Drosophila Wash and the Wash regulatory complex function in nuclear envelope budding

Jeffrey M. Verboon, Mitsutoshi Nakamura, Kerri A. Davidson, Jacob R. Decker, Vivek Nandakumar and Susan M. Parkhurst
DOI: 10.1242/jcs.243576

Editor: Daniel Billadeau

Review timeline
Original submission: 5 January 2020
Editorial decision: 10 February 2020
First revision received: 28 April 2020
Accepted: 28 May 2020

Original submission

First decision letter

MS ID#: JOCES/2020/243576

MS TITLE: Wash and the WASH Regulatory Complex function in Nuclear Envelope budding

AUTHORS: Susan M Parkhurst, Jeffrey M Verboon, Mitsutoshi Nakamura, Jacob Decker, Kerri A Davidson, and Vivek Nandakumar
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Research during the last few years has established that egress from nuclei of large macro-molecular complexes can take place by nuclear envelope budding. How cells use this interesting alternative to traffic mediated by nuclear port complexes is not fully understood nor are the mechanics of budding. This paper from the Parkhurst lab investigates the role of the nuclear lamina and WASH, a multi-functional protein that can regulate Arp2/3-mediate F-actin branching, among other things. The results presented support the conclusion that WASP interacts with Lamin B and is important for the organization of the nuclear lamina. Mutations in either Wasp or Lamin B result in loss of nuclear envelope budding and a wrinkled nucleus phenotype. Separately, Wash and it regulatory SHRC complex are needed to form buds implicating the F-actin cytoskeleton in bud membrane architecture. However, perturbation of SHRC complex members did not disrupt the nuclear lamina or cause a wrinkled nucleus phenotype.

Generally, the results are well presented, controlled and quantified, and the conclusions drawn are justified. The super-resolution imaging and use of different tissues (salivary glands indirect flight muscles, larval body wall neurons and cultured cells) strongly support the conclusions. Given the evidence invoking Arp2/3 function, it was disappointing that the nuclear F-actin cytoskeleton was not analyzed at the forming buds. Nonetheless, the paper provides valuable new insight into the role of structural elements in forming nuclear envelope buds.

Comments for the author

The text will benefit from significant editing. Specific comments follow.
1. Page 1 last line: Fig. 1B-C does not show mega RNPs as stated in the sentence. The figure shows the C-terminal fragment of dFz, which marks nuclear envelope buds. Re-write for clarity.
2. Page 4, line 88: The topic sentence for this paragraph does not relate to the contents of the paragraph in any obvious way. Either make a connection, or provide a better topic sentence.
3. Page 6, line 130: Again, the figures do not show megaRNPs – better to say they show “dFz2C-containing buds”.
4. Page 7, line 179: state that ATP-Synthetase alpha is a mitochondrial activity marker here instead of on page 11, line 317.
5. Line 132: Fig. S1A shows a western blot, not confocal microscopy.
6. Line 152, Figure 1H-I: buds are not discernable at this magnification, so it is not justified to conclude that wash knockdown salivary gland cells lack nuclear envelope buds.
7. Page 10, line 263: what cells are being analyzed?
8. Line 274: I see a Lamin B band in the CCDC53 pull down lane, calling into question the conclusion that “none of the SHRC subunits co-immunoprecipitated with Lamin B”.
9. Page 11, line 302: I disagree with the interpretation of the microscope images provided. The wash-WT rescue nucleus looks noticeably wrinkly, and it is not obvious that the wash-deltaLamB rescue phenotype is intermediate.
10. Page 12, line 332: missing word – “both wash and aPKC mutations almost completely…”.
11. Line 338: spell out INM before abbreviating for the first time.
12. Lind 339: what does “megaRNP collared necks” mean?
13. Page 14, line 399: missing words – “…and that Wash functions with Lamin B and independently of the…”.
14. Page 15, first paragraph: The summary of the main results of the paper in the paper needs to include Lamins. Also, nuclear envelope budding appears to be a normal way for big complexes to exit the nucleus, not a way to “bypass” the NPCs.
15. Line 407: missing word – “…like in the cytoplasm, it is likely involved…”.
16. Page 16, line 450: Not a great topic sentence since the paragraph is not about megaRNP components.

