2013-37108V1: Answer to the reviewers

Thank you for conveying the reviews on our manuscript entitled **"The myotubularin-amphiphysin 2 complex in membrane tubulation and centronuclear myopathies".** We have carefully read the suggestions of the reviewers, who we thank for their insightful and constructive comments. We have taken the time to conduct several critical additional experiments requested by the reviewers, as well as critically reevaluate some of our data, and are happy that the study is much improved. We hope you will now find it suitable for publication in *EMBO reports*. Below are our point-by point responses to each of the reviewer's comments. Thank you again for consideration of this work in *EMBO reports* and we are looking forward to the final decision on manuscript.
To summarize, our work documents a novel functional relationship between myotubularin (MTM1) and amphiphysin 2 (BIN1), two proteins mutated in different forms of centronuclear myopathy. We identified MTM1 as a binding

partner of BIN1 and as a regulator of BIN1-mediated membrane tubulation, supporting a common pathway in these forms of centronuclear myopathy.

Referee# 1:

In this manuscript, Royer et al characterize a novel interaction between Myotubularin (MTM1) and amphiphysin 2 (BIN1). They went on to show that MTM1 enhances BIN1-mediated membrane tubulation. Interestingly, MTM1 and BIN1 are two proteins mutated in different forms of centronuclear myopathy (CNM) and BIN1 mutations found in CNM patients alter its binding and regulation by MTM1.
This is a very intriguing manuscript supporting a common pathological mechanism in different forms of CNM, nevertheless additional experiments are required to set the conclusions on firmer ground.

Major points

Figure 1B indicates that the BAR or the SH3 domains of BIN1 are sufficient to interact with MTM1. Nevertheless, when the BAR and SH3 domains are together (construct called delta PI in Figure) no interaction is detected. This is a very surprising result that must be explained.

Answer of the authors:. We showed in Figure 1B that MTM1 interacts more efficiently with the BAR and SH3 domains alone than with the wild-type full length BIN1 protein containing the BAR, PI and SH3 domains, suggesting an involvement of the conformation of BIN1 in the interaction with MTM1. We did not detect an interaction between MTM1 and the BIN1 delta PI construct (that contains the BAR and SH3 domains but depleted from PI motif) under similar experimental conditions. This can be explained by a differential conformational state between the two isoforms with or without the PI motif. Indeed, the PI motif is localised between the BAR and the SH3 domains and could modulate the relative position of the two domains within the protein. As MTM1 is interacting both with the BAR domain and the SH3 domain of BIN1, the correct relative position of the two domains could be critical for the binding of MTM1. In addition, as BIN1 undergoes complex tissuespecific splicing events, we hypothesize that inclusion of the PI motif is a physiological determinant of BIN1 conformation. The fact that the PI motif is mainly found in skeletal muscle isoforms of BIN1 (Butler et al., J Cell Biol 1997; Ramjaun et al., J Neurochem 1998; Wechsler-Reya et al., Mol Cell Biol 1998; Toussaint *et al.*, Acta Neuropathol, 2011) supports the hypothesis that the precise regulation of BIN1 conformation and interactions are especially important in this tissue. This point is now included in the text (part "MTM1 and BIN1 interact both in vitro and endogenously in skeletal muscle").

While the IF staining clearly indicates that BIN1 and MTM1 are present on the triad of skeletal muscle, only marginal co-localization is seen on merged images of Figure 1E and Figure S1B-C. This result suggests that only a small fraction of BIN1 and MTM1 interact on the triad. Similarly, a very limited BIN1/MTM1 co-localization is shown in Figure 2. This discrepancy must be discussed.

Answer of the authors: We agree that BIN1 and MTM1 only partially colocalize. Nevertheless, the confocal images presented in Figure 1E show that the highest signal detected for MTM1 and BIN1 colocalize and that these two proteins display a similar pattern. In order to strengthen our data we added on the revised Figure 1E quantitative measurement of MTM1 and BIN1 signals (intensity plots) on both transversal and longitudinal orientations, showing that the maximal signal intensity of both proteins mostly colocalize. Moreover, according to co-staining with triad makers and to muscle subcellular fractionation (Figure 1 and S2), we showed that BIN1 and MTM1 are both present at the triads. We now clarify in the text (part "MTM1 and BIN1 interact both in vitro and endogenously in skeletal muscle") that a fraction of MTM1 and BIN1 is in complex, and propose this interaction occurs mainly

at the triads based on imaging and fractionation experiments.

