EMBO reports

Myosin 1C uses a novel phosphoinositide-dependent pathway for nuclear localization

Ilya Nevzorov, Ekaterina Sidorenko, Weihuan Wang, Hongxia Zhao and Maria K. Vartiainen

Review timeline:

Submission date:	28 March 2017
Editorial Decision:	3 May 2017
Revision received:	29 August 2017
Editorial Decision:	5 October 2017
Revision received:	6 November 2017
Editorial Decision:	4 December 2017
Revision received:	5 December 2017
Accepted:	11 December 2017

Editor: Martina Rembold

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 3 May 2017

Thank you for the submission of your manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, while the referees agree that the study is potentially interesting, they also all point out that it requires significant revision before it can be considered for publication here. The major concerns regard the association of myosin 1C with the ER and chromatin and that a possible requirement of the NLS has not been unambiguously ruled out. Moreover, the referees suggest several control experiments and consider further biochemistry regarding protein localization, protein expression levels or the role of calmodulin necessary to fully support the conclusions drawn.

From the referee comments it is clear that, as the study stands, the data are rather preliminary and publication of the manuscript in our journal can therefore not be considered at this stage. On the other hand, given the potential interest of your findings, I would like to give you the opportunity to address the concerns and would be willing to consider a revised manuscript with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board.

Should you decide to embark on such a revision, 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. Please contact us if a 3-months time frame is not sufficient

for the revisions so that we can discuss the revisions further.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EVx. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Regarding data quantification, please ensure to specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

****
REFEREE REPORTS

Referee #1:

Nevzorov et al describe a potentially novel nuclear import pathway for myosin 1c. Based on a combination of mutated forms of myosin 1c and fluorescence-based methods (FRAP and FLIP), they suggest that myosin 1c is primarily imported in the cell nucleus through an importin-independent mechanism. They suggest that nuclear import requires an interaction with the endoplasmic reticulum followed by phosphoinositide binding. This mechanism responsible for import but not for nuclear retention of myosin 1c. Interestingly, the authors identify the NLS-like sequence, previously identified by the Hozak's lab as the main driver for nuclear import, as being the site that specifically interacts with phosphoinositide molecules.

Overall the study is of potential interest. However, although PIP2 seems to be involved in the localization of myosin 1C, in my opinion the study is at a rather preliminary phase. The data presented do not rule out the previously suggested import mechanism for myosin 1c. The authors do not convincingly show association of myosin 1c with the ER and, possibly, with the ER lumen. This should be addressed by co-localizations of myosin 1c with ER markers and PIP2 antibodies by IF/confocal microscopy and corroborated by EM. Association/localization with the ER lumen can be studied biochemically. My second concern is that the authors discuss phosphoinositide-dependent association of myosin with the chromatin but this point is not thoroughly investigated. What kind of chromatin are we discussing, transcriptionally active or inactive? Further, association of myosin 1c (NM1) with chromatin at both gene and genome level has been shown and the authors should put their findings in the context of published work. My third concern is terminology. The study is performed on myosin 1c isoform b also known as NM1. So the authors should refer their findings to that specific isoform and not to the general myosin 1c unless they prove that their findings are valid for all three isoforms.

Specific points

line 154 - import of Myo1C is slower than actin so it is concluded that the two proteins are imported

through different mechanisms. There are several alternative explanations for these findings, including the transcriptional state of the cell.

In figure 2, the authors show different mutants and their nuclear localizations. The strongest localization is shown for the construct where PK is fused with the PH domain in the myosin 1c tail. This construct still contains the previously described NLS in the neck domain (Dzijak et al). The same construct was used by Dzijak et al with the same results. The authors should therefore prepare a construct with PK attached to the tail and analyze its nuclear localization.

In figure 3 panel a there is some confusion. Many cells expressing GFP-Myo1c with a mutation in the PH domain display nuclear localization which somehow is not consistent with the dominant effect of PIP2 binding. Please explain this apparent contradiction. Is it possible that the NLS-like sequence is still in the construct that was used? Myo1c is generally described as a modular protein with head, neck and tail domain. In the present study, neck and tail seem to be referred altogether as the tail domain which would then argue that the NLS-like sequence is still there.

In figure 3 panel d, quantifications are performed on 10 cells and the standard deviations are very high. A larger number of cells analyzed would improve the statistics. Panel b is not informative; the blots should have loading controls.