Reviewer 2

Advance summary and potential significance to field

This is an interesting submission that aims to elucidate the molecular mechanism of nuclear budding, a relatively recently described process of importance in diverse areas of basic cell biology, development, and biomedicine. While the molecular underpinnings of this process are only beginning to be sketched out, identification of the function of WASH complex (of the Wiskott-Aldrich Syndrome Protein family) that is undertaken in this work can make a solid contribution to this field. The manuscript is well written. The pertinent literature is cited thoroughly. The authors have previously described the WASH mutant phenotype that includes nuclear envelope deformation resembling premature aging, and the present work that assesses the complex's role in physiological nuclear budding extension is a logical extension of their approach.

The main findings are 1) Drosophila Wash participates in the spatial organization of the lamin A/C and B meshes that is conducive to budding; 2) through a distinct interaction with the SHRC regulatory complex's component CCDC53, Wash stimulates budding; 3) Wash effects budding through a direct interaction with and the activity of nuclear Arp2/3 complex (an actin polymerization nucleation molecule). While the last finding may appear unexpected to some readers, it in fact meshes well with the long list of documented functions of intranuclear actin, including its roles in intranuclear transport and egress phases of nuclear export. Thus, the paper can be a valuable addition not only to the literature on nuclear envelope budding, but also to the field of nuclear actin biology.

Comments for the author

While the included dataset is impressive and very thoroughly assembled there are some critical questions that have not been addressed and that could potentially impact the interpretation of the data. These are listed below. The authors can decide if the existing data are sufficient to answer them as required to remove any ambiguity vis-à-vis the paper's conclusions or if additional experimental results might need to be incorporated to this aim.

1) Throughout the present manuscript, the foci/bud count (similarly taken statically, per nucleus, at a given time) is used as a positive measure of functionality of the budding-mediated nuclear export pathway. What supports this interpretation? Alternatively (and similarly to what is encountered in studies of exocytosis), accumulation of the marker-enriched foci and buds/nascent buds could be a sign of inhibition of the further downstream steps in the budding-mediated nuclear export pathway (i.e., completion of budding, nuclear envelope lumen transit, etc.).

2) The authors showed previously (see p. 12, line 334) that knocking down Wash reduces the expression of the SHRC components (CCDC53, etc.) and vice versa. Does this impact the interpretation of the effects of the numerous Wash and SHRC component mutation and knock-down experiments in this paper (which have been used to differentiate between Wash and SHRC-mediated functions)?

3) Does the essentially equal effect that is observed downstream of nuclear envelope budding (the mitochondrial effects—p. 11, line 318) in experiments perturbing the lamin and SHRC/CCDC53 interactions of Wash, despite budding being reduced to a markedly different ($p<0.0001$) extent in the lamin and SHRC/CCDC53 experiments (p. 11, lines 303, 306), suggest that the bud count as implemented here is not a measure of functionality or carrying capacity of the budding-mediated nuclear export pathway?

Reviewer 3

Advance summary and potential significance to field

This is an interesting study that addresses the function of the class I NPF WASH in nuclear envelope budding, a recently described process wherein large macromolecular complexes are packaged and transferred through the nuclear membrane by bypassing nuclear pores. Aside for the role of WASH,

effects are also observed concerning phenomena that are considered to rely on NE-budding, which is neuromuscular junction development and mitochondrial integrity in Drosophila, so additional assays repeatedly employed to explore changes in these processes that would correlated with dysfunction of NE budding.
In general, this is a well-done study that describes a number of interesting, thought-provoking experiments, but that I would promote for straight-forward publication in due time. While most of the experiments and derived conclusions look interesting and believable, one complication is though that the authors postulate the existence of two pools of WASH in the nucleus if I understand them correctly. One pool of WASH seems to co-operate with nuclear lamins and to contribute to the organization of lamin isoforms relative to each other in the nuclear lamina. This pool does not seem to associate with other WASH Regulatory Complex (SHRC) subunits or with Arp2/3 complex, and would affect NE budding indirectly. The second pool is incorporated into SHRC and requires Arp2/3 complex likely for Arp2/3-dependent actin assembly, which is proposed (but not demonstrated) to be directly required for NE-bud formation. In conclusion, the study is well-executed, but requires just a few clarifications and key experiments to make it even more comprehensive and relevant for a broader community.