I'm not convinced that the results of Figures 2 and S2 indicate that the catalytic activity of MTM1 is required to regulate BIN1-mediated membrane tubulation. For example, authors cannot exclude the possibility that the MTM1 mutations impair MTM1 ability to interact with BIN1. This important control must be performed.

Answer of the authors: We appreciate this suggestion that helped us to further complete our data. Additionnal experiments have been included to clarify the impact of the binding vs the catalytic activity of MTM1 on the enhancement of BIN1 tubulation.
First, we showed that MTM1 catalytically inactive mutants are not able to enhance BIN1 tubulation, contrary to MTM1 wild type, but we did not exclude that MTM1 mutations can potentially impair the BIN1 membrane tubulation by altering the binding of MTM1 to BIN1. We thus performed pull down experiments that show that, while the p.C375S MTM1 is not able to bind the BAR domain of BIN1 under our experimental conditions, other catalytically inactive mutants p.D278A and R421Q are still interacting with the BAR domain of BIN1, at the same level as MTM1 wild type (displayed in Figure S3). This points further to the importance of the phosphatase activity. We comment in the text that the inability of the p.C375S MTM1 mutant to fully rescue the *Mtm1*-null mice phenotype (Amoasii et al. PLoS Genet 2012) could be due either to lack of enzymatic activity or decreased BIN1 binding.
Then, we tested additional myotubularins, that have similar catalytic activity and substrate specificity as MTM1 (the closest homologs MTMR1 and MTMR2) or that are catalytically inactive (MTMR10 and MTMR11), for their interaction with BIN1 and for their impact on BIN1-mediated tubulation. None of these myotubularins (active or inactive) could interact with BIN1 or affect tubulation (displayed in Figure 2C and novel Figure S4). These data suggest that activity alone without binding is not enough regulate the membrane remodeling properties of BIN1. We cannot however excluded that MTMR1 and MTMR2 are dephosphorylating different phosphoinositides subpools than MTM1.
Last, we tested MTM1 domains (C-terminal, GRAM and ΔGRAM constructs) for their interaction with BIN1 and their impact on BIN1-mediated tubulation. We found that the phosphatase domain lying between the GRAM and C-terminal region is the binding domain of BIN1 (displayed in novel Figure S5B). The ΔGRAM construct binds to BIN1, is catalytically inactive, and has no significant impact on BIN1-mediated membrane tubulation (displayed in Figure 2D and novel Figure S5), suggesting activity is the most important regulation.
Taken together, these results sustain the conclusion that MTM1 specifically enhances BIN1 membrane tubulation and that phosphatase activity and to a lesser extent binding are needed for enhancing BIN1 membrane tubulation. These results are now commented in the text in the paragraph "MTM1 enhances BIN1-mediated membrane tubulation in cells through binding and phosphatase activity".
Among the different MTM1 mutants available in our lab or in the literature, we were not able to identify one MTM1 mutant that would decrease BIN1 binding but retains a normal enzymatic activity.

Input for GST proteins must be shown in Figure 5B-C.

Answer of the authors: We apologize for this omission and have included the GST proteins input in Figure 5B and C.

Minor points.

Legend to Figure 1, panels E and F has been swapped.

Answer of the authors: We apologize for this mistake. The legends is corrected.

For non-experts, it would be useful to include a description of the different fractions

used in subcellular fractionation of Figure 1 (how have the fractions be obtained? what do they contain? what the letter indicating them stands for?).

Answer of the authors: We have now included these informations in the supplementary information and in the legend of Figure 1.

Referee #2:

Here Laporte and colleagues continue their examination of the regulation and function of myotubularins. The manuscript specifically defines the interaction between MTM1 and amphiphysin 2 (BIN1) in the biology and pathology of centronuclear myopathy. This is clearly an interesting study. However, further supporting data is required to validate some of the stated conclusions. Without this detailed analysis the study remains mostly observational.

Major points:

Through out the manuscript there is a need to be more clear with the details of the analysis that was performed to define the impact of MTM1 on BIN1 trigger tubulation. For example, number of tubules analysed, in what number of cells etc. In legend to Fig. 2 what precisely is 'tubules were considered long when they were more that twice longer than large".