Line 287 and figure 4, the authors introduce calmodulin while discussing the results of the in vitro import system. This is misleading as it looks like they incubate permeabilized cells with lysates. To connect their results with the role of calmodulin they have to block calmodulin in the lysates for instance by pre-incubating them with anti-calmodulin antibodies or by producing cytoplasmic extracts from calmodulin-silenced cells

Figure 5, changes in the localization cannot be taken as proof of binding to ER. The authors must label cells with ER markers (see also above for main concerns)

Referee #2:

The manuscript entitled "Phosphoinositide-binding dependent nuclear localization of Myosin 1C: Novel view on nuclear import of soluble cargos" by Nevzorov" et al. describes a novel mechanism for nuclear import of Myosin 1C (Myo1C) that involves phosphoinositide binding via a pleckstrin homology (PH) domain and membrane association, but does not employ soluble protein factors. The mechanism is reminiscent of the diffusion-retention mechanism used by inner nuclear membrane proteins. In particular, the data show that Myo1C is imported into the nucleus and interacts with chromatin, but a key PH domain mutant of Myo1C, Myo1C K892A, previously shown to inhibit binding to phos-phatidylinositol-4,5-bisphosphate (PIP2) in vitro and membrane association in vivo, is impaired for import. Critically, in an in vitro import assay, lysate, containing soluble transport factors and other proteins, is not required for import of Myo1C. In addition, Myo1C associates with ER membrane prior to import and the Myo1C K892A PH domain mutant shows reduced binding to phospholipid vesicles in a co-sedimentation assay. Overall, the data on the phosphoinositide-binding dependent import of Myo1C is novel, quite compelling, and has implications for the nuclear import of numerous lipid binding proteins. However, a few experiments are required to strengthen the conclusion that the C-terminal PH domain of Myo1C is sufficient to mediate nuclear import and that the Myo1C K892A mutant is impaired for import. As a result, this manuscript is currently not ready for publication in EMBO Reports, but could be reconsidered upon revision.

Major Points:

1. In Figure 2a, comparison of Myo1c WT and Myo1C K892A would benefit from enhanced contrast and DAPI staining. The authors should also perform an immunoblot of the Myo1C proteins to ensure that the Myo1C mutant proteins are expressed to a similar level as Myo1C WT.

2. In Figure 2c, the authors should perform a standard nucleocytoplasmic fractionation of U2OS cells expressing Myo1C and Myo1C K892A to visualize the nuclear and cytoplasmic amounts of the Myo1C proteins and support the conclusion that Myo1C K892A shows reduced nuclear import.

3. In Figure 3, the reduced import of GFP-Myo1C Tail K892A relative to GFP-Myo1C Tail WT requires further support. The authors should perform FRAP measurements on these Myo1C Tail proteins, which appear to have higher nuclear abundance than the full-length Myo1C proteins. In Figure 3b, a loading control and quantitation are also required to confirm that the Myo1C K892A level is similar to Myo1C WT.

4. In Figure 5b, the authors should co-stain the cells with an ER marker to confirm that Myo1C co-localizes with the ER.

5. To support the conclusion that the PH domain of Myo1C is sufficient for nuclear import, the authors should assess the localization of a GFP-GFP-PH construct, containing two GFPs fused to the Myo1C PH domain. The dual GFP molecular weight should serve to prevent passive diffusion into the nucleus. Testing the import of the Myo1C PH domain alone would be important since the reduced import of the Myo1C K892A mutant could be caused by the K892A substitution exerting a change in the folding/interactions of another domain of Myo1C.

Minor Points:

1. Line 194: "Hemagglutinine" should be "Hemagglutinin."
2. Line 225: "inside the" should not be in italics.
3. Line 389: "mechanisms" should be "mechanism."
4. Figure 4a: label for 4th row panels - "lysate/enerrgy/RanQL" should be "lysate/energy/RanQL"

Referee #3:

The paper "Phosphoinositide-binding dependent nuclear localization of myosin 1C: Novel view on nuclear import of soluble cargos" by Nevzorov et al. describes the molecular mechanism of Myo1C nuclear import. They show that Myo1C constantly shuttles between the nucleus and the cytoplasm using photobleaching techniques. Furthermore, their data suggested that the nuclear import of Myo1C is dependent on phosphoinositide-binding but not on soluble factors, and is preceded by its interaction with the endoplasmic reticulum. This paper contains interesting data suggesting the existence of a novel nuclear import mechanism. However, their current data does not fully support the hypothesis as stated. The following points should be addressed before further consideration.