Comments for the author

Specific points:
I think the experiments are well done and controlled, so I don’t have much to criticize on those at this point, but I would like see a small number of major issues clarified:
1) Aside from CCDC53, the authors don’t find any enrichment of other SHRC components at the nuclear periphery and coincident with NE-buds, and they postulate that this is for technical reasons due to epitope masking on respective components when associating with these structures (see page 8 1st para). I feel that it would be an important control to find a few additional SHRC-components localize at these particular subcellular locations. If the antibodies don’t work, would it be possible to ectopically express a simple tagged SHRC-component, such as equipped with a myc or HA-tag, both of which should work very well with immunolabelings.
2) All studies on SHRC subunits (other than WASH) were performed upon RNAi, but without controlling the extent of knockdown at given experimental timepoint at the protein level. Would it be possible to perform some sort of control experiment to prove that the RNAi of these subunits (that show milder phenotypes in most cases than seen for WASH) does cause a significant reduction of SHRC subunit expression? How about making genetic knockouts for at least one or two additional SHRC subunits to confirm the RNAi phenotypes?
Are they equally modest as compared to RNAi?
I am also asking this because the authors claim that WASH-KO or RNAi will reduce the expression of all SHRC-subunits, and vice versa (see 2nd sentence on page 12 top), but if that was correct, the differential effects seen upon WASH versus SHRC subunit knockdown would not be observable because SHRC subunit knockdown should also reduce WASH equally efficiently. So my question is: can the authors show a differential (i.e. less severe)
suppression of WASH expression upon SHRC subunit knockdown as compared to WASH knockdown or as compared to SHRC subunit suppression upon WASH knockdown? Something like this would actually have to be assumed if the authors’ proposal that WASH knockdown has more severe effects in their assays than SHRC knockdown was correct.
3) In the discussion, the authors elaborate on the conclusion that one of the two WASH pools, i.e. the one associated with other SHRC subunits is required for NE-budding specifically, and associates with Arp2/3 complex in this process. So my question is: can the authors actually show that NE-buds are enriched for Arp2/3 complex and actin somehow? This would be very interesting!
4) I was wondering how or why the WASH that binds to lamins does not require actin or Arp2/3 binding? What happens actually if WASH binds to lamin B? Are we supposed to assume that this WASH population does not at all activate Arp2/3 complex-dependent actin assembly, or is it only that “isolated” WASH does not require Arp2/3 complex for its function? Is lamin binding incompatible perhaps with Arp2/3 complex activation, or did I misunderstand something? If correct, could this actually be shown? These points should at least be addressed more clearly in the discussion!
5) What about heterodimeric capping protein? The authors did not mention capping protein at all in this manuscript, although I understand that it should be an important associate of SHRC during Arp2/3-mediated actin assembly. Do the authors see effects similar to SHRC knockdown/knockout if

targeting capping protein, or are effects seen even stronger perhaps than obtained with other SHRC subunits? If the authors have data on this, they should at least be mentioned.

One more minor point:
I think the abstract (in particular the last four lines or so) is quite cryptic, and does not well reflect the data shown, so I would recommend rewording this and summarizing the results more clearly and concisely.

First revision

Author response to reviewers' comments

Response to Reviews
MS ID#: JOCES/2020/243576
MS TITLE: Wash and the WASH Regulatory Complex function in Nuclear Envelope budding
AUTHORS: Jeffrey M. Verboon, Mitsutoshi Nakamura, Jacob R. Decker, Kerri A. Davidson, Vivek Nandakumar, and Susan M. Parkhurst

Changes made to the manuscript as described below are in red text.

Reviewer 1
1. Page 1 last line: Fig. 1B-C does not show mega RNPs as stated in the sentence. The figure shows the C- terminal fragment of dFz, which marks nuclear envelope buds. Re-write for clarity.

We have revised the text to make this point clear and now reference Fig.1B-C in a more appropriate place.

2. Page 4, line 88: The topic sentence for this paragraph does not relate to the contents of the paragraph in any obvious way. Either make a connection, or provide a better topic sentence.

We take the point of the Reviewer and include a new topic sentence.

3. Page 6, line 130: Again, the figures do not show megaRNPs - better to say they show "dFz2C-containing buds".

We have amended the text to clarify this point.

4. Page 7, line 179: state that ATP-Synthetase alpha is a mitochondrial activity marker here instead of on page 11, line 317.

We have amended the text as suggested.

5. Line 132: Fig. S1A shows a western blot, not confocal microscopy.

Fig. S1A was included as it shows dFz2C antibody specificity. We have amended the text so that Fig. S1A does not refer to confocal microscopy.

6. Line 152, Figure 1H-I: buds are not discernable at this magnification, so it is not justified to conclude that wash knockdown salivary gland cells lack nuclear envelope buds.

