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Cargo-mediated recruitment of the endocytic adaptor protein Sla1 in *S. cerevisiae*

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MS TITLE: Cargo-Mediated Recruitment of the Endocytic Adaptor Protein Sla1

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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 found the study interesting and well organized and they also appreciated that experiments were well controlled and carefully quantified. From the reviewers' comments, you will see that there are few important points that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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.

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Reviewer 1

Advance summary and potential significance to field

The present study from the Di Pietro lab examines the contributions of discrete functional domains in plasma membrane recruitment of the endocytic adaptor protein, Sla1. Like other adaptor proteins, Sla1 interacts with sequence-based motifs (such as NPFxD) or ubiquitin modifications on cargos, as well as with coat proteins including clathrin itself as well as Pan1 and End3. The variety of interactions likely can occur simultaneously and may together play important roles in recruitment/stabilization of cargo at an endocytic site, as well as progression of the site toward formation of a clathrin-coated vesicle. The importance of these interactions is not fully understood, and this study begins to address these important questions.

In general, this is a well-organized, logical study that adds to our understanding of adaptor protein function.
Experiments are well-controlled and easy to understand from a reader's perspective. The idea that Sla1 acts as an adaptor for ubiquitinated cargo, in addition to its ability to recognize the NPFxD signal, will expand our knowledge of this adaptor's contribution to selective cargo internalization. As the authors point out in the discussion, the role of Sla1 interaction with ubiquitinated cargos vs. interaction with ubiquitinated endocytic machinery is not clear, and may be important for the overall function of the protein. I look forward to future studies aimed at addressing this key issue.

At this point, there are a few minor concerns that can hopefully be easily addressed; however, this study should be of interest to J. Cell Sci. Given the impact of the COVID-19 pandemic on research activity, I do not consider any of these suggestions to be essential for acceptance if they require significant additional experimentation. If they can be completed using data already in hand, they would make a nice addition to the paper.

Comments for the author

Minor concerns:

1. At the bottom of page 6, the last two sentences describing quantification of NPFxD mutations for Fig. 1B are imprecise. The second-last sentence suggests that mutation of SHD1 reduces maximal intensity at endocytic patches, while the last sentence states that the F507L and I531E mutations, which abolish NPFxD binding, gave the most severe reduction. The last part of this does appear to be the case, but the K525A mutation, which strongly reduces (but does not abolish) NPFxD binding, shows no significant difference in intensity compared to wild-type Sla1. This should be stated more clearly.

2. The quantification for Fig. 1B shows changes in peak patch/cytosol ratio, and total membrane/cytosol ration. Other useful metrics for quantification may include the total number of endocytic patches (or number of patches/surface area) or relative distribution of patches into the mother and bud. If they authors can use their current imaging data to provide these measurements, it would provide additional context.

3. Figure 3 reports FRAP using Sla1ΔSR-GFP. How does the recovery rate compare to that of wild-type Sla1?

4. In Figs. 5 and 6, the authors use a W391A mutation to impair association of Sla1 with ubiquitin, and show a reduction in membrane/cytosol ratio that is less severe than the I531E that abolishes NPFxD association. In both of these mutations, there is still a significant amount of protein at the cell surface. What happens if both of these mutations are combined?

5. Results page 11 and Fig. 7: the authors state that "SHD1-NPFGF* was unable to bind to GST-3xNPFSD," but the blot in Fig. 7A shows a clear band in the pulldown (lane 4). This is perhaps an overstatement, and should be revised to say that the binding is much weaker than what is seen for SHD1-NPAGF*.

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Reviewer 2

Advance summary and potential significance to field

Current models suggest that the recruitment of endocytic adaptors to membrane sites is regulated, at least in part, by binding to cargo proteins. Here, the authors test this model by taking advantage of the well-defined site binding site in the endocytic adaptor Sla1 for “NPxY” cargo. They show that mutation of this binding site reduces Sla1 recruitment to the plasma membrane, yet the SHD1 domain that contains this site does not bind strongly to the PM on its own, suggesting other sites must contribute to PM recruitment. The results presented in this paper identify two other regions of Sla1 that influence membrane binding in addition to the previously characterized Sla1 C-terminal “SR” region that binds other components of the endocytic site. They show that the third SH3 domain, which was previously shown to bind ubiquitin, helps promote PM association. It is not clear which ubiquitinated proteins are recognized at the PM, and as far as I know no ubiquitinated cargo proteins have been identified that require Sla1 for their uptake, but the idea that Sla1 binds ubiquitinated cargos is a plausible one. The authors also discover that Sla1 has a C-terminal “NPXY” motif that competitively inhibits the binding of cargo signals to the SHD1 domain. I found this to be an intriguing finding, although no evidence was presented to show this presumed autoinhibitory interaction alters rates of endocytosis, or coat formation/progression. Instead, the authors show that mutation of the NPxY site influences the dynamics of the Sla1-interacting Pan1 protein at the endocytic site, which supports the idea that cargo binding is coupled to endocytic progression.