Specific points:
1. (Fig 1) To show that GFP-Myo1c used for photobleaching experiment is not degraded, the western blot should be presented.
2. (Fig 2e, Page 11 line 236-238) What causes the specificity of the rescue effect of PH-domain? Is the other domain of Myo1C, such as IQs (NLS-like sequence) or Post IQ, involved in its nuclear delivery? This could be examined by creating constructs of PK- (IQs or PostIQ) -PH.
3. (Fig 3) How long does it take for GFP-Myo1c Tail K892A to enter nucleus? A longer time course experiment should be performed.
4. (Fig 3a) Could the authors quantify the data, as performed in supplementary Fig 1.
5. (Fig 3b) A loading control is needed for each cell line.
6. (Page 13 line 287 and 289) What is the role of Calmodulin? It is not clear why the authors have to use calmodulin in this study, since IQ2 mutant showed the similar nuclear localization as WT (Fig 2). In addition, the authors stated that calmodulin binding is not crucial for nuclear transport of Myo1C (Page 9, line 205-206).
7. In in vitro transport assay using GFP-Myo1C Tail WT (Fig 4), the size of nucleus is quite different depending on the conditions (ex. Compare between "no lysate/energy" and "no lysate/no energy"). In contrast, these differences were not observed in the experiment using GST-GFP-NLS. Could the authors explain why this occurs? In addition, the authors should examine the nuclear integrity, for example, by using TRITC-labeled dextran.
8. (Fig 5b) A higher-magnification image of the cell supplemented with energy mix should also be presented.
9. (Fig 6d) The author should perform the experiment using the phospholipid vesicles without PIP2. This is an important experiment to validate the specificity of PI-Myo1C Tail (WT, K892A, NLSmut) interaction. Again, what is the role of calmodulin?

1st Revision - authors' response 29 August 2017

Referee #1:

Nevzorov et al describe a potentially novel nuclear import pathway for myosin 1c. Based on a combination of mutated forms of myosin 1c and fluorescence-based methods (FRAP and FLIP), they suggest that myosin 1c is primarily imported in the cell nucleus through an importin-independent mechanism. They suggest that nuclear import requires an interaction with the endoplasmic reticulum followed by phosphoinositide binding. This mechanism responsible for import but not for nuclear retention of myosin 1c. Interestingly, the authors identify the NLS-like sequence, previously identified by the Hozak's lab as the main driver for nuclear import, as being the site that specifically interacts with phosphoinositide molecules.
Overall the study is of potential interest. However, although PIP2 seems to be involved in the localization of myosin 1C, in my opinion the study is at a rather preliminary phase. The data presented do not rule out the previously suggested import mechanism for myosin 1c. The authors do not convincingly show association of myosin 1c with the ER and, possibly, with the ER lumen. This should be addressed by co-localizations of myosin 1c with ER markers and PIP2 antibodies by IF/confocal microscopy and corroborated by EM. Association/localization with the ER lumen can be studied biochemically.

To strengthen the notion that Myo1C associates with the ER, we now show by confocal microscopy that it partially colocalizes with the ER marker calreticulin (Figure EV3a). Furthermore, we show by cell fractionation that Myo1C can be found in the ER fraction (Figure EV3b). This data is now discussed in the text on page 13, lines 287 - 290.

The localization of phosphotidylinositides to the ER is reviewed in:

Van Meer G, Voelker DR, Feigenson GW. Membrane lipids: where they are and how they behave. Nature reviews Molecular cell biology. 2008;9(2):112-124.

We find it highly unlikely that Myo1C would be localized to the ER lumen, since it does not contain a signal sequence, which is crucial for luminal targeting of cytoplasmic proteins. Furthermore, luminal localization would hardly contribute to nuclear localization.

My second concern is that the authors discuss phosphoinositide-dependent association of myosin with the chromatin but this point is not thoroughly investigated. What kind of chromatin are we discussing, transcriptionally active or inactive? Further, association of myosin 1c (NM1) with chromatin at both gene and genome level has been shown and the authors should put their findings in the context of published work.

Our data do not support phosphoinositide-dependent association of Myo1C with chromatin, because Myo1C Tail K892A construct (with mutated PH domain), as well as the similar construct with NLS mutations, interact with chromatin as efficiently as the WT Tail construct (Figure 6B and C). Due to this finding, we have not explored the chromatin-binding here further, and would like to restrict the manuscript to the import mechanism of Myo1C. However, we now highlight Myo1C chromatin-binding properties in the discussion (page 19, lines 440-442).

My third concern is terminology. The study is performed on myosin 1c isoform b also known as NM1. So the authors should refer their findings to that specific isoform and not to the general myosin 1c unless they prove that their findings are valid for all three isoforms.

We show in Expanded view (EV) figure 2a (EV2a) that also Myo1C isoform c (the shortest isoform, with no N-terminal peptide) shows similar dependency of an intact PH domain for its nuclear localization. It is therefore extremely likely that the nuclear import mechanism described here applies to all Myo1C isoforms. Hence we would prefer to keep the nomenclature as it is. Of note, already the data by Dzijak et al (Plos One, 2012) suggested that all Myo1C isoforms share the same nuclear localization signal, and that this process is not dependent on the N-terminal peptide that distinguishes the isoforms from each other.

Specific points

line 154 - import of Myo1C is slower than actin so it is concluded that the two proteins are imported through different mechanisms. There are several alternative explanations for these findings, including the transcriptional state of the cell.

We find that this is highly unlikely, since we randomly chose cells for analysis. Furthermore, our subsequent data confirm that Myo1C indeed uses a very different mechanism than actin for its nuclear localization.