The conclusion is based on analyzing the entire periphery of 50 nuclei at 8,000x magnification for each genotype. We now include TEM images of wash null and wash RNAi at 8,000X to show that the nuclear periphery in these genotypes is devoid of NE-buds (Figs. 1H' and 1I').

7. Page 10, line 263: what cells are being analyzed?

The text has been amended to indicate that the nuclear extracts are from fly Kc cells.

8. Line 274: I see a Lamin B band in the CCDC53 pull down lane, calling into question the conclusion that "none of the SHRC subunits co-immunoprecipitated with Lamin B".

We have performed this IP many times and do not generally observe a band in the CCDC53 pull down lane. In the same figure, we do not observe a band in the Lamin B pull down lane for the top panel probed with anti-CCDC53. We have replaced this panel with that from another of the IPs performed.

9. Page 11, line 302: I disagree with the interpretation of the microscope images provided. The wash-WT rescue nucleus looks noticeably wrinkly, and it is not obvious that the wash-deltaLamB rescue phenotype is intermediate.

We appreciate the Reviewer's comment and have amended several things. We have replaced the image with one that better represents the average of 6.6 dFz2C foci/nucleus and the non-wrinkled phenotype (Fig. 5A-A") and we have also increased the number of nuclei included in our analysis of the number of buds (dFz2C foci) per nucleus (Fig. 5D). We have clarified in this section that by "intermediate" we were referring to this mutation having a smaller effect on the number of buds. We show that $wash^{\Delta\Delta LamB}$ is significantly different from *wash* null, $wash^{\Delta SHRC}$, and wildtype. Finally, we now discuss modeling the effect on the count of buds from *wash* mutations. We treat Wash as having two independent effects: disrupted Lamin activity or disrupted actin related activity (as there is no difference in the effect of $wash^{\Delta Arp2/3}$ or $wash^{\Delta SHRC}$ on nuclear buds, p=0.9). The *wash* null mutation exhibits both effects. Using poisson regression, we find a much smaller effect on the count of nuclear buds in $wash^{\Delta\Delta LamB}$ (disrupted Lamin activity) versus $wash^{\Delta Arp2/3}$ or $wash^{\Delta SHRC}$ (disrupted actin activity), where the log of expected buds decreases by 0.99 ($p < 0.0001$) for disrupted lamin activity versus 3.14 ($p < 0.0001$) for disrupted actin activity. This multivariate modelling supports our hypothesis that Wash affects NE-budding in two independent ways.

10. Page 12, line 332: missing word – "both wash and aPKC mutations almost completely…".

We have amended the text include the missing word.

11. Line 338: spell out INM before abbreviating for the first time.

We have amended the text to spell out INM.

12. Line 339: what does "megaRNP collared necks" mean?

"megaRNP collared necks" refers to sites of contact between the megaRNP and inner nuclear membrane. We have removed this phrase and amended the text as follows:
Torsin, an AAATPase, has been proposed to function in NE-bud scission from the inner nuclear membrane (INM) as Torsin accumulates at sites of contact between the megaRNP and INM, and *torsin* mutants exhibit accumulation of megaRNPs within the perinuclear space (Jokhi et al., 2013).

13. Page 14, line 399: missing words – "…and that Wash functions with Lamin B and independently of the…".

We have amended the text to include the missing words.

14. Page 15, first paragraph: The summary of the main results of the paper in the paper needs to include Lamins. Also, nuclear envelope budding appears to be a normal way for big complexes to exit the nucleus, not a way to "bypass" the NPCs.

We have revised this paragraph to include Lamins and to more precisely define NE-budding.

15. Line 407: missing word - "…like in the cytoplasm, it is likely involved…".

We have amended the text to include the missing word (now line 409).

16. Page 16, line 450: Not a great topic sentence since the paragraph is not about megaRNP components.

We take the point of the Reviewer and have removed this sentence.

Reviewer 2

1) *Throughout the present manuscript, the foci/bud count (similarly taken statically, per nucleus, at a given time) is used as a positive measure of functionality of the budding-mediated nuclear export pathway. What supports this interpretation? Alternatively (and similarly to what is encountered in studies of exocytosis), accumulation of the marker-enriched foci and buds/nascent buds could be a sign of inhibition of the further downstream steps in the budding-mediated nuclear export pathway (i.e., completion of budding, nuclear envelope lumen transit, etc.).*

The Budnik lab has shown that nuclear dFz2C foci constitute large RNP granules that become encapsulated by lamins and the inner nuclear envelope – which they termed NE-buds. In their original paper, they also showed these granules leaving the nucleus (Speese et al., 2012). We are similarly using dFz2C foci/lamin buds to define NE-buds and show that we can observe the same phenotypes that they have linked to loss of NE-budding.