Answer of the authors: In novel Figure 2A-B, the number of cells that express the transfected constructs and that present tubules have been counted, but not the number of tubules. An explanation is included in the section "Material and methods". In order to clarify what is considered as short tubule and as long tubule, images were included in the novel Figure S3B. In additional experiments we performed on COS cells, where BIN1 membrane tubules are usually longer and can be measured more easily than in C2C12 cells, we measured membrane tubule length using imageJ software and a fixed length was established for what we consider short (0.5 to 1 µm) or long tubule (5 to 15 µm). These data are now included in the corresponding Figures 2C-D, S4 and S5, referenced in the main text and explained in the section "Material and methods".

The interaction between BIN1 and MTM1 needs to be mapped in more detail. Through the subsequent generation of interacting deficient mutants the importance of the interaction for tubulation will be more definitively defined. How important is the localised activity of MTM1 for the effect on BIN1 tubulation. If a PI3P/PI3,5P2 phosphatase that does not associate with BIN1 is overexpressed does this affect tubulation? The idea of a localised microdomain mediated regulation of the phosphoinositide environment is an important aspects of the manuscript.

Answer of the authors: We appreciate this suggestion that helped us to further complete our data.
We previously showed that both the BAR and SH3 domain of BIN1 bind to MTM1 and that differential conformational state of full-length BIN1 regulate this binding (Figure 1). The domain of interaction of MTM1 with BIN1 has now been mapped more precisely. The deletion of the GRAM domain does not affect the interaction of BIN1, and the C-terminal domain alone binds BIN1 very weakly, showing that the phosphatase domain lying between the GRAM and C-terminal domains triggers the interaction of MTM1 with BIN1. This phosphatase domain is globular based on the structure of the closest homolog MTMR2 (Begley et al., Mol Cell 2003) and cannot be investigated further with deletion mutants that will impact on the general structure, while alanine scaning of the surface of this 380 aminoacid domain was not envisaged. These data have been included in Figure S5B and referenced in the main text.

Additionnal experiments have also been included to clarify the impact of the binding vs the catalytic activity of MTM1 on the enhancement of BIN1 tubulation.
First, we showed that MTM1 catalytically inactive mutants are not able to enhance BIN1 tubulation, contrary to MTM1 wild type, but we did not exclude that MTM1 mutations can potentially impair the BIN1 membrane tubulation by altering the binding of MTM1 to BIN1. We thus performed pull down experiments that show that, while the p.C375S MTM1 is not able to bind the BAR domain of BIN1 under our experimental conditions, other catalytically inactive mutants p.D278A and R421Q are still interacting with the BAR domain of BIN1, at the same level as MTM1 wild type (displayed in Figure S3). This points further to the importance of the phosphatase activity. We comment in the text that the inability of the p.C375S MTM1 mutant to fully rescue the *Mtm1*-null mice phenotype (Amoasii et al. PLoS Genet 2012) could be due either to lack of enzymatic activity or decreased BIN1 binding.
Then, we tested additional myotubularins, that have similar catalytic activity and substrate specificity as MTM1 (the closest homologs MTMR1 and MTMR2) or that are catalytically inactive (MTMR10 and MTMR11), for their interaction with BIN1 and for their impact on BIN1-mediated tubulation. None of these myotubularins (active or inactive) could interact with BIN1 or affect tubulation (displayed in Figure 2C and novel Figure S4). These data suggest that activity alone without binding is not enough regulate the membrane remodeling properties of BIN1. We cannot however excluded that MTMR1 and MTMR2 are dephosphorylating different phosphoinositides subpools than MTM1.

Last, we tested MTM1 domains (C-terminal, GRAM and ΔGRAM constructs) for their impact on BIN1-mediated tubulation. (displayed in novel Figure S5B). The binding domain to BIN1 ΔGRAM construct binds to BIN1, is catalytically inactive, and has no significant impact on BIN1-mediated membrane tubulation (displayed in Figure 2D and novel Figure S5), suggesting activity is the most important regulation.
Taken together, these results sustain the conclusion that MTM1 specifically enhances BIN1 membrane tubulation and that phosphatase activity and to a lesser extent binding are needed for enhancing BIN1 membrane tubulation. These results are now commented in the text in the paragraph "MTM1 enhances BIN1-mediated membrane tubulation in cells through binding and phosphatase activity".