In figure 2, the authors show different mutants and their nuclear localizations. The strongest localization is shown for the construct where PK is fused with the PH domain in the myosin 1c tail. This construct still contains the previously described NLS in the neck domain (Dzijak et al). The same construct was used by Dzijak et al with the same results. The authors should therefore prepare a construct with PK attached to the tail and analyze its nuclear localization.

To clarify these issues, we now study the localization of two new constructs: 2GFP-PK-PH_{Myo1C} and GFP-Myo1C Tail NLSmut (see Figure EV1e for the data and outline for these constructs). The first construct, containing only the PH domain of Myo1C, fails to localize to the nucleus. This demonstrates that PH domain is not sufficient for nuclear localization of Myo1C, and the result is in line with the finding that the PH domain from PLC δ is cytoplasmic as well (Figure EV1d). On the other hand, a construct containing also the IQ and post-IQ regions, but with a mutated NLS, still localizes to the nucleus. This indicates that this region of Myo1C, but not the NLS, could be important for nuclear retention of Myo1c. Data from Dzijak et al., showing no nuclear localization for GFP-postIQ-PH_{Myo1C}, points to a role for the IQ here. Of note, structural studies have shown that calcium binding to calmodulin induces drastic conformational changes in Myo1C IQ-postIQ, which probably indicates that this domain is a hotspot of protein-protein interactions. The potential role of IQ-post-IQ in nuclear retention of Myo1C is now discussed on page 19, lines 449 - 456.

In figure 3 panel a there is some confusion. Many cells expressing GFP-Myo1c with a mutation in the PH domain display nuclear localization which somehow is not consistent with the dominant effect of PIP2 binding. Please explain this apparent contradiction. Is it possible that the NLS-like sequence is still in the construct that was used? Myo1c is generally described as a modular protein with head, neck and tail domain. In the present study, neck and tail seem to be referred altogether as the tail domain which would then argue that the NLS-like sequence is still there.

First, all of our "tail" constructs also contain the neck domain, but are called just "tail" for simplicity. Figure 1f shows the schematic of the key constructs used in this study.

In figure 3, we use Myo1C Tail constructs, which are export incompetent (Figure 1c and 1d), allowing us to focus only on nuclear import. In a steady-state culture, with cells at random cell cycle phases, the GFP-Myo1C Tail with mutated PH domain indeed shows nuclear localization in majority of cells. However, when we measure how fast this protein accumulates in the nucleus after cell division (Figure 3c and 3d), there is very drastic delay compared to the wild type Myo1C tail construct. Since the construct still contains the NLS-like sequence, this likely contributes to residual import. However, since the import is very slow, it is clear that the NLS-like sequence is not sufficient for full nuclear import rate of Myo1c.

In figure 3 panel d, quantifications are performed on 10 cells and the standard deviations are very high. A larger number of cells analyzed would improve the statistics. Panel b is not informative; the blots should have loading controls.

We have now analyzed 22 cells per condition (Figure 3d) and added a loading control for the blow in Figure 3b.

Line 287 and figure 4, the authors introduce calmodulin while discussing the results of the in vitro import system. This is misleading as it looks like they incubate permeabilized cells with lysates. To connect their results with the role of calmodulin they have to block calmodulin in the lysates for instance by pre-incubating them with anti-calmodulin antibodies or by producing cytoplasmic extracts from calmodulin-silenced cells

The recombinant Myo1C Tail used here as an import cargo is expressed and purified as a complex with calmodulin, and this is absolutely essential in order to obtain any functional and soluble Myo1C for biochemical assays. Since our Myo1C has already calmodulin bound, its presence (or absence) in the lysate (which is not even needed for nuclear import of Myo1C) does not make any difference to the result.

Figure 5, changes in the localization cannot be taken as proof of binding to ER. The authors must label cells with ER markers (see also above for main concerns)

As stated above, we now show by microscopy and fractionation (EV3a and b) that Myo1C can be found on the ER.

Referee #2:

The manuscript entitled "Phosphoinositide-binding dependent nuclear localization of Myosin 1C: Novel view on nuclear import of soluble cargos" by Nevzorov" et al. describes a novel mechanism for nuclear import of Myosin 1C (Myo1C) that involves phosphoinositide binding via a pleckstrin homology (PH) domain and membrane association, but does not employ soluble protein factors. The mechanism is reminiscent of the diffusion-retention mechanism used by inner nuclear membrane proteins. In particular, the data show that Myo1C is imported into the nucleus and interacts with chromatin, but a key PH domain mutant of Myo1C, Myo1C K892A, previously shown to inhibit binding to phos-phatidylinositol-4,5-bisphosphate (PIP2) in vitro and membrane association in vivo, is impaired for import. Critically, in an in vitro import assay, lysate, containing soluble transport factors and other proteins, is not required for import of Myo1C. In addition, Myo1C associates with ER membrane prior to import and the Myo1C K892A PH domain mutant shows reduced binding to phospholipid vesicles in a co-sedimentation assay. Overall, the data on the phosphoinositide-binding dependent import of Myo1C is novel, quite compelling, and has implications for the nuclear import of numerous lipid binding proteins. However, a few experiments are required to strengthen the conclusion that the C-terminal PH domain of Myo1C is sufficient to mediate nuclear import and that the Myo1C K892A mutant is impaired for import. As a result, this manuscript is currently not ready for publication in EMBO Reports, but could be reconsidered upon revision.