2) *The authors showed previously (see p. 12, line 334) that knocking down Wash reduces the expression of the SHRC components (CCDC53, etc.) and vice versa. Does this impact the interpretation of the effects of the numerous Wash and SHRC component mutation and knock-down experiments in this paper (which have been used to differentiate between Wash and SHRC-mediated functions)?*

We note the Reviewer's point and agree that this is an essential question in this field. Our goal was to show that the effects we are describing are indeed related to Wash-SHRC function – and not SHRC or Wash doing this on their own and indirectly by knocking each other down. We believe we have shown this in several ways. First, we show that SHRC and capping protein knockdowns do not exhibit all of Wash's nuclear phenotypes (i.e., wrinkled nucleus) (Fig.3N-Q" and Fig. 8A-B"). Secondly, we show that Ccdc53 and SWIP are enriched at nuclear buds supporting these players' involvement in NE-budding (Fig. 3G-M"). We also show that Arp2/3 has similar NE-budding phenotypes, and that Arp2/3 relies on Wash (Fig. 7). Finally, we show that the *wash*$^{\Delta SHRC}$ point mutation – Wash that cannot bind the SHRC – exhibits phenotypes similar to Wash and the SHRC with respect to NE-budding (Fig. 5). We now include staining of *wash*$^{\Delta SHRC}$ nuclei with antibodies to Wash and show that Wash is still present in the nucleus (even though it doesn't bind the CCDC53 SHRC subunit) (Fig. S2J-K"). We also include staining of *wash*$^{\Delta SHRC}$ nuclei with antibodies to CCDC53 and show that it is also present in the nucleus, but similar to aPKC RNAi nuclei, it does not accumulate in foci at the nuclear periphery (Fig. S2H-I"). Thus, the same phenotypes observed with the loss of the Wash and/or SHRC proteins are also observed when these proteins are present in the nucleus but cannot interact with each other.

3) Does the essentially equal effect that is observed downstream of nuclear envelope budding (the mitochondrial effects—p. 11, line 318) in experiments perturbing the lamin and SHRC/CCDC53 interactions of Wash, despite budding being reduced to a markedly different ($p<0.0001$) extent in the lamin and SHRC/CCDC53 experiments (p. 11, lines 303, 306), suggest that the bud count as implemented here is not a measure of functionality or carrying capacity of the budding-mediated nuclear export pathway?

While we did not observe a significant difference in mitochondrial integrity between *wash*$^{\Delta SHRC}$ and *wash*mutants when assaying the activity dependent mitochondrial marker ATP-Synthetase α (Fig. 5L- O), we did observe an intermediary phenotype for *wash*$^{\Delta\Delta LamB}$ mutants when assaying poly-ubiquitin aggregates as a marker of mitochondrial damage (Fig. 5P). Examining the different phenotypes requires the use of different Gal4 drivers. Since we observe all-or-none phenotypes for the other genotypes we assayed, it is possible that there is a threshold for ATP-Synthetase α activity. We now examine neuromuscular junction integrity in *wash*$^{\Delta\Delta LamB}$ mutants as an additional assay of NE-budding function. Consistent with an intermediary phenotype for dFz2C foci/nucleus and poly-ubiquitin aggregates/mitochondrial integrity, we also observe an intermediary phenotype for the number of ghost boutons present in *wash*$^{\Delta\Delta LamB}$ mutants (Fig. 5Q- S).

Reviewer 3

1) Aside from CCDC53, the authors don't find any enrichment of other SHRC components at the nuclear periphery and coincident with NE-buds, and they postulate that this is for technical reasons due to epitope masking on respective components when associating with these structures (see page 8, 1st para). I feel that it would be an important control to find a few additional SHRC-components localize at these particular subcellular locations. If the antibodies don't work, would it be possible to ectopically express a simple tagged SHRC-component, such as equipped with a myc or HA-tag, both of which should work very well with immunolabelings.