Overall, I found this paper to be well written and of high technical quality. I appreciated the fact that most experiments were carefully quantified, and that the paper uses a mix of in vitro binding assays and localization data. The results as a whole are interesting, and support the model that cargo interactions are important for adaptor recruitment and endocytic patch progression. The role of the C-terminal motif, while not thoroughly characterized here, will be an interesting topic for future study.

Comments for the author

Specific comments:

The point mutations studied in this paper were previously shown to reduce binding to an NPXY peptide without causing gross changes in overall protein structure. However, the mutations may cause more subtle alterations in protein folding, and any protein instability would invalidate the conclusions of the imaging studies. While a supplemental figure is included to show the mutant proteins are indeed stable, this figure is not quantified (unlike most others in the paper) and to my eye at least one of the mutants (F507L) has reduced stability. Thus it will be important to do this quantification to ensure all mutants are indeed stable.

Potential extensions of the study:

1. The discovery of a putatitve autoinhibitory interaction was intriguing and it would be interesting to see more followup studies, although these may be beyond the scope of the current manuscript. For example, does mutation of this autoinhibitory sequence help rescue mutants with a partial defect in NPxY binding, and does it alter the progression of the endocytic patch as measured by Pan1 dynamics?

2. The delta-SR mutant isn't completely diffuse - instead it appears to be at patches around the perimeter of the cell. The yeast plasma membrane has many subdomains. Do these patches correspond to a specific type of PM domain, where critical cargo are believed to reside?

Additional minor comments:

1. The schematic diagram showing the various fragments examined in Figure 5 are illustrated in Figure 1. It would be more helpful to instead have a diagram explaining the fragments in Figure 5 itself.

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2. Details of the statistical tests used should be given in the Figure legends or Materials and Methods

3. Pg8 has a typo: “Wisc1-GFP”

Reviewer 3

Advance summary and potential significance to field

In this manuscript, the authors address the mechanisms by which endocytic adaptor proteins get recruited to the site of endocytosis. Specifically, they focus on the role of cargo interactions in recruiting endocytic adaptors. This is an important question, which has remained quite poorly understood. The key challenge has been that adaptors likely use multiple different interactions with both other endocytic proteins and cargo molecules to get recruited to the plasma membrane. This has made it difficult to go from knowledge of individual protein-protein interactions to functional conclusions at cell biology level.
The authors take yeast Sla1 protein as an example of an endocytic cargo adaptor and use genetics biochemistry and quantitative live cell imaging to show that cargo interactions, via SHD1 and SH3 domains have a significant role in recruiting Sla1 to the plasma membrane. The experiments are well performed and controlled, the conclusions are well supported by the data. The findings are interesting and significantly add to our knowledge of endocytic mechanisms by suggesting that the cargo interactions may directly influence the assembly of the endocytic coat proteins.

Comments for the author

I have only a few minor suggestions that the authors may want to consider.

Is the fluorescence recovery in the FRAP experiment corrected for the loss of fluorescence due to the photobleaching? It seems that the bleached area is relatively large and therefore a large fraction of the total cellular pool of the fluorescent protein is bleached. If this is not taken in consideration the true recovery is underestimated. Eyeballing from the micrographs in the figure 3A it seems that the actual recovery would be much higher that about 25%. I.e. at 40s the signal in the bleached area seems quite similar to the signal on the unbleached side of the cell, not just 25%. In any case this would not change the conclusions drawn by the authors but would emphasize the effect observed.

Fig1A: the amino acids that are point mutated could be marked in the Sla1 protein cartoon, so the reader can easily see which domains are affected (SHD1 and SH3).

Personally, I would not end the discussion section with a paragraph about Sla1’s nuclear localization. This paper does not add much to the understanding of Sla1’s possible nuclear function. Otherwise the discussion is clear and to the point.