The enzymology of the lack of effect of BIN1 on MTM1 phosphatase activity requires more robust quantification. Similarly, do the experiments in Fig. 5 allow one to conclude that the affinity of any interaction is not perturbed? This needs to be more robustly quantified.

Answer of the authors: In order to complement the results obtained from our in vitro lipid phosphatase assay, we performed independent experiments using a second technique, the malachite green assay, based on the protocol published by Taylor and Dixon in Methods in Enzymology 2003. We first determined the saturating concentration of MTM1 for optimal activity, then tested a wide range of BIN1 concentration. This confirmed the lack of effect of BIN1 on MTM1 phophatase activity. In parallel, a co-IP performed after the malachite green assay confirmed that BIN1 and MTM1 interact in the condition of the experiments. These novel data have been included as Figure 4B and S6B and referenced in the main text.

Minor points:

Material and Methods: co-ip assay "300Mm" ?

Answer of the authors: This mistake has been corrected

Referee #3:

The manuscript from Royer et al. presents an thorough molecular analysis of Bin1, a relatively little understood skeletal muscle protein that has been implicated in an complex human disease, central nuclear myopathy. Bin1 is very modular with a BAR domain, which mediates membrane interactions and membrane curvatures and a structurally unique SH3 domain, which is not as well defined (in terms of proteinprotein interactions and biologic function). The manuscript focuses on the unique activity imparted by the SH3 domain and the data strongly suggests that there is a relevant functional interaction between MTM1 and Bin1. Collectively the experiments form a convincing argument in establishing this protein interaction, which has bearing on the molecular pathology that derives from central nuclear myopathy. Despite the general enthusiasm for the manuscript, there is are a number of concerns that need to be addressed prior to acceptance for publication.

1. The data in figure 1 shows that the Bin1 SH3 domain can interact with MTM1, yet the interaction is noticeably reduced compared to full length Bin1. This raises the issue as to the significance of the SH3 interaction in the absence of the BAR domain. What is puzzling in discussing prominent SH3 interactions for Bin1, there is no mention of the reported interactions between the Bin1 SH3 domain and actin/myosin (as reported in Fernando et al. 2009). To be fair, this paper makes use of SH3 overexpression models, which may be difficult to interpret in a physiologic context, yet the authors of this manuscript present compelling biochemical data to demonstrate that the Bin1 SH3 domain mediates a functional interaction with actin and myosin. Moreover, a transgenic over-expression of the SH3 domain leads to measurable disruptions in sarcomeric structure. As such, the author should discuss their observations in light of this prior paper. 2. Regarding the issue raised above, Figure 1E needs further clarification. The authors imply that this immuno-localization of Bin1 and MTM1 has a Triad/t-tuble like distribution. However, the low magnification images indicate a sarcomeric distribution as well. Confocal imaging should be utilized to limit confusion and/or misinterpretation here. Perhaps the authors should think of co-staining with other markers to confirm the exact sub-cellular location of MTM1 (and what happens to MTM1 localization when the mutant Bin1 SH3 domain protein (from Figure 5) is over-expressed). 3. The images in Figure 3 confirm the concerns noted above. For example, Bin1 distribution in the Wt scenario has a very sarcomeric orientation, yet the authors claim a Triad exclusive distribution. Again, these observations need to be discussed in light of Fernando et al. 2009.

Answer of the authors: We apologize for having omitted the important work of Fernando et al. and are now discussing this previous report in light of our data. We report that BIN1 and MTM1 are both present at the triads as they partially colocalise with markers of this structure, RYR1 and DHPR (Figure S2 and magnification) and they do colocalize in the typical double rows pattern of triads (Figure 1E and magnification). Similarly, the typical double rows pattern of triads is evident from Figure 3 and magnification and is in agreement with previous reports (Butler et al., J Cell Biol 1997; Toussaint et al. Acta Neuropathol 2011). This localization data is also in agreement with our subcellular fractionation experiments depicted in Figure 1F. To clarify Figure 1E, that are confocal images, we have added on the revised figure quantitative measurement of MTM1 and BIN1 signals (intensity plots) on both transversal and longitudinal orientations that emphasize MTM1 and BIN1 are present on the same localization. In addition, we have now clarified (part "MTM1 and BIN1 interact both in vitro and endogenously in skeletal muscle") that this is a partial colocalization and that "In addition to a role at the triad, BIN1 could play a pleiotropic role in the organization/functioning of the sarcomere, as it was proposed to mediate interaction with proteins of the structure of the sarcomere, actin and myosin (Fernando et al 2009)." Moreover, triads are a part of the sarcomere (Figure S2D), so both triadic and sarcomeric localizations/functions of BIN1 and MTM1 are not exclusive. In support to reviewer's suggestion we added confocal images of Bin1/sarcomeric actin and Bin1/α-actinin (Z-line marker) staining, which showed no extensive colocalisation of BIN1 with these markers and point to a localization of