We have tried optimizing our staining with SWIP, Strumpellin, and FAM21 antibodies in larval salivary gland nuclei. We've been successful with antibodies recognizing the SWIP subunit – super-resolution microscopy with these show enrichment of SWIP at NE-buds (Fig. 3L-M"). Consistent with Wash-SHRC working at NE- buds, we also find that Arp2/3 and capping protein are also enriched at NE-buds (see points 3 & 5 below).

2) All studies on SHRC subunits (other than WASH) were performed upon RNAi, but without controlling the extent of knockdown at given experimental timepoint at the protein level. Would it be possible to perform some sort of control experiment to prove that the RNAi of these subunits (that show milder phenotypes in most cases than seen for WASH) does cause a significant reduction of SHRC subunit expression? How about making genetic knockouts for at least one or two additional SHRC subunits to confirm the RNAi phenotypes? Are they equally modest as compared to RNAi?
I am also asking this because the authors claim that WASH-KO or RNAi will reduce the expression of all SHRC-subunits, and vice versa (see 2nd sentence on page 12 top), but if that was correct, the differential effects seen upon WASH versus SHRC subunit knockdown would not be observable because SHRC subunit knockdown should also reduce WASH equally efficiently. So my question is: can the authors show a differential (i.e. less severe) suppression of WASH expression upon SHRC subunit knockdown as compared to WASH knockdown or as compared to SHRC subunit suppression upon WASH knockdown? Something like this would actually have to be assumed if the authors' proposal that WASH knockdown has more severe effects in their assays than SHRC knockdown was correct.

We now include staining in wildtype and CCDC53 RNAi larval salivary gland nuclei to show that the CCDC53 RNAi line effectively knockdowns CCDC53 expression in this tissue (Fig. S1I-J") and that, as shown previously in other tissues, this leads to loss of Wash expression (Fig. S1K-L").
As discussed in point 2 from Reviewer 2: The loss of Wash or the SHRC per se is not needed for the NE- budding phenotypes observed. The *wash*$^{\Delta SHRC}$ point mutation exhibits phenotypes similar to Wash and the SHRC with respect to NE-budding (Fig. 5). We now include staining of *wash*$^{\Delta SHRC}$ nuclei with antibodies to Wash and show that Wash is still present in the nucleus (even though it doesn't bind the CCDC53 SHRC subunit) (Fig. S2J-K"). We also include staining of *wash*$^{\Delta SHRC}$ nuclei with

antibodies to CCDC53 and show that it is also present in the nucleus, but similar to aPKC RNAi nuclei, it does not accumulate in foci at the nuclear periphery (Fig. S2H-I”). Thus, the same phenotypes observed with the loss of the Wash and/or SHRC proteins are also observed when these proteins are present in the nucleus but cannot interact with each other.

3) In the discussion, the authors elaborate on the conclusion that one of the two WASH pools, i.e. the one associated with other SHRC subunits is required for NE-budding specifically, and associates with Arp2/3 complex in this process. So my question is: can the authors actually show that NE-buds are enriched for Arp2/3 complex and actin somehow? This would be very interesting!

We have not been able to find a nuclear actin specific antibody that works well in *Drosophila* larval salivary gland nuclei. However, we now include staining of larval salivary gland nuclei with antibodies recognizing the Arpc1 subunit of the Arp2/3 complex, where we do observe Arpc1 enrichment around NE-buds (Figure 7C-D”)., supporting a role for Wash-SHRC-Arp2/3 in NE-budding. We agree with the Reviewer that this is very interesting!

4) I was wondering how or why the WASH that binds to lamins does not require actin or Arp2/3 binding? What happens actually if WASH binds to lamin B? Are we supposed to assume that this WASH population does not at all activate Arp2/3 complex-dependent actin assembly, or is it only that “isolated” WASH does not require Arp2/3 complex for its function? Is lamin binding incompatible perhaps with Arp2/3 complex activation, or did I misunderstand something? If correct, could this actually be shown? These points should at least be addressed more clearly in the discussion!