First revision

Author response to reviewers' comments

Reviewer 1

1. At the bottom of page 6, the last two sentences describing quantification of NPFxD mutations for Fig. 1B are imprecise. The second-last sentence suggests that mutation of SHD1 reduces maximal intensity at endocytic patches, while the last sentence states that the F507L and I531E mutations, which abolish NPFxD binding, gave the most severe reduction. The last part of this does appear to be the case, but the K525A mutation, which strongly reduces (but does not abolish) NPFxD binding,

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shows no significant difference in intensity compared to wild-type Sla1. This should be stated more clearly.

We agree with the reviewer and adjusted the text accordingly (page 6 of the revised manuscript).

2. The quantification for Fig. 1B shows changes in peak patch/cytosol ratio, and total membrane/cytosol ration. Other useful metrics for quantification may include the total number of endocytic patches (or number of patches/surface area) or relative distribution of patches into the mother and bud. If they authors can use their current imaging data to provide these measurements, it would provide additional context.

We followed the suggestion and quantified the number of patches per cell. Results are consistent with the peak patch/cytosol ratio and total membrane/cytosol ratio quantification showing no defect for Sla1K525A-GFP and a reduction for Sla1F507L-GFP and Sla1I531E-GFP (pages 6 and 7 of the revised manuscript).

3. Figure 3 reports FRAP using Sla1ΔSR-GFP. How does the recovery rate compare to that of wild-type Sla1?

While Sla1ΔSR-GFP is largely immobile and displays fairly constant intensity at the cell surface, wild type Sla1-GFP is present in dynamic patches with intensity that is not constant but rather increases over time, reaches a peak and quickly decreases. Thus, the recovery phase would give different results depending on the stage of the endocytic patch at the time of photobleaching and would not reach a plateau we could quantify as observed for Sla1ΔSR-GFP. Thus, it would be difficult to compare FRAP experiments between wild type Sla1-GFP and Sla1ΔSR-GFP. Given the limited ability to perform additional experiments we dedicated our efforts to other aspects of the revision.

4. In Figs. 5 and 6, the authors use a W391A mutation to impair association of Sla1 with ubiquitin, and show a reduction in membrane/cytosol ratio that is less severe than the I531E that abolishes NPFxD association. In both of these mutations, there is still a significant amount of protein at the cell surface. What happens if both of these mutations are combined?

The reviewer suggests an interesting experiment and we tried to carry it out. Unfortunately, our attempts to generate the double mutant cells failed or produced strains in which additional mutations were also introduced in the SLA1 gene. Therefore, we were unable to carry out the imaging experiment.

5. Results page 11 and Fig. 7: the authors state that “SHD1-NPFGF* was unable to bind to GST-3xNPFSD,” but the blot in Fig. 7A shows a clear band in the pulldown (lane 4). This is perhaps an overstatement, and should be revised to say that the binding is much weaker than what is seen for SHD1-NPAGF*.

We agree with the reviewer and modified the text accordingly (page 11 and 28 of the revised manuscript).

Reviewer 2

1. The point mutations studied in this paper were previously shown to reduce binding to an NPXY peptide without causing gross changes in overall protein structure. However, the mutations may cause more subtle alterations in protein folding, and any protein instability would invalidate the conclusions of the imaging studies. While a supplemental figure is included to show the mutant proteins are indeed stable, this figure is not quantified (unlike most others in the paper) and to my eye at least one of the mutants (F507L) has reduced stability. Thus it will be important to do this quantification to ensure all mutants are indeed stable.

The reviewer’s comment is well considered. Even though we did not include the data in the original manuscript, we had performed multiple independent cell extracts and immunoblotting experiments to corroborate all Sla1 mutants are indeed stable. We now show quantification of these replicates

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along with the immunoblotting analysis and quantification of two new mutant strains generated for this revised manuscript (Figure S1 of the revised manuscript).
As a side note, even wild type Sla1-GFP is prone to some degradation during yeast cell extract preparation and immunoblotting analysis. This is probably due to its large size and combination of multiple folded domains with unstructured linker regions. Thus, when analyzing multiple samples simultaneously in any given experiment, it is difficult to obtain all of them as intact full-size bands. In any case, when multiple biological replicates were analyzed, the only trend we noticed was that some mutants appeared to show slightly increased rather than decreased levels relative to wild type Sla1-GFP. However, none of the differences reached statistical significance.

2. The discovery of a putative autoinhibitory interaction was intriguing and it would be interesting to see more follow up studies, although these may be beyond the scope of the current manuscript. For example, does mutation of this autoinhibitory sequence help rescue mutants with a partial defect in NPxY binding, and does it alter the progression of the endocytic patch as measured by Pan1 dynamics?