BIN1 mainly at triad next to the Z line, in the I-band of the sarcomere. We now clarify in the text (part "MTM1 and BIN1 interact both in vitro and endogenously in skeletal muscle") that a fraction of MTM1 and BIN1 is in complex, and propose this interaction occurs mainly at the triads based on imaging and fractionation experiments.

Correspondence - editor 20 June 2013

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, the referees are now all positive about its publication in EMBO reports. Before we proceed with the official acceptance of your study I would like to draw your attention to two minor things:

1. Please indicate for each experiment how many times it has been repeated (biological, not technical replicates) and please note that if an experiment has only been performed twice, error bars should not be calculated.

2. We have also started encouraging authors to submit the raw data for western blots (i.e. original scans) and for microscopical images to our editorial office. These data will be published online as part of the supplementary information. This is voluntary at the moment, but if you agree that this would be useful for readers I would like to invite you to supply these files when submitting the revised version of your study.

I would suggest you simply send us the final version of your text, along with the raw data of the figures (if you choose to show them) by email. We will then upload it to the rest of the manuscript.

Please do not hesitate to contact me if you have any further questions at this point.

I congratulate you on a very nice piece of work and look forward to seeing it published in EMBO reports soon.

REFEREE REPORTS:

Referee #1:

The authors have fully addressed my concerns. This leads to a significant improvement of the manuscript.

Referee #2:

In the revised version of this manuscript Laporte and colleagues have presented significant additional data that have addressed most of the issues that arose from their original submission. These data have considerably strengthened the manuscript.

Referee #3:

The authors have fully addressed all concerns noted. The additional data regarding

© European Molecular Biology Organization

the co-localization of Bin1 in the Triad region vs the sarcomere is very convincing, well controlled. The manuscript is addressing an important aspect of skeletal muscle biology and following the revisions is now suitable for publication.

Correspondence - author 27 June 2013

Please find attached the final version of the text, together with the TIFF files of the figures.
For each quantification, we have added the number of independent experiments in the legends and in the material and method section. We removed the error bars on Fig2C and 2D and in Fig 4B as these quantifications were based on experiments performed twice independently. Other quantifications in Fig. 2B, 4A and 5F are based on experiments performed at least 3 times independently.
The number of experiments performed for the supplementary figures has been added in the supplementary material and methods.
Figures 1,3 and 5 were not modified and are not sent in this mail.

Correspondence - editor 28 June 2013

Many thanks for sending the revised figures and for clarifying the number of independent experiments. I appreciate that you removed the error bars from Figure 2C and D, but it looks a bit odd to then still show the statistical significance between the different conditions. What I would suggest to show is the following:

take n as the number of cells within one experiment (in this case n will be very large I assume) and calculate the error bars and the statistical significance based on this number (and show both the error bar and the statistical significance). Then in the figure legend you can mention that you performed the actual experiment (ie the transfection) twice and that the statistical significance was calculated based on n number of cells from one of the two independent experiments, but that the second set looks similar.

I think this makes more sense and if you agree please send us the final version of the text and figure by email again.

I am sorry for this inconvenience and thank you for your cooperation.

Correspondence - author 17 July 2013

Thank you for your suggestions and sorry for the delay.

We have now modified the text and figure 2C and 2D to show, as suggested, the error bars and statistical significance based on data obtained on "n" cells within one experiment. We now precise in the legend the number n and that a second experiment was performed with similar findings: "For (C) and (D), about 200 transfected cells (with/without tubules) and 20 to 30 tubules/cell in about 90 transfected cells (ratio long tubules/short tubules) were counted. The statistical significance was calculated based on number of cells from one of two independent experiments; the second set looked similar."

Attached are the novel text file and figure 2 file. The other figure files sent did not change nor the supplemental informations.

© European Molecular Biology Organization

2nd Editorial Decision 18 July 2013

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

© European Molecular Biology Organization