We have shown previously that *Drosophila* Wash encodes several independent biochemical activities (actin nucleation, actin bundling, microtubule bundling, actin/microtubule crosslinking) and that the use of these activities is context-dependent (Liu et al., 2009; Verboon et al, 2015a; Verboon et al, 2015b; Verboon et al, 2018). For example, when Wash interacts with Rho GTPase, it is independent of its association with the SHRC and does not require its actin nucleation activity (Liu et al., 2009; Verboon et al, 2015a). When Wash associates with its SHRC, it uses its actin nucleation activity and requires Arp2/3 (Verboon et al. 2018). When Wash interacts with Lamin, it does not require Wash’s association with its SHRC or Arp2/3 (Verboon et al. 2015b). Our working hypothesis is that Wash’s interaction with Lamin B is required for organizing the cortical nucleoskeleton, such that Wash mutants that cannot bind Lamin result in separation of the Lamin B and Lamin C meshes from each other at the nuclear periphery. This could require Wash’s actin bundling and/or cross-linking activities rather than its actin nucleation activity. We have revised the discussion to make Wash’s context-dependence more clear.

5) What about heterodimeric capping protein? The authors did not mention capping protein at all in this manuscript, although I understand that it should be an important associate of SHRC during Arp2/3-mediated actin assembly. Do the authors see effects similar to SHRC knockdown/knockout if targeting capping protein, or are effects seen even stronger perhaps than obtained with other SHRC subunits? If the authors have data on this, they should at least be mentioned.

As suggested by the Reviewer, we examined RNAi knockdowns of capping protein (Cpa and Cpb in *Drosophila*) for their effects on NE-bud formation. Knockdown of Cpa and Cpb result in a decrease in dFz2C foci/NE-bud/nucleus (~1 dFz2C foci-lamin bud/nucleus compared to ~7 dFz2C foci-lamin bud/nucleus in wildtype) (Fig. 8A-C). Similar to SHRC and Arp2/3 knockdowns, these nuclei are spherical rather than wrinkled. In addition, we stained larval salivary gland nuclei with Cpa antibodies and find that, along with a general nuclear distribution, Cpa is enriched at lamin buds (Fig. 8D-G”).

<u>*One more minor point*</u>*:*
I think the abstract (in particular the last four lines or so) is quite cryptic, and does not well reflect the data shown, so I would recommend rewording this and summarizing the results more clearly and concisely.

We take the point of the Reviewer and have re-written the abstract to better reflect the data presented.

Second decision letter

MS ID#: JOCES/2020/243576

MS TITLE: Wash and the WASH Regulatory Complex function in Nuclear Envelope budding

AUTHORS: Jeffrey M Verboon, Mitsutoshi Nakamura, Kerri A Davidson, Jacob R Decker, Vivek Nandakumar, and Susan M Parkhurst
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The revised version of this study is significantly improved and does an excellent job of identifying how WASH and its regulatory complex participate in nuclear envelope budding. The addition of information about Arp2/3 association with buds strengthens the evidence for a role of the actin cytoskeleton in the process. The authors have satisfactorily addressed my concerns about the original submission.

Comments for the author

n/a

Reviewer 2

Advance summary and potential significance to field

This is an interesting submission that aims to elucidate the molecular mechanism of nuclear budding, a relatively recently described process of importance in diverse areas of basic cell biology, development, and biomedicine. While the molecular underpinnings of this process are only beginning to be sketched out, identification of the function of WASH complex (of the Wiskott-Aldrich Syndrome Protein family) that is undertaken in this work can make a solid contribution to this field. The manuscript is well written. The pertinent literature is cited thoroughly. The authors have previously described the WASH mutant phenotype that includes nuclear envelope deformation resembling premature aging, and the present work that assesses the complex's role in physiological nuclear budding extension is a logical extension of their approach.

The main findings are 1) Drosophila Wash participates in the spatial organization of the lamin A/C and B meshes that is conducive to budding; 2) through a distinct interaction with the SHRC regulatory complex's component CCDC53, Wash stimulates budding; 3) Wash effects budding through a direct interaction with and the activity of nuclear Arp2/3 complex (an actin polymerization nucleation molecule). While the last finding may appear unexpected to some readers, it in fact meshes well with the long list of documented functions of intranuclear actin, including its roles in intranuclear transport and egress phases of nuclear export. Thus, the paper can be a valuable addition not only to the literature on nuclear envelope budding, but also to the field of nuclear actin biology.

Comments for the author

The authors have appropriately addressed all my concerns and questions in the revision. I have no further comments and recommend publication.

Reviewer 3

Advance summary and potential significance to field

The authors have addressed my previous points and criticisms in a satisfactory fashion, and I was also intrigued to see addition of the capping protein knockdown data, which nicely mirrors phenotypes obtained with SHRC and Arp2/3 complex knockdowns.
So in conclusion, I am happy with the revised version of the manuscript!

Comments for the author

No further changes requested.