Major Points:

1. In Figure 2a, comparison of Myo1c WT and Myo1C K892A would benefit from enhanced contrast and DAPI staining. The authors should also perform an immunoblot of the Myo1C proteins to ensure that the Myo1C mutant proteins are expressed to a similar level as Myo1C WT.

We have now included images that contain also the DAPI channel (Figure 2a), and show a Western blot of the relative expression levels of the key constructs (Figure EV1b).

2. In Figure 2c, the authors should perform a standard nucleocytoplasmic fractionation of U2OS cells expressing Myo1C and Myo1C K892A to visualize the nuclear and cytoplasmic amounts of the Myo1C proteins and support the conclusion that Myo1C K892A shows reduced nuclear import.

We now include data from a fractionation experiment using hypotonic buffer and sucrose cushions (Figure EV1c), which also shows decreased nuclear localization of the Myo1C K892A construct.

3. In Figure 3, the reduced import of GFP-Myo1C Tail K892A relative to GFP-Myo1C Tail WT requires further support. The authors should perform FRAP measurements on these Myo1C Tail proteins, which appear to have higher nuclear abundance than the full-length Myo1C proteins. In Figure 3b, a loading control and quantitation are also required to confirm that the Myo1C K892A level is similar to Myo1C WT.

Unfortunately, we cannot perform FRAP experiments to measure nuclear import rate of the tail constructs. The FRAP and FLIP techniques used in Figure 1 require sufficient amount of fluorescence in both compartments. The tail constructs are very nuclear, so if we bleached the

nucleus, there would not be enough cytoplasmic fluorescence to reliably measure the recovery of nuclear fluorescence. For this reason, we decided to measure the nuclear import rate of these constructs after mitosis. Since we are using the tail constructs, which are export incompetent (Figure 1c and d), this gives a good measure of the nuclear import rate of these constructs. We have added a loading control for the blot in Figure 3b to show that the two constructs used in this figure are expressed at similar levels.

4. In Figure 5b, the authors should co-stain the cells with an ER marker to confirm that Myo1C co-localizes with the ER.

As already stated in response to reviewer 1, we now show by microscopy and fractionation (Figure EV3a and b) that Myo1C localizes to the ER.

5. To support the conclusion that the PH domain of Myo1C is sufficient for nuclear import, the authors should assess the localization of a GFP-GFP-PH construct, containing two GFPs fused to the Myo1C PH domain. The dual GFP molecular weight should serve to prevent passive diffusion into the nucleus. Testing the import of the Myo1C PH domain alone would be important since the reduced import of the Myo1C K892A mutant could be caused by the K892A substitution exerting a change in the folding/interactions of another domain of Myo1C.

We actually do not claim that Myo1C PH domain is sufficient for nuclear localization of Myo1C, since it is clear from our data that while it is required, it is not sufficient. This has now been clarified in the text, and we further show that similarly to the PH domain of PLC δ, the PH domain of Myo1C alone cannot confer nuclear localization (Figure EV1e). This suggests that the PH domain may not contain the sequences required for nuclear retention; also this aspect is further discussed (page 19, lines 438 – 439, 449 – 456).

Minor Points:

1. Line 194: "Hemagglutinine" should be "Hemagglutinin."
2. Line 225: "inside the" should not be in italics.
3. Line 389: "mechanisms" should be "mechanism."
4. Figure 4a: label for 4th row panels - "lysate/enerrgy/RanQL" should be "lysate/energy/RanQL"

All corrected

Referee #3:

The paper "Phosphoinositide-binding dependent nuclear localization of myosin 1C: Novel view on nuclear import of soluble cargos" by Nevzorov et al. describes the molecular mechanism of Myo1C nuclear import. They show that Myo1C constantly shuttles between the nucleus and the cytoplasm using photobleaching techniques. Furthermore, their data suggested that the nuclear import of Myo1C is dependent on phosphoinositide-binding but not on soluble factors, and is preceded by its interaction with the endoplasmic reticulum. This paper contains interesting data suggesting the existence of a novel nuclear import mechanism. However, their current data does not fully support the hypothesis as stated. The following points should be addressed before further consideration.

Specific points:
1. (Fig 1) To show that GFP-Myo1c used for photobleaching experiment is not degraded, the western blot should be presented.