We agree with the reviewer and performed the suggested experiment. We chose the Sla1F507L-GFP mutant, which shows a recruitment defect but less severe than the defect observed for Sla1I531E-GFP. We created a strain carrying both the F507L mutation and a deletion of the C-terminal NPFGF-COOH inhibitory sequence (Sla1F507LΔNPFGF-GFP). For comparison, we also created a strain carrying only a deletion of the C-terminal NPFGF-COOH inhibitory sequence (Sla1ΔNPFGF-GFP). Fluorescence microscopy analysis showed the plasma membrane levels of Sla1F507LΔNPFGF-GFP are higher than Sla1F507L-GFP suggesting that mutation of the NPFGF-COOH sequence indeed rescued the recruitment defect although not completely. This result is consistent with the idea of NPFGF-COOH acting as an autoinhibitory motif.
Interestingly, mutation of the NPFGF sequence alone (Sla1ΔNPFGF-GFP) causes a modest but statistically significant decrease in plasma membrane recruitment relative to Sla1-GFP. This result implies the NPFGF-COOH sequence may also contribute positively to Sla1 recruitment indicating a more complex function. As the reviewer pointed out, full functional characterization of the NPFGF-COOH motif will necessitate significant additional work outside the scope of this manuscript, but these new data shows it is in fact functionally important in the context of full length Sla1.
The new results are shown in Figure S3 (described in pages 11 and 12, and discussed in page 14 of the revised manuscript).

3. The delta-SR mutant isn't completely diffuse - instead it appears to be at patches around the perimeter of the cell. The yeast plasma membrane has many subdomains. Do these patches correspond to a specific type of PM domain, where critical cargo are believed to reside?

We agree with the reviewer. In fact, Figure 3B shows that the patchy localization of Sla1ΔSR-GFP in part coincides with Pan1-mCherry patches, which mark CME sites, but other patches do not colocalize with Pan1-mCherry. We modified the text to indicate that while Sla1ΔSR-GFP is more diffuse than wild type Sla1-GFP, it does show a patchy localization. We also included the idea that this Sla1ΔSR-GFP localization may be driven by higher cargo concentration at various plasma membrane regions (page 8 of the revised manuscript).

Minor comments:
1. The schematic diagram showing the various fragments examined in Figure 5 are illustrated in Figure 1. It would be more helpful to instead have a diagram explaining the fragments in Figure 5 itself.

We made the change as suggested by the reviewer (Figures 1 and 5 of the revised manuscript).

2. Details of the statistical tests used should be given in the Figure legends or Materials and Methods

Following the reviewer's suggestions, we added a separate section on Statistical Analysis under Materials and Methods (page 21 of the revised manuscript).

3. Pg8 has a typo: "Wisc1-GFP"

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The typo was corrected (page 9 of the revised manuscript).

Reviewer 3

1. Is the fluorescence recovery in the FRAP experiment corrected for the loss of fluorescence due to the photobleaching? It seems that the bleached area is relatively large and therefore a large fraction of the total Page 5 of 5 cellular pool of the fluorescent protein is bleached. If this is not taken in consideration the true recovery is underestimated. Eyeballing from the micrographs in the figure 3A it seems that the actual recovery would be much higher that about 25%. I.e. at 40s the signal in the bleached area seems quite similar to the signal on the unbleached side of the cell, not just 25%. In any case this would not change the conclusions drawn by the authors but would emphasize the effect observed.

We agree with the reviewer and corrected the data accordingly (Figure 3A of the revised manuscript).

2. Fig1A: the amino acids that are point mutated could be marked in the Sla1 protein cartoon, so the reader can easily see which domains are affected (SHD1 and SH3).

We added the SHD1 and SH3 point mutations in the Sla1 cartoon (Figure 5A of the revised manuscript).

3. Personally, I would not end the discussion section with a paragraph about Sla1's nuclear localization. This paper does not add much to the understanding of Sla1's possible nuclear function. Otherwise the discussion is clear and to the point.

We agree with the reviewer and eliminated the paragraph.

Second decision letter

MS ID#: JOCES/2020/247684

MS TITLE: Cargo-Mediated Recruitment of the Endocytic Adaptor Protein Sla1

AUTHORS: Thomas O Tolsma, Hallie P Febvre, Deanna M Olson, and Santiago M Di Pietro
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.

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