We show a Western blot of GFP-Myo1C to prove that it is not degraded in cells (Figure 1e).

2. (Fig 2e, Page 11 line 236-238) What causes the specificity of the rescue effect of PH-domain? Is the other domain of Myo1C, such as IQs (NLS-like sequence) or Post IQ, involved in its nuclear delivery? This could be examined by creating constructs of PK- (IQs or PostIQ) -PH.

Rescue by the PH domain from PLC δ is explained by the fact that it introduces a phosphoinositide-binding site to the Myo1C construct with abolished PIP_2 binding due to a point mutation in Myo1C PH domain.

As stated above in response to reviewer 1, we now study the localization of two new constructs: 2GFP-PK-PH_{Myo1C} and GFP-Myo1C Tail NLSmut (see Figure EV1e for the data and outline for these constructs). The first construct, containing only the PH domain of Myo1C, fails to localize to the nucleus. This demonstrates that PH domain is not sufficient for nuclear localization of Myo1C, and the result is in line with the finding that the PH domain from PLC δ is cytoplasmic as well. On the other hand, the construct containing also the IQ and post-IQ regions, but with a mutated NLS still localizes to the nucleus. This indicates that this region of Myo1C, but not the NLS, could be important for its nuclear retention. Data from Dzijak et al., showing no nuclear localization for GFP-postIQ-PH_{Myo1C}, points to a role for the IQ here. Of note, structural studies have shown that calcium binding to calmodulin induces drastic conformational changes in Myo1C IQ/post-IQ, which probably indicates that this domain is a hotspot of protein-protein interactions. The potential role of IQ/post-IQ in nuclear retention of Myo1C is now discussed on page page 19, lines 449 – 456.

3. (Fig 3) How long does it take for GFP-Myo1c Tail K892A to enter nucleus? A longer time course experiment should be performed.

We actually image the cells for up to 12 hours, and have found that it takes Myo1c Tail K892A around 5-7 hours to regain fully nuclear localization. The time points in Figure 3c were chosen to reflect the distinct behavior of the two constructs, and the rate of nuclear signal recovery was measured from early time points, which in general are less variable than later time points.

4. (Fig 3a) Could the authors quantify the data, as performed in supplementary Fig 1.

In principle we could, but this would most likely not result in statistically significant difference between the constructs. This is because, upon random sampling of cells, the fraction that has just undergone mitosis, and thus display less nuclear Myo1C Tail K892A, is rather small.

5. (Fig 3b) A loading control is needed for each cell line.

As requested also by the other reviewers, we have now added a loading control for the cell lines in Figure 3b.

6. (Page 13 line 287 and 289) What is the role of Calmodulin? It is not clear why the authors have to use calmodulin in this study, since IQ2 mutant showed the similar nuclear localization as WT (Fig 2). In addition, the authors stated that calmodulin binding is not crucial for nuclear transport of Myo1C (Page 9, line 205-206).

The only reason for including calmodulin here is that we need to express and purify Myo1C as a complex with calmodulin to obtain any functional protein to serve as a cargo for the import assay.

7. In in vitro transport assay using GFP-Myo1C Tail WT (Fig 4), the size of nucleus is quite different depending on the conditions (ex. Compare between "no lysate/energy" and "no lysate/no energy"). In contrast, these differences were not observed in the experiment using GST-GFP-NLS. Could the authors explain why this occurs? In addition, the authors should examine the nuclear integrity, for example, by using TRITC-labeled dextran.

We do not have a very good explanation, why the size of the nucleus is different in these conditions. Nuclear integrity is actually controlled by GST-GFP-NLS, which is used at exactly same conditions as Myo1C import cargo, and does not accumulate in the nucleus for example upon RanQ69L and no lysate/energy conditions, which both show robust nuclear accumulation of Myo1C.

8. (Fig 5b) A higher-magnification image of the cell supplemented with energy mix should also be presented.

Higher magnification images of both GST-GFP-NLS and GFP-Myo1C at condition of “no lysate/energy” is now shown in Figure EV3c.

9. (Fig 6d) The author should perform the experiment using the phospholipid vesicles without PIP2. This is an important experiment to validate the specificity of PI-Myo1C Tail (WT, K892A, NLSmut) interaction. Again, what is the role of calmodulin?

We include the vesicle binding experiment without PIP2. As can be seen, the amount of the NLSmut protein in the pellet fraction does not significantly increase upon PIP2 addition, suggesting that this protein has indeed lost its ability to interact with PIP2. As stated above, we have to express Myo1C as a complex with calmodulin to obtain functional protein for biochemical analysis.

2nd Editorial Decision 5 October 2017

Thank you for the submission of your revised manuscript to our journal.
We have meanwhile received a complete set of reviews from all referees, which I include below for your information.

As you will see, the referees are very positive about the study. However, referee 1 has some remaining concerns regarding the suggested role for the (former) NLS as a retention signal and the co-localization between Myo1C and the ER. Upon further discussion the other referees agree with referee 1 and I think that these two concerns should be experimentally addressed to further strengthen and clarify these points. Please also address the other concerns of referee 1.

I look forward to seeing a final version of your manuscript as soon as possible.

****
REFEREE REPORTS

Referee #1:

During the revision, the authors have made an effort to respond to the issues raised by this reviewer either by extra clarifications or by including new experiments. However, I still have a few concerns that require attention before publication.

In particular, the use of new constructs suggests a role for NLS and IQ/post-IQ region in nuclear retention. This experiment, although very interesting, brings more questions (considering previous work on the identification of the myo1c NLS) and I am surprised that the authors did not test their hypothesis. Claiming that PIP2 binding is needed for import while the former NLS is just a retention signal can be clarified by blocking nuclear export with Leptomycin B and see whether NLS mutants and PIP2 mutants still go to the nucleus.
One of my major concerns was myo1c association with the ER. The authors now show partial co-localization of overexpressed HA-tagged myo1c with calreticulin, a protein that localizes to the ER and it is also known to enter the nucleus to become part of the nuclear matrix. These new results seem to be indicative of a possible localization/association of myo1c with the ER. However, previous work from the same authors shows that myo1c regulates actin filaments associated with the ER and manipulation (by overexpression or depletion) of myo1c induces ER phenotypes (Joensuu et al 2014). In my opinion, therefore, to convincingly show association of myo1c with the ER the authors should study co-localization of the endogenous myo1c with ER markers. I also think that it is important to expand the number of ER markers and use those which do not enter the nucleus. Furthermore, from a technical point of view staining of the HA-tagged myo1c construct is not optimal and anyway much weaker compared to the calreticulin signal. This requires improvement prior to publication. The authors should possibly include insets with magnifications.

Clarifications
- The authors claim that the PIP2 mutant is able to bind to the chromatin. However they should clarify this point. In their model PIP2 is required to enter the nucleus and therefore one would not expect to see the PIP2 mutant still associated with the chromatin (fig 2c and fig EV3b)
- There is no clear significant difference between graphs 2b and 2e

Suggestion
In the introduction, the authors should follow the myosin field and use proper nomenclature when referring to myosin domains such as head, neck and tail domain. It is really confusing to read the paper and not being able to refer to the broad myosin field brings further confusion to those new to the field.

Referee #2:

The revised manuscript entitled "Phosphoinositide-binding dependent nuclear localization of Myosin 1C: Novel view on nuclear import of soluble cargos" by Nevzorov" et al. has addressed all my major concerns and is now much improved. The conclusion that the PH domain of Myo1C mediates novel nuclear import independent of classical transport factors is greatly strengthened and the results will be extremely valuable to the field. The revised manuscript is now highly suitable for publication in EMBO Reports.

Referee #3:

The authors have addressed my major concerns.

2nd Revision - authors' response 6 November 2017

Referee #1:

During the revision, the authors have made an effort to respond to the issues raised by this reviewer either by extra clarifications or by including new experiments. However, I still have a few concerns that require attention before publication.

In particular, the use of new constructs suggests a role for NLS and IQ/post-IQ region in nuclear retention. This experiment, although very interesting, brings more questions (considering previous work on the identification of the myo1c NLS) and I am surprised that the authors did not test their hypothesis. Claiming that PIP2 binding is needed for import while the former NLS is just a retention signal can be clarified by blocking nuclear export with Leptomycin B and see whether NLS mutants and PIP2 mutants still go to the nucleus.

We do not claim anywhere in the manuscript that the "former" NLS would be a retention signal. On the contrary, data in EV1e suggest a role for the IQ, but not the NLS within the IQ, in retention. This figure shows that while PH-domain of Myo1c alone is not sufficient to confer nuclear localization, addition of the IQ and post-IQ regions of Myo1C, even when the putative NLS is mutated, confers nuclear localization to the construct. Since earlier studies had shown that GFP-postIQ-PHMyo1c (so lacking the IQ) is not nuclear (Dzijak et al. 2012), collectively, these results suggest that the IQ contributes to the retention, but it is not the NLS within the IQ that does this. The discussion on this has been slightly modified regarding this on page 19-20, lines 452-457.

Regarding the experiment suggested by the reviewer, we would have loved to utilize Leptomycin B to block export in our experiments, since it would have simplified things. However, in our hands, Leptomycin B does not cause nuclear accumulation of Myo1c at conditions, where it robustly blocks nuclear export of control proteins, for example MAL/MKL1 (Vartiainen et al. 2007). We can provide the data for the reviewers, if needed. This data was not included in the manuscript, because we wanted concentrate this manuscript specifically on Myo1c import.

One of my major concerns was myo1c association with the ER. The authors now show partial co-localization of overexpressed HA-tagged myo1c with calreticulin, a protein that localizes to the ER and it is also known to enter the nucleus to become part of the nuclear matrix. These new results seem to be indicative of a possible localization/association of myo1c with the ER. However, previous work from the same authors shows that myo1c regulates actin filaments associated with the ER and manipulation (by overexpression or depletion) of myo1c induces ER phenotypes (Joensuu et al 2014). In my opinion, therefore, to convincingly show association of myo1c with the ER the

authors should study co-localization of the endogenous myo1c with ER markers. I also think that it is important to expand the number of ER markers and use those which do not enter the nucleus.

Furthermore, from a technical point of view staining of the HA-tagged myo1c construct is not optimal and anyway much weaker compared to the calreticulin signal. This requires improvement prior to publication. The authors should possibly include insets with magnifications.

We fully agree that this is an important point. However, showing the co-localization of endogenous Myo1c with ER is technically very difficult. First of all, despite trying several Myo1c antibodies, we are not particularly satisfied with how they perform in immunofluorescence. Second, Myo1c is very strongly localized to the plasma membrane, which (due to limitations in z-resolution) makes it very difficult to distinguish the signal coming from the ER especially from flat regions of the cell. Hence, microscopy is not the ideal assay to prove this point, and despite serious effort, we did not get any clearer images.

However, to provide evidence that endogenous Myo1c can interact with ER, we now show that it is found in the ER fraction in the fractionation experiment by using an antibody against Myo1c. In this experiment, we also used another marker for ER, calnexin, to further prove that the fractionation was successful. This data is now shown in EV3c, and mentioned in the text on page 13, lines 289-291.

Clarifications

- The authors claim that the PIP2 mutant is able to bind to the chromatin. However they should clarify this point. In their model PIP2 is required to enter the nucleus and therefore one would not expect to see the PIP2 mutant still associated with the chromatin (fig 2c and fig EV3b)
There is some misunderstanding here. Both experiments (Figure 2c and quantification in 2d, and EV3b) show that in the context of full length Myo1C, the PIP2 mutant K892A interacts less with chromatin than the wild type protein. In figure 6b (with quantification in 6c), we use the tail constructs, which are nuclear, and here we can uncouple the nuclear import from chromatin binding. In this experiment, the PIP2 and NLS mutants interact with chromatin as efficiently as the wild type tail domain.

- There is no clear significant difference between graphs 2b and 2e
I am not entirely sure what the reviewer means here, but we did not want to make a graph with too many bars.

Suggestion
In the introduction, the authors should follow the myosin field and use proper nomenclature when referring to myosin domains such as head, neck and tail domain. It is really confusing to read the paper and not being able to refer to the broad myosin field brings further confusion to those new to the field.
We clearly mention the head, neck and tail domain in the introduction. We now further highlight in the results and the figure legend 1 that our tail-constructs contain also the neck.

3rd Editorial Decision 4 December 2017

Thank you for your patience while former referee 1 has seen your revised manuscript. As you will see from the reports below, this referee is all positive about its publication in EMBO reports and requests only some further clarification to the text.

Thank you for your contribution to EMBO reports.

****
REFEREE REPORT

Referee #1:

I am satisfied by the authors'responses and the extra experiments included and I support publication

of the manuscript.

In my opinion, however, it is important to mention in the text that NM1 export is independent of leptomycin B as this suggests that the export mechanism is also independent from CRM1 and RanGTP.

3rd Revision - authors' response 5 December 2017

Referee #1:

I am satisfied by the authors'responses and the extra experiments included and I support publication of the manuscript.

In my opinion, however, it is important to mention in the text that NM1 export is independent of leptomycin B as this suggests that the export mechanism is also independent from CRM1 and RanGTP.

We would rather no mention this, since I feel we cannot do this, without actually showing the data. We would prefer to keep this paper focused on the import.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Maria Vartiainen
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-44296V1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Exploratory experiments were performed to evaluate mean and standard deviation in each case.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded from analysis
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Cells were randomly chosen for analysis (e.g. in terms of import/export rate in Figure 1, localization in Figure 2, 4 and 5, live-cell imaging in Figure 3).
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	As stated in 2 and 3, cells were chosen randomly and no samples were exluded from analysis
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes, this is indicated in respective figure legends, and collectively in Materials and methods
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Reported in materials and methods
Is there an estimate of variation within each group of data?	All quantified data include standard devision.
Is the variance similar between the groups that are being statistically compared?	Reported in materials and methods section. Variance is not similar, and this is taken into account in the statistical analysis.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Catalog numbers for all antibodies are specified in the methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Source of cell lines indicated in materials and methods section. Our cell lines are routinely tested for mycoplasma by using two different assays.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Programs used for quantitative analysis are indicated in materials and methods section

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA