JCB

A selective transmembrane recognition mechanism by a membrane-anchored ubiquitin ligase adaptor

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Review Timeline:

Submission Date:	2020-01-19
Editorial Decision:	2020-02-11
Revision Received:	2020-09-10
Editorial Decision:	2020-10-21
Revision Received:	2020-10-25

Monitoring Editor: William Prinz

Scientific Editor: Tim Spencer

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.)

DOI: https://doi.org/10.1083/jcb.202001116

February 11, 2020

Re: JCB manuscript #202001116

Dr. Ming Li
University of Michigan
Molecular, Cellular, and Developmental Biology
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1105 N. University Ave.
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Dear Dr. Li,

Thank you for submitting your manuscript entitled "A selective transmembrane recognition mechanism by a membrane-anchored ubiquitin ligase complex". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

Both reviewers felt your study was well done and convincing but share significant concerns about how some of your findings are presented and interpreted. All of the reviewers' concerns should be addressed. Many do not require additional experimentation; both reviewers felt you should tone down or refine a number of your conclusions. However, some additional work will be necessary. It is particularly important to address these four issues. First, if possible, please measure the transport activity of the most important mutants to understand how transport defects might impact your conclusions (Reviewer 1, point 1). Second, determine whether the Ssh4 mutants that do not degrade Ypq1 affect the clearance of other transporters (Reviewer 1, point 3). Third, determine whether the oligomeric state of Ypq1 is regulated by lysine and, if so, how this affects your conclusions (Reviewer 2, point 2). Fourth, both reviewers ask for additional evidence that endogenous Ypq1 and Ssh4 interact directly (Reviewer 1, points 5, 6; Reviewer 2, point 4).

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

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When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

William Prinz, Ph.D.
Monitoring Editor

Marie Anne O'Donnell, Ph.D.
Scientific Editor

Journal of Cell Biology

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

In this study, Arines and colleagues investigate how abundance of Ypq1, a lysine transporter, of the yeast vacuole is regulated by lysine availability. More specifically, they investigate how Ypq1 is recognized by Ssh4, a transmembrane protein that serves as an adaptor for the recruitment of the Rsp5 ubiquitin E3 ligase to Ypq1. Taking advantage of structural models based on related transporters as well as extensive unbiased mutagenesis experiments, biochemistry experiments and imaging assays; the authors identify residues within transmembrane domains 5 and 7 of Ypq1 that are required for Ssh4 interactions and Ssh4-dependent down-regulation in response to prolonged lysine starvation. The relevance of key amino acids in transmembrane domain 5 of Ypq1 for interactions with Ssh4 is supported by experiments that generate and test complementary pairs of mutations at putative sites of contact between the two proteins. Collectively, these experiments provide interesting new insights into how changes in lysine availability and resulting changes in transport activity of Ypq1 may control interactions between Ypq1 and Ssh4 and the Rsp5-dependent ubiquitylation and down-regulation of Ypq1. The manuscript is well written, data quality is high and data presentation is effective. The concerns outlined below are generally modest but are important for maximum clarity and for aligning conclusions more narrowly with the limitations of

the data.

1. Is lysine transport activity required for Ypq1-Ssh4 interaction? In addition to the Crapeau et al, 2014 study that is cited in this manuscript, additional studies have proposed that yeast plasma membrane nutrient transporters undergo transport-dependent, adaptor-mediated, ubiquitylation and degradation (PMIDs: 28965784 and 20002879). In these studies, the authors demonstrated that transporter transmembrane mutants which were defective in substrate transport were also impaired in their vacuolar degradation. As the current manuscript did not perform transport assays on the mutants implicated in Ssh4 interactions, the authors should discuss the impact that transport defects would have on their conclusions. Measuring transport activity of key mutants would help to address this concern. However, this may be beyond the scope of the current study and is made challenging by the vacuole localization of Ypq1.
2. Suppression scanning mutagenesis results show that TM5 and TM7 of Ypq1 are critical for the putative interaction with Ssh4. However, other residues (e.g. in loop 1-2 and even 2 residues in TM3) are also important for this interaction and/or Ypq1 degradation. This suggests a more complex explanation than what is proposed by the authors. Indeed, 55 out of 99 mutants affecting Ypq1 degradation occur outside of transmembrane domains. Although the abstract directly states that the "binding site" for Ssh4 is formed by TM5 and TM7, it is not obvious that additional contacts between Ypq1 and Ssh4 can be excluded and thus whether the "binding site" is fully defined.
3. Are Ssh4 TMD mutants that fail to interact with and degrade Ypq1 also deficient in supporting the clearance of other transporters that were recently proposed to be recognized by cytoplasmic regions on Ssh4 (Sardana, Zhu and Emr; PMID 30361468). The answer to this question would help to test conclusions about specificity and directness of proposed TMD interactions between Ypq1 and Ssh4.
4. Two different templates were used for homology modeling (4CTG, OsSWEET in Fig. 1 and E. coli semisweet in Fig. 6). Does use of the different templates have a major impact on the models for Ypq1? It would be helpful to have some explanation on this topic.
5. Page 12. It is claimed that an immunoprecipitation demonstrates a direct interaction between Ypq1 and Ssh4. This conclusion may be correct but over-reaches what can be formally concluded as the possibility of other interactors cannot be ruled out from this type of experiment.
6. Over-expression of mutant proteins was required to detect the weak interaction between Ypq1 and Ssh4. Can an interaction between the endogenous proteins be detected in the absence of Rsp5?
7. In addition to Li et al (Mol. Cell 2015) which reported a role for Ssh4 in the degradation of Ypq1, the subsequent study which clarified mechanisms of ESCRT-dependent clearance of ubiquitinated Ypq1 at the vacuole (PMID 28661397) should also be cited and explained as it is relevant for explaining how Ypq1 is cleared following ubiquitination.
8. It was recently shown that PQ motif mutations in the human PQLC2 transporter prevent interactions with the cytoplasmic WDR41 protein (PMID 31851326). Thus, it should also be mentioned that transport associated changes within transmembrane regions can influence the interactions of this family of transporters with cytoplasmic binding partners.
9. In many fluorescence microscopy experiments, vacuole fragmentation is observed following lysine starvation for a long period (6h). Is there any physiological relevance or explanation for this phenomenon?

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

The manuscript by Ming and colleagues studies the mechanism of substrate recognition by SSh4, which is substrate adaptor for the E3 ubiquitin ligase Rsp5. They used the multi-spanning lysine

transporter Ypq1 as the substrate. Ypq1 is localized to the membrane of the yeast vacuole, but translocated into the vacuole lumen for degradation when lysine concentration in the cytosol is low. The authors first used structure-based mutagenesis to identify a few Ypq1 mutants that undergo constitutive degradation in a SSh4 dependent manner. Using these mutants, they performed a suppressor screen, which revealed critical roles for certain residues in the PQ motifs, the TM5 and TM7 regions for both constitutive degradation of Ypq1 mutants and lysine dependent degradation of wild type Ypq1. They then performed scanning mutagenesis, focusing on TM5 and TM7, which confirmed the role of these TM domains in Ypq1 degradation. They hypothesized that these TM domains may interact with the transmembrane domain of SSh4, so they did a similar scanning mutagenesis study on the TM domain of SSh4, which showed that the essential residues are all clustered on one size of the TM domain. To further prove their model that the TM of Ssh4 interacts with the TM5 and TM7 of Ypq1, they performed charge complementation experiments, which showed that when opposite charges are introduced to these TM domains of these proteins, respectively they can rescue the degradation defects caused by the introduction of a single charged residue. Lastly, they showed that the effect of charge complementation could be abolished if they mutated the PQ motifs.

Overall, the study was elegant and well controlled. Experimental data are beautiful and convincing. The main problem I have is regarding writing data presentation. I feel that the paper needs to be thoroughly edited to improve the clarity. There are a few places where the authors need to tone down their conclusions. Lastly, a few experiments need to include some additional controls.

Specific points:

1. The title is misleading. The study does not touch upon Rsp5. They cannot claim that they have examined the interaction of Ypq1 with an E3 ligase complex.
2. Figure 1C shows a structural model of Ypq1, but Figure 6 shows another model of Ypq1. What is the difference between these models? Were they made from the same template? If so, does the model in Figure 1C represent the outward-open, inward-open, or the occluded conformation? The model in Figure 6E shows was based on the E. coli transporter SemiSWEET. Since SemiSWEET functions as a homo-dimer, I am surprised that the authors never comment on the oligomeric state of Ypq1. If it also functions as a dimer, how will that affect the relative positions of TM5 and TM7? Is the oligomeric state of Ypq1 regulated by lysine transport? Will the proposed model still be valid if it works as a dimer?
3. Figure 2B, they need to show what these residues have been mutated to in the screen. It is also puzzling why the authors never comments on where the identified TM5, TM7 mutations are on their structural models. Are these residues consistent with the ones identified by scanning mutagenesis? My quick scanning of Figure 2B and Figure 3D suggests that the two methods only give rise to partial overlapping results. For example, for TM5, G216, L224, S226 were identified as suppressor residues from the screen, but could not be confirmed by scanning mutagenesis. Please comments on this discrepancy.
4. Figure 2G shows a co-IP experiment, suggesting that Ypq1 mutants defective in SSh4-mediated degradation cannot interact with SSh4. Please explain why these three mutants were chosen to represent the identified mutants. Additionally, the authors conclude on page 11 that the experiment demonstrates a direct interaction of the two proteins, but co-IP cannot be taken as a proof for direct interaction. They need to tone down their conclusion. This experiment also misses a key gel showing that WT Ypq1 and mutants are expressed at the same level. They also need to comment on why the loop mutant F49I also affects the binding, or at least mention that their experiments did not exclude the involvement of other sequences in the binding.
5. Figure S6 (related to Figure 4) shows that the SSh4 mutants generated are properly localized to the vacuole, but the authors never comments on why some mutants are in the lumen while other are on the membrane.
6. Figure 6B shows the positions of the charged residues that had been introduced to Ypq1. Can

the authors comment on where the membrane is? Are these residues exposed to the cytosol when the interaction occurs? If these residues are embedded in the membrane, how can these charged residues become protonated or deprotonated given that there should be no water molecules in the lipid bilayer? Also, why was the occluded conformation never mentioned in the discussion?
7. Even though the charge complementation assay in Figure 5 provides strong evidence, supporting the conclusion that direct interaction likely occurs between the transmembrane domains of Ypq1 and that of SSh4, the authors should include a statement saying that other regions outside of the membrane may still contribute to the interaction. Otherwise, why does SSh4 have such a large cytosolic domain, but only needs a small motif to recruit Rsp5.
8. While discussing the model, they propose that the transporter may be rapidly shuttling between different conformations during lysine transport, making it difficult to interact with Ssh4. Since they can generate structural models of Ypq1, can they comment on which conformation is mostly like the one that bind Ssh4 (e.g. TM5 would be more likely exposed)? If the binding occurs in one the three conformations during lysine transport, that would indicate that Ypq1 in the absence lysine transport should more or less take the same conformation. Is that true?
Other points:
1. Page 4, "two pioneer studies....". The earlier work from Juan Bonifacino on TCRalpha degradation should be cited as well. It was shown by J. Bonifacino (EMBO 1991) that a single charged residue in the TM domain of a type I membrane protein is sufficient to retain the protein in the ER for ER.
2. Page 7, "The first and last three TMs form two parallel triple helix bundles....". I don't quite get this. How can 4 TM domains form two triple helix bundles?
3. Page 15, "Among these, six strongly blocking mutants....". Please list these six mutants here. Same page down, "we mutated the first residue....". The first residue is not clear,
4. Page 17, "consistently, we saw an increased sorting of.....". This statement is not accurate because endogenous level of Ssh4 S52R only increase sorting in the absence of lysine.
5. Page 17, "the occurence of charge complementation....." is contradictory to the observation that under overexpression conditions, complementation was also seen in the presence of lysine.

1st Revision - Authors' Response to Reviewers: September 10, 2020

Reviewer #1:

1. Is lysine transport activity required for Ypq1-Ssh4 interaction? In addition to the Crapeau et al, 2014 study that is cited in this manuscript, additional studies have proposed that yeast plasma membrane nutrient transporters undergo transport-dependent, adaptor-mediated, ubiquitylation and degradation (PMIDs: 28965784 and 20002879). In these studies, the authors demonstrated that transporter transmembrane mutants which were defective in substrate transport were also impaired in their vacuolar degradation. As the current manuscript did not perform transport assays on the mutants implicated in Ssh4 interactions, the authors should discuss the impact that transport defects would have on their conclusions. Measuring transport activity of key mutants would help to address this concern. However, this may be beyond the scope of the current study and is made challenging by the vacuole localization of Ypq1.

We thank the reviewer for this suggestion. We regret that we were not able to do the transport assays as our lab was not yet equipped with the necessary set-up as of this paper's review. We pursued some on-campus and off-campus collaborations previously, [redacted] but the lab shutdowns during the pandemic made it unlikely. However, we agree that the possibility of a direct relationship between lysine transport and Ypq1 recognition is very exciting, and we plan to explore this in our future work. We cited the suggested papers in the Introduction (**page 3, lines 42-43**).

2. Suppression scanning mutagenesis results show that TM5 and TM7 of Ypq1 are critical for the putative interaction with Ssh4. However, other residues (e.g. in loop 1-2 and even 2 residues in TM3) are also important for this interaction and/or Ypq1 degradation. This suggests a more complex explanation than what is proposed by the authors. Indeed, 55 out of 99 mutants affecting Ypq1 degradation occur outside of transmembrane domains. Although the abstract directly states that the "binding site" for Ssh4 is formed by TM5 and TM7, it is not obvious that additional contacts between Ypq1 and Ssh4 can be excluded and thus whether the "binding site" is fully defined.

We agree that Ypq1 and Ssh4 likely rely on several regions in addition to TM helices for their interaction. In this paper, we only studied transmembrane interactions in detail. In doing so, we ended up overstating the importance of TM helices over non-TM regions, when they all may play important roles. We have modified the text in several places to reflect this point:

In the **Abstract, line 25**, we changed "We show the binding site is formed…" to "We show evidence of an interaction between two transmembrane…"

In **Results, page 13, lines 278-280 (re: TM3):**
"*Although we do not know the role of the two suppressor residues in TM3 yet*, here we underscore the importance of TM5 and TM7 in mediating Ypq1 degradation ..."

Indeed, 55 out of 99 mutants affecting Ypq1 degradation occur outside of transmembrane domains.

Out of 99 mutants isolated from our screen, 17 mutants were trapped in the ER due to ER exit signal mutations, 9 were trapped in the ER due to folding defects or other mechanisms, and 7 had weak phenotypes. This leaves 66 Ypq1-GFP mutants that localized on the vacuole membrane, which are most informative in our study. Out of these, 44 had mutations on

transmembrane helices, specifically TM5 or TM7. This represents two-thirds of the instances and we believe that this is significant.

To reflect this point, we changed “44 out of 99 mutants” to “44 out of 66 VM-localized mutants” (**page 12, line 257**).

3. Are Ssh4 TMD mutants that fail to interact with and degrade Ypq1 also deficient in supporting the clearance of other transporters that were recently proposed to be recognized by cytoplasmic regions on Ssh4 (Sardana, Zhu and Emr; PMID 30361468). The answer to this question would help to test conclusions about specificity and directness of proposed TMD interactions between Ypq1 and Ssh4.

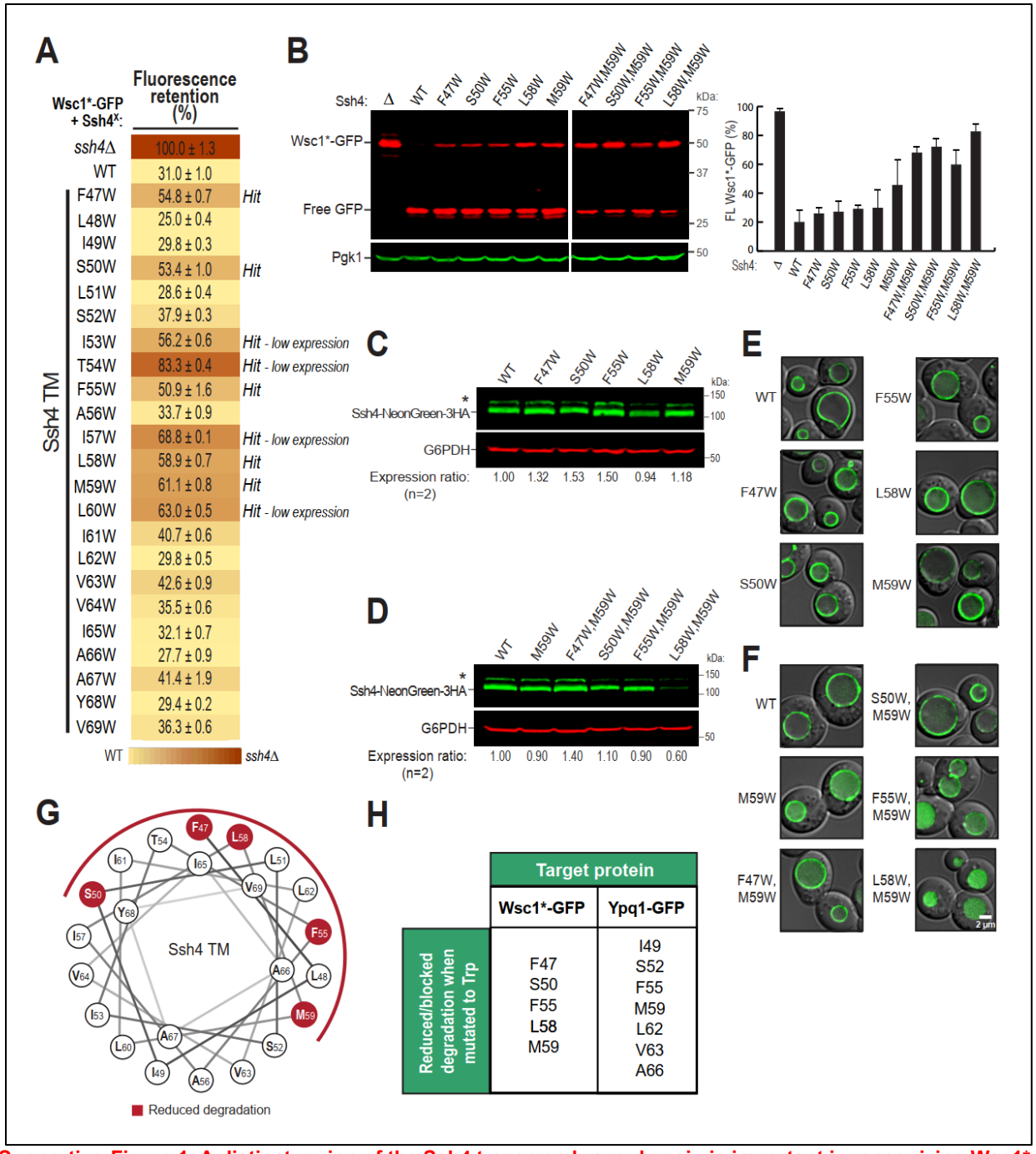
We thank the reviewer for this insight. We generated the Wsc1-EQSPLL-GFP (hereafter, Wsc1*-GFP) mutant described in Sardana et al.[1] and combined it with Ssh4 TM mutants. Wsc1 is a plasma membrane protein, while Wsc1*-GFP harbors an artificial di-leucine motif that redirects it to the vacuole membrane, where it is recognized by Ssh4 for luminal degradation. Using flow cytometry, we surveyed the degradation of Wsc1*-GFP by Ssh4 mutants (**Supporting Figure 1A**). Since Wsc1*-GFP is constitutively degraded, the fluorescence retention values for this experiment were lower than what we observed for Ypq1-GFP. We found several Ssh4 TM mutants that conferred a partial block. Mutants that did not localize properly or were unstable based on Fig. S6 were excluded.

We confirmed our flow cytometry results with western blot and found partial stabilization of the full-length Wsc1*-GFP (**Supporting Figure 1B**). Of note, similar to Sardana et al., we also initially found Wsc1*-GFP to appear as multiple bands following a trichloroacetic acid (TCA)-based protein extraction protocol. However, we were able to collapse Wsc1*-GFP into a single band by using a non-TCA-based de-glycosylation buffer system (NEB Cat. No. P0704S) and by swapping the secondary antibodies (800CW goat anti-mouse and 680LT goat anti-rabbit).

We also tested double mutants and found the double mutants further increased the stabilization of Wsc1*-GFP. We also showed that the single mutants and most of the double mutants are well-expressed and localized properly on the vacuole membrane (**Supporting Figure 1C-F**).

These findings suggest that the transmembrane helix of Ssh4 indeed plays a role in its recognition of Wsc1*-GFP, although not as great a role as in the recognition of Ypq1-GFP. Furthermore, mapping the identified residues on a helical wheel suggests that Ssh4 probably uses a distinct face to recognize different cargo (**Supporting Figure 1G-H**, compare Fig. 4F). This presents a possibility that Ssh4 assigns different regions in its transmembrane helix to recognize various motifs, thereby achieving both specificity and range, consistent with its role as an E3 adaptor for a wide range of cargo[1–3]. Elucidating the details regarding multiple cargo recognition is beyond the scope of our current study, but we are interested in studying this further in the future.

We have incorporated these data as **Fig. S5**, and a description is added to the main text (**Results page 16, lines 336-348** and **Discussion page 25, lines 539-543**).



Supporting Figure 1. A distinct region of the Ssh4 transmembrane domain is important in recognizing Wsc1*-GFP. A) Flow cytometry heat map showing the degradation defect on Wsc1-EQSPLL-GFP (hereafter, Wsc1*-GFP) imparted by Ssh4 transmembrane domain mutants (cutoff = 50%). B) *Left*: Western blot showing the degradation of Wsc1*-GFP in the presence of single-residue and double-residue mutants. *Right*: Quantification (± SD, *n*=3). C-D) Protein levels of NeonGreen-3HA-tagged single-residue and double-residue Ssh4 mutants. E-F) Subcellular localization of NeonGreen-3HA-tagged single-residue and double-residue Ssh4 mutants. G) Helical wheel showing the position of residues conferring partial degradation block when mutated to Trp. H) Summary of Ssh4 TM residues that block/reduce Wsc1*-GFP or Ypq1-GFP degradation when mutated to Trp.

4. Two different templates were used for homology modeling (4CTG, OsSWEET in Fig. 1 and E. coli semisweet in Fig. 6). Does use of the different templates have a major impact on the models for Ypq1? It would be helpful to have some explanation on this topic.

OsSWEET was used as a preliminary model because it had the highest homology based on unbiased/automatic homology modeling. This was a good template because similar to Ypq1, it is a eukaryotic protein and also localizes on the vacuolar membrane (in rice). However, for the purpose of linking Ypq1 degradation to conformational changes during transport (as in Fig. 6), OsSWEET is not a suitable model because its structure had only been solved in one conformation (inward-open)[4].

So far, only a few members of the PQ-loop family have been solved in different conformations, and among the most studied is SemiSWEET. This protein occurs in several species of bacteria, and had been crystallized in *E. coli*[5], *L. biflexa*[6,7], *Vibrio sp.*[6], *B. japonicum*, and *T. yellowstonii* [8]. For this purpose, we used the inward-open (PDB ID: 4x5m) and outward-open (PDB ID: 4x5n) crystals from *E. coli* to model Ypq1 in two different conformations.

We apologize that this shift in templates have caused confusion. We have added an explanation of this in our main text **(page 20, lines 440-447)**.

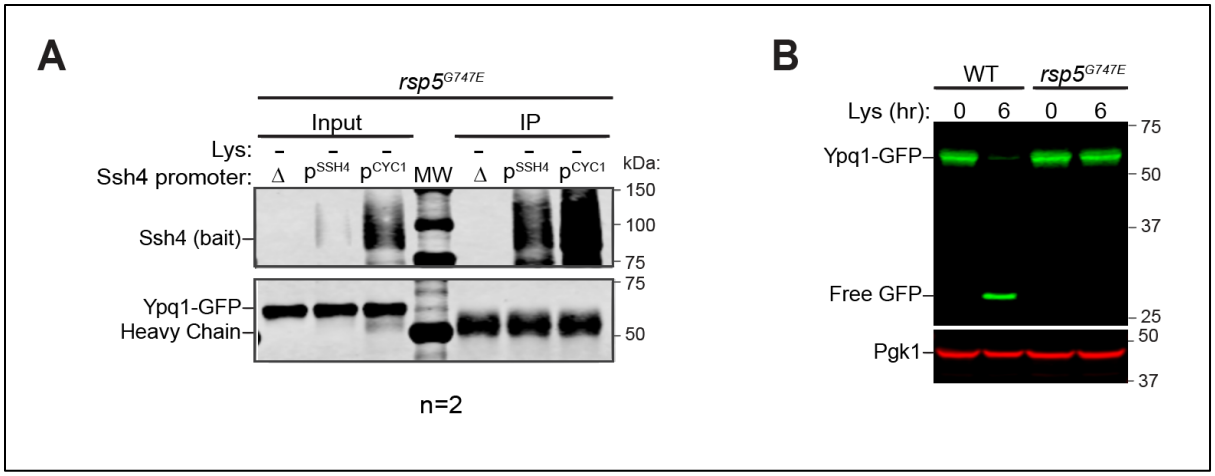
5. Page 12. It is claimed that an immunoprecipitation demonstrates a direct interaction between Ypq1 and Ssh4. This conclusion may be correct but over-reaches what can be formally concluded as the possibility of other interactors cannot be ruled out from this type of experiment.

Thank you for this feedback. We have toned down the text accordingly **(page 12, lines 241-248)**. We also changed mentions of “interact” or “directly interact” with “associate”.

6. Over-expression of mutant proteins was required to detect the weak interaction between Ypq1 and Ssh4. Can an interaction between the endogenous proteins be detected in the absence of Rsp5?

This is a great point but a difficult one. We have been trying for several years and failed numerous times to show the native interaction between Ypq1 and Ssh4. There are several possible reasons: 1) endogenous Ssh4 expression level is very low, 2) the Ypq1-Ssh4 interaction is transient and weak, 3) the Ssh4-Ypq1 interaction quickly leads to the ubiquitination and degradation of Ypq1. The conditions presented in the original manuscript (i.e. overexpressing Ssh4 and mutating the PY motifs, using Ssh4 antibody for IP, enriching Ssh4 by using it as a bait) are the best ones we have tested.

Thank you for the suggestion. However, Rsp5 is an essential gene in yeast and cannot be completely knocked out[9]. We have instead repeated the co-IP in Fig. 2F in a hypomorphic *rsp5*^{G747E} mutant strain[10,11] to reduce Ypq1 ubiquitination and degradation after interacting with Ssh4. The G747E mutation blocked Ypq1 degradation (**Supporting figure 2B**). It allowed us to use wild-type Ssh4 (i.e. PY motifs are intact) as a bait to pull down Ypq1-GFP. We expressed Ssh4 at both endogenous (P^{SSH4}) and overexpression (P^{CYC1}) levels and performed the IP experiments. However, using these conditions, we failed to pull down Ypq1-GFP, even after overexpressing Ssh4. It could be possible that Rsp5 recruitment to Ypq1 may sterically compete with the binding between Ypq1 and Ssh4.



Supporting Figure 2. Co-immunoprecipitation of Ypq1 and Ssh4 in the presence of an Rsp5 mutant. A) Co-IP of Ypq1-GFP with wild-type Ssh4 (bait) expressed under its native promoter or an overexpression promoter in a weak Rsp5 mutant background. B) Degradation of Ypq1-GFP before (0 hr) or after (6 h) in the presence of wild-type Rsp5 or rsp5^{G747E} mutant.

7. In addition to Li et al (Mol. Cell 2015) which reported a role for Ssh4 in the degradation of Ypq1, the subsequent study which clarified mechanisms of ESCRT-dependent clearance of ubiquitinated Ypq1 at the vacuole (PMID 28661397) should also be cited and explained as it is relevant for explaining how Ypq1 is cleared following ubiquitination.

Thank you for this comment. We have included a discussion of the ESCRT-dependent sorting of Ypq1-GFP into the vacuole lumen in our Introduction (**page 5, lines 91-92**) and cited Zhu et al., 2017.

8. It was recently shown that PQ motif mutations in the human PQLC2 transporter prevent interactions with the cytoplasmic WDR41 protein (PMID 31851326). Thus, it should also be mentioned that transport associated changes within transmembrane regions can influence the interactions of this family of transporters with cytoplasmic binding partners.

We apologize for this oversight, as this manuscript had been prepared and submitted shortly after the publication of the PQLC2 paper. It is an important study on the lysosomal PQ loop proteins and should be cited in our study.

We referenced this information in our Discussion (**page 24, lines 536-539**):

"*Transmembrane events within PQ loop proteins may also drive cytosolic interactions with their binding partners that are not necessarily embedded in the membrane, such as the interaction between PQLC2 and the cytosolic C9orf72 complex that can be abolished by mutations in the PQ motif (Amick et al., 2020).*"

9. In many fluorescence microscopy experiments, vacuole fragmentation is observed following lysine starvation for a long period (6h). Is there any physiological relevance or explanation for this phenomenon?

Thank you for this comment. We do not fully understand the physiological relevance of this interesting phenotype, but there are several possible explanations. At normal growth conditions, the yeast vacuole undergoes constant fission and fusion[14]. To more effectively

visualize the localization of our proteins on the vacuole, we resuspend yeast cells in water. This hypotonic environment promotes vacuole fusion and thus we achieve larger, more circular vacuoles. For starved cells, it is possible that after a long period of lysine starvation, the vacuole loses its ability to fuse efficiently. It could be that the fusion machinery is naturally turned over (or is not as competent) because protein synthesis is halted when there is no fresh supply of lysine.

Another explanation could be that $PI(3,5)P_2$ levels are regulated by lysine starvation. An increase in $PI(3,5)P_2$ during osmotic or salt stress have been shown to promote vacuolar fission[15–17]. At the moment, we do not have enough experimental evidence to support this claim, and this may be beyond the scope of our study.

Reviewer #2:
Specific points:
1. The title is misleading. The study does not touch upon Rsp5. They cannot claim that they have examined the interaction of Ypq1 with an E3 ligase complex.

This is a fair point, we thank the reviewer for this comment. Our first title was to "A selective transmembrane recognition mechanism by a membrane-anchored ubiquitin ligase **complex**". We have now replaced "complex" with "**adaptor**".

Initially, we chose our title because it is well-established that Ssh4 and Rsp5 form a complex. Rsp5 has three WW domains that are known to interact with the PPxY motif, which is a signature motif in Rsp5 targets and adaptors[13,18,19]. Ssh4 has two cytosolic PPxY motifs and mutating both abolishes its interaction with Rsp5[20]. Furthermore, mutating any of these PPxY motifs abolishes Ypq1 ubiquitination and degradation.

2. Figure 1C shows a structural model of Ypq1, but Figure 6 shows another model of Ypq1. What is the difference between these models? Were they made from the same template?

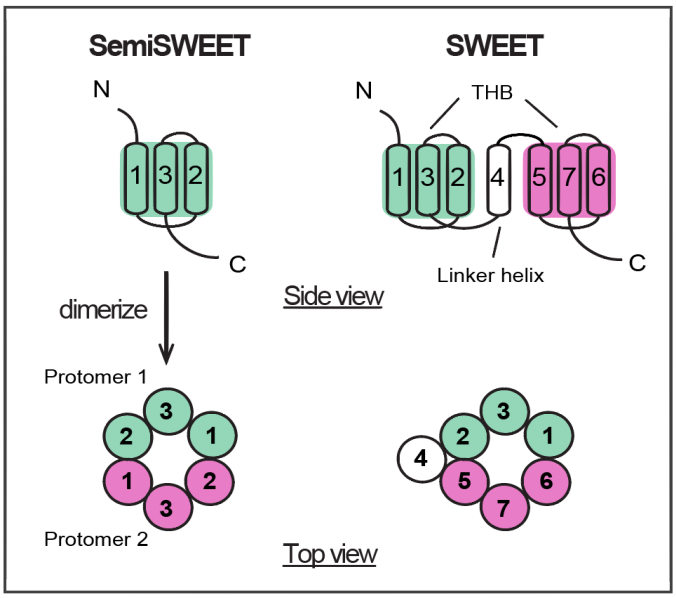
No, Figure 1C was modeled on OsSWEET2b (PDB ID: 5cth.1.B)[4], while Figure 6 was modeled on two crystals of *E. coli* SemiSWEET: inward-open (PDB ID: 4x5m) and outward-open (PDB ID: 4x5n)[5]. We agree that the shift in templates was not explained properly in the main text. We have modified our manuscript to clarify this **(page 20, lines 440-447)**. For more details, please see **response to Reviewer #1, point 4**.

If so, does the model in Figure 1C represent the outward-open, inward-open, or the occluded conformation?

Ypq1 was modeled on the inward-open crystal of OsSWEET2b, which is the only conformation available. We have indicated this in our original manuscript as "Using homology modeling, we generated a three-dimensional model of Ypq1 (Fig. 1C) based on the inward-open structure of rice glucose transporter OsSWEET2b".

The model in Figure 6E shows was based on the E. coli transporter SemiSWEET. Since SemiSWEET functions as a homo-dimer, I am surprised that the authors never comment on the oligomeric state of Ypq1. If it also functions as a dimer, how will that affect the relative positions of TM5 and TM7? Is the oligomeric state of Ypq1 regulated by lysine transport? Will the proposed model still be valid if it works as a dimer?

Through the work of Liang Feng (Stanford), Wolf B. Frommer (Stanford), Osamu Nureki (University of Tokyo), among others, the structures solved among the SWEET proteins have generated great insight on the evolution of the PQ-loop family. Bacterial SemiSWEETs are known to have 3 transmembrane helices that homodimerize to form a functional transporter (please see **Supporting Figure 3**). Eukaryotic SWEETs evolved via duplication of the triple-helix bundle, which are connected by an inversion linker helix (TM 4)[21]. In essence, a dimer of SemiSWEET has a similar configuration as a monomer of SWEET, and likely Ypq1.



Supporting Figure 3. Architecture of prokaryotic SemiSWEET and eukaryotic SWEETs, which are well-studied members of the PQ-loop protein family. SemiSWEET protomers and SWEET THBs are colored in pink and green. THB: triple helix bundle. (*adapted from Feng and Frommer, Trends in Biochem. Sci., 2015*).

Indeed, we agree that laying out the architectural distinction between eukaryotic SWEETs and bacterial SemiSWEETs would provide better clarity, especially since we used both to generate our models. We included Supporting Figure 3 into our main figures (**Fig. 6A**) and an explanation is provided in **page 19, lines 409-417**.

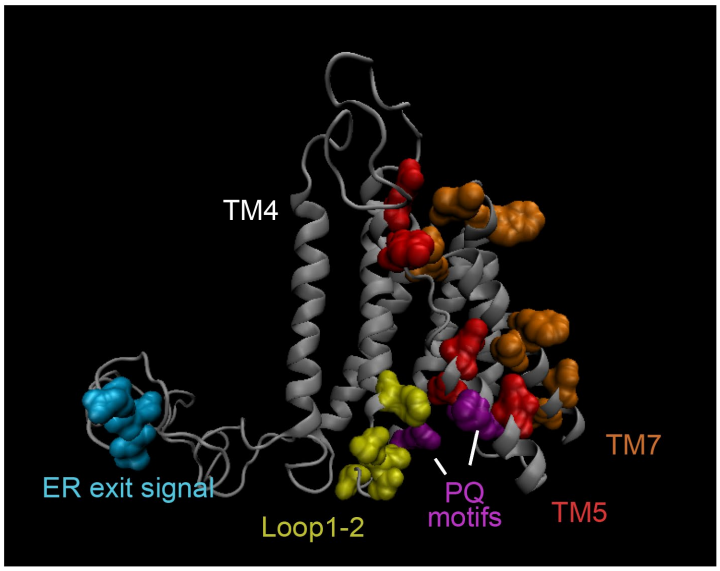
[Text and figure redacted]

3. Figure 2B, they need to show what these residues have been mutated to in the screen.

Agreed. We replaced **Fig. 2B** in the revised manuscript.

It is also puzzling why the authors never comments on where the identified TM5, TM7 mutations are on their structural models.

In our original manuscript, we showed a cartoon to illustrate where the suppressor residues were located (**Fig. 2H**). We did this mainly for simplicity, especially since Fig. 2 was already data-heavy. Also, we wanted to highlight how TM5 and TM7 are adjacent to each other based on the conserved architecture of the PQ-loop protein family. In **Supporting Fig. 5**, we show the suppressor residues from the critical regions mapped onto the Ypq1 model. Here we see that even in the 3D model, TM5 and TM7 are still adjacent to each other. We have added this as **Fig. 2I**, and included a movie to show the model at all angles (**Movie S2**).



Supporting Figure 5. Residues mutated in the suppressor screen mapped onto the 3D model of Ypq1. TM4 is also labeled to serve as a reference point.

Are these residues consistent with the ones identified by scanning mutagenesis? My quick scanning of Figure 2B and Figure 3D suggests that the two methods only give rise to partial overlapping results. For example, for TM5, G216, L224, S226 were identified as suppressor residues from the screen, but could not be confirmed by scanning mutagenesis. Please comments on this discrepancy.

Thank you for this point. Alanine scanning was performed because it is a commonly used method in structure-function studies, due to alanine's ability to mimic the removal of native side chain properties while keeping secondary structures intact and limiting severe steric disruptions due to its small size[22–24]. Scanning with other residues have also been done in the literature, for example, cysteine[25], glycine[26], phenylalanine[27], proline[28], and tryptophan[29,30], and were found to have varying effects.

A paper by Lemmon et al. (1992) meticulously substituted residues on the transmembrane helix of Glycophorin A (GpA) to all other amino acids[31]. They showed that subtle differences in side chain properties can introduce large changes in GpA's ability to

dimerize, provided that the disruption occurs on the dimerization interface. In their study, alanine, a small hydrophobic amino acid, mostly disrupted the larger residues L and I, and the polar residue T on the putative dimerization interface. It is possible that the effect of alanine on our scanning mostly affected polar residues or residues that have large side chains.

Furthermore, the mutants randomly generated in our suppressor screen were derived from error-prone PCR using unbalanced dNTP levels (please see Materials and Methods for details). It is possible that this caused a preference to randomly produce R mutations (29 instances), S mutations (9 instances), C mutations (6 instances), and so on.

Considering that no major alanine mutations were detected in our screen, it is possible that the disruption caused by alanine may not be as severe as R, S, C, etc. mutations. And so, transformants that harbored severe mutations grew colonies more quickly, and were the only ones identified in our screen. Had we allowed our plates to grow longer, then maybe we would have detected alanine mutations.

Regardless, alanine scanning was informative because it allowed us to systematically analyze all residues in TM5 and TM7, and confirm these TMs' relevance in comparison to other helices such as TM3. At the same time, it revealed new residues that did not appear in the screen.

Therefore, 1) the disruption of the function of a residue depends on the property of the substituting amino acid, and there is no catch-all amino acid that can disrupt all residues, and 2) we view the results from our screening and alanine mutagenesis as complementary to each other, rather than competing. We have modified **Fig. 3D** and added a supporting statement (**page 13, line 268-269**):

"*Through this scanning, we identified additional suppressor mutations in all TMs tested*."

4. Figure 2G shows a co-IP experiment, suggesting that Ypq1 mutants defective in SSh4-mediated degradation cannot interact with SSh4. Please explain why these three mutants were chosen to represent the identified mutants.

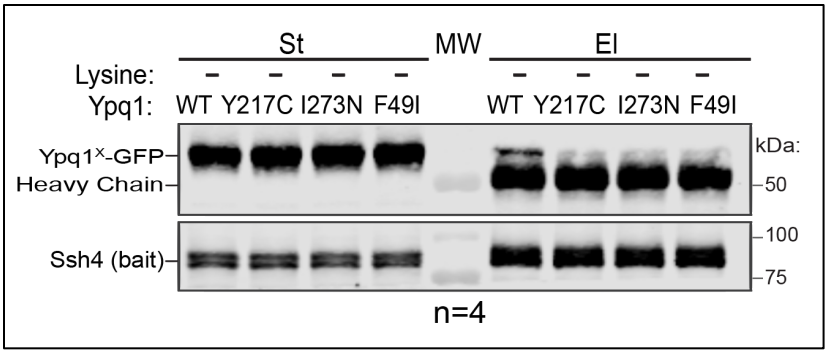
These were randomly selected among the mutants in each region.

Additionally, the authors conclude on page 11 that the experiment demonstrates a direct interaction of the two proteins, but co-IP cannot be taken as a proof for direct interaction. They need to tone down their conclusion.

Thank you for this feedback, Reviewer# 1 has also commented on this. We have toned down our conclusion and modified our text accordingly **(page 11, lines 241-248)**. For details, please also refer to the **response to Reviewer #1, Point 5.**

This experiment also misses a key gel showing that WT Ypq1 and mutants are expressed at the same level.

Thank you for pointing out this control. Indeed, similar to our experience on Ssh4 mutants, Ypq1-GFP mutants could also express at different levels and thus confound the results of the co-IP. We repeated the co-IP to include the input. Our results show that these mutants expressed at similar levels and so the decrease in pull-down is indicative of a loss of association between Ypq1 mutants and Ssh4. We have updated our figure to reflect this point (**Fig. 2G**).



Supporting Figure 6. Co-IP of Ypq1-GFP suppressor mutants with Ssh4 (bait) in lysine-starved conditions. St: Starting material, El: Elution. Cell lysates were harvested from 1000 OD cells.

They also need to comment on why the loop mutant F49I also affects the binding, or at least mention that their experiments did not exclude the involvement of other sequences in the binding.

We agree that we failed to expound on this finding in the original manuscript, because our initial goal was to shift the focus to TM interactions. We have toned down overstatements regarding the importance of TM interactions over cytosolic interactions, as we agree that they could both be important. We have modified the text **(page 12**, **lines 251-259)**.

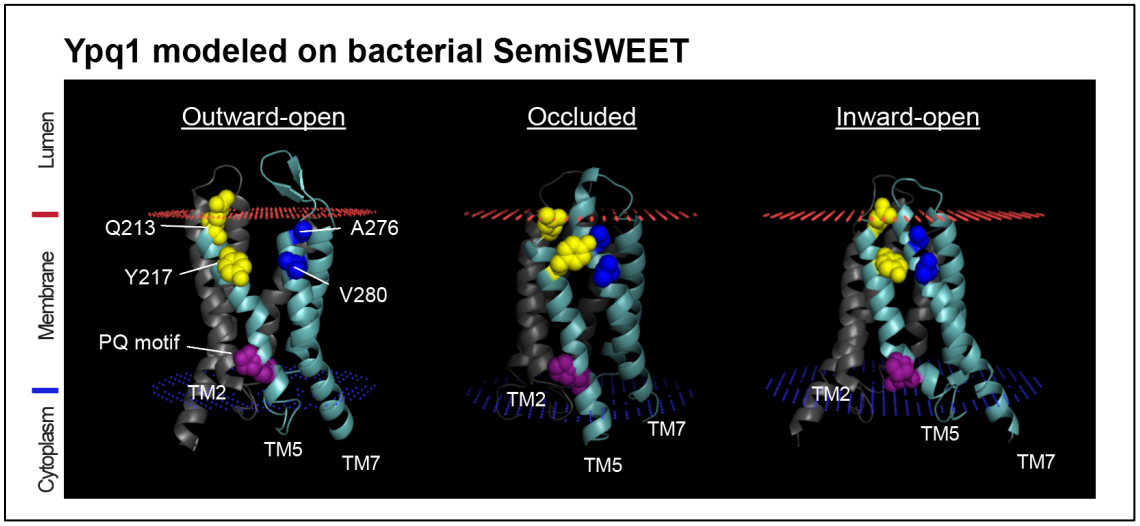
5. Figure S6 (related to Figure 4) shows that the Ssh4 mutants generated are properly localized to the vacuole, but the authors never comments on why some mutants are in the lumen while other are on the membrane.

In our original manuscript, we wrote “Among these, six strongly blocking mutants expressed to near-WT levels and localized on the vacuole membrane, whereas four others (I53, T54, I57, and L60) had low expression levels.”

We have modified “had low expression levels” to “were either expressed at low levels or mislocalized in the vacuole lumen” (**page 15, lines 325-326**).

6. Figure 6B shows the positions of the charged residues that had been introduced to Ypq1. Can the authors comment on where the membrane is? Are these residues exposed to the cytosol when the interaction occurs? If these residues are embedded in the membrane, how can these charged residues become protonated or deprotonated given that there should be no water molecules in the lipid bilayer?

This is an excellent point. We haven't really thought about this. To predict the position of the residues on Fig. 6 relative to the membrane, we uploaded our models to the Positioning of Proteins in Membranes (PPM) server (https://opm.phar.umich.edu/ppm_server)[32]. For this revision, we also added a model of Ypq1 based on *L. biflexa* SemiSWEET (PDB ID: 4qnc), which is the only occluded crystal so far[6]. According to PPM predictions, Q213 localizes at the membrane interface while Y217 occurs right below the interface.



Supporting Figure 7. Critical residues on TM5 are predicted to localize at or just below the membrane interface. A) Ypq1 models were placed into the membrane using the Positioning of Proteins in Membranes (PPM) server. Highlighted are the PQ motifs on TM5 and suppressor residues on TM5 and TM7. Q213 and Y217 were mutated to Asp in charge complementation experiments.

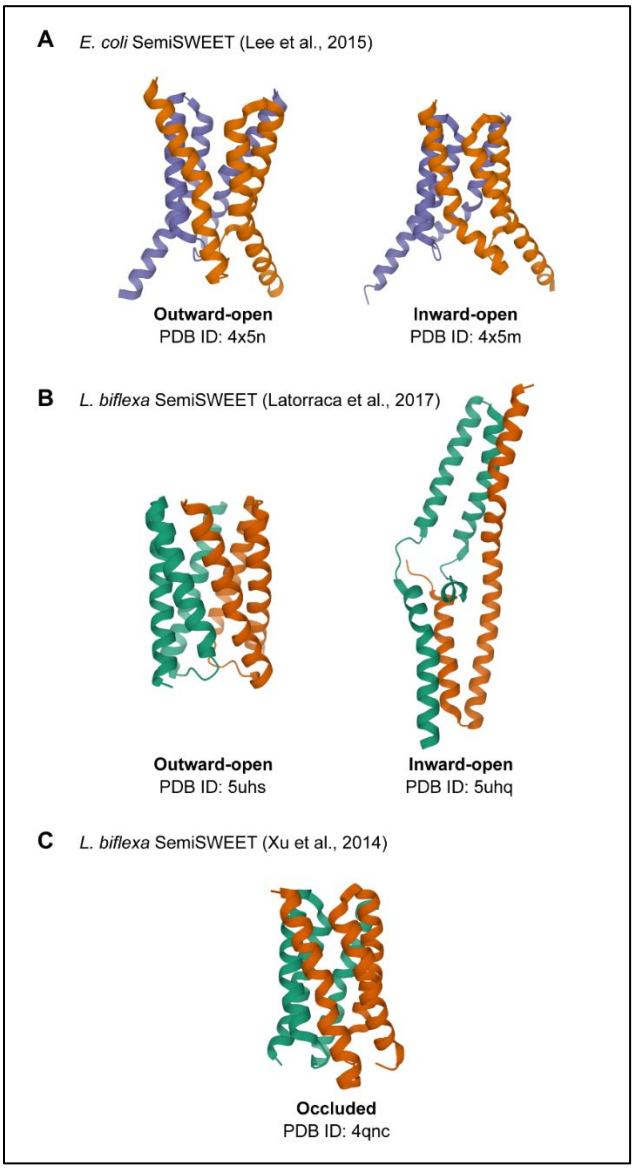
Based on their position in the membrane, it is possible that these residues can snorkel to the interface or aqueous regions to allow protonation/deprotonation. When mutated to charged amino acids, Q213 and Y217 can thus form salt bridges with the corresponding residues on Ssh4 TM.

Alternatively, it is possible that these residues do not need to snorkel. It is increasingly recognized that integral membrane proteins can bend their surrounding membrane[33–36]. For channels, transporters, and translocons, membrane thinning is observed more towards the cavity[33,36,37]. If the same occurs for Ypq1, it could be possible that the residues at the ends of its transmembrane helices may be partially accessible to the aqueous environment.

Also, why was the occluded conformation never mentioned in the discussion?

We did mention the occluded conformation in the discussion of the original manuscript (page 21): "Possibly, the loss of kinking at residue 229 straightens TM5, stabilizing Ypq1 at an outward-open or occluded conformation". At the time of submission, we generated models based on the inward-open and outward-open crystals of SemiSWEET from *E. coli*[5]. A different group also reported inward-open and outward-open crystals of SemiSWEET, but these were

from *L. biflexa* (5uhq and 5uhs, respectively)[7]. However, the inward-open crystal from *L. biflexa* had a domain-swapped configuration and was not amenable to homology modeling (**Supporting Figure 8**).



Supporting Figure 8. Crystals of SemiSWEET at different conformations. A) Crystals of *E. coli* SemiSWEET. B-C) Crystals of *L. biflexa* SemiSWEET. Images were obtained from RCSB Protein Data Bank (http://rcsb.org).

Since we chose the *E. coli* set as a template, we did not generate a model in the occluded conformation because the only available crystal was coming from a different species of bacteria (i.e. *L. biflexa*) and we thought that this could introduce variability. However, this new model provides new information (discussed more in response to **Reviewer#2, Point 8**) and so we will include this in the revised manuscript **(Fig. 6E)**. We thank the reviewer for bringing attention to this point.

7. Even though the charge complementation assay in Figure 5 provides strong evidence, supporting the conclusion that direct interaction likely occurs between the transmembrane domains of Ypq1 and that of SSh4, the authors should include a statement saying that other regions outside of the membrane may still contribute to the interaction. Otherwise, why does SSh4 have such a large cytosolic domain, but only needs a small motif to recruit Rsp5.

We completely agree, and we acknowledged the complexity of the interaction between Ypq1 and Ssh4 in our original manuscript. In our original manuscript, we mentioned: "Of course, what we found is likely only one cog in a multi-faceted regulation. For example, the isolation of suppressor mutants in the Loop1-2 region suggested the importance of cytosolic interactions, which will be characterized in our future studies."

For this revision, we kept these statements, but also toned down or added new statements to highlight this point:

- page 12, **lines 251-259** (also referenced in response to **Reviewer#2, Point 4, re: F49I**),

- page 14, lines 293-295:

"While it is likely that Ssh4 simultaneously uses several regions to recognize Ypq1, we wanted to test the possibility that its transmembrane helix plays a functional role."

8. While discussing the model, they propose that the transporter may be rapidly shuttling between different conformations during lysine transport, making it difficult to interact with Ssh4. Since they can generate structural models of Ypq1, can they comment on which conformation is mostly like the one that bind Ssh4 (e.g. TM5 would be more likely exposed)?

In our original manuscript, we proposed that Ypq1 is recognized when it is at the inward-open conformation. Our basis was the interpretation that mutating proline in the PQ motif to alanine removes the kink in TM5, and so straightens TM5 as in the outward-open and occluded conformation (Fig. 6A-B, original manuscript). In our experiments, PQ mutants showed a degradation block and abolished charge complementation. We associated the loss of recognizability of Ypq1 to the straightening of TM5, and so claimed that the inward-open state (which retains the kink at the PQ motif) as the conformation that exposes the critical residues more favorably to Ssh4. Furthermore, we proposed that during lysine starvation, Ypq1 may be arrested in the cytosol-facing (i.e. inward-open) conformation while it waits to bind lysine.

However, including the occluded conformation in the comparison (**Supporting Figure 7**) provides new information. In both the occluded and inward-open states, Q213 and Y217 seem to pack more into the residues on TM7. This goes against our previous hypothesis that the kink (or the loss of it) at proline determines the position of Q213 and Y217. In this comparison, only the outward-open conformation has a significantly different positioning of Q213 and Y217. Furthermore, as this reviewer has hinted, it would seem that Q213 and Y217 in the outward-open conformation are more sterically "free" or exposed because they are not attached to the residues at TM7. Beyond modeling, however, we do not have enough evidence to confirm this. A more detailed biophysical examination would be necessary. We have modified our discussion to reflect this (**page 23, lines 504-513**).

We also changed previous claims of preference for the inward-open conformation to "one of these conformations".

If the binding occurs in one the three conformations during lysine transport, that would indicate that Ypq1 in the absence lysine transport should more or less take the same conformation. Is that true?

Yes, this is our current model. However, we can only predict and propose a conformation that is preferred by Ssh4, but we concede that this paper does not have experimental evidence to definitively choose one over the others. The key point of this paper, however, is that Ypq1 needs to be stabilized into one of these three conformations to stably expose its critical transmembrane residues to Ssh4.

Accordingly, we have toned down our claims and revised our discussion to acknowledge this ambiguity.

Page 23, lines 516-518:
"Although active lysine transport is coupled to conformation changes that antagonize Ssh4 recognition, lysine starvation may stabilize Ypq1 in a conformation that favors the recognition."

We have also emphasized our model by adding **Fig. 7B**:

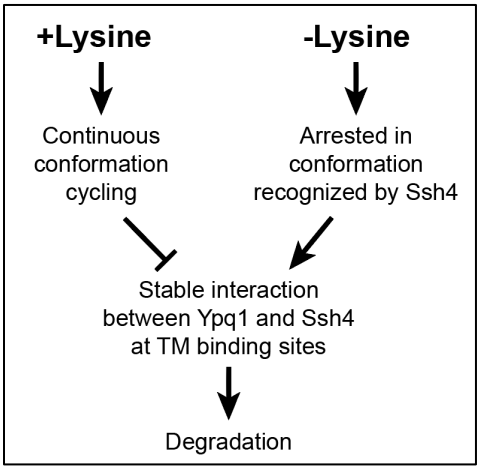


Fig. 7B. **Proposed model for Ypq1 recognition.** When lysine is present, constant conformation cycling of Ypq1 prevents the stable exposure of its transmembrane recognition sites. Absence of lysine arrests Ypq1 in a conformation favored by Ssh4 and leads to Ypq1 degradation.

Other points:
1. Page 4, "two pioneer studies....". The earlier work from Juan Bonifacino on TCRalpha degradation should be cited as well. It was shown by J. Bonifacino (EMBO 1991) that a single charged residue in the TM domain of a type I membrane protein is sufficient to retain the protein in the ER for ER.

Thank you, we have cited the Bonifacino paper accordingly (**Introduction, page 4, line 72**).

2. Page 7, "The first and last three TMs form two parallel triple helix bundles....". I don't quite get this. How can 4 TM domains form two triple helix bundles?

Thank you for this comment and we apologize for the confusion. We have clarified the text accordingly (**page 7, line 130**):

"TMs 1-3 and TMs 5-7 form triple helix bundles (THB)…"

3. Page 15, "Among these, six strongly blocking mutants....". Please list these six mutants here.

Agreed. We have modified the text (**page 15, line 323**).

Same page down, "we mutated the first residue....". The first residue is not clear,

Agreed. We have modified the text (**page 17, lines 356-357)**:

"We mutated *one* residue at the putative interface *on Ypq1* to aspartic acid, and the opposite residue *on Ssh4* to arginine."

4. Page 17, "consistently, we saw an increased sorting of.....". This statement is not accurate because endogenous level of Ssh4 S52R only increase sorting in the absence of lysine.

Thank you for noticing this error. We have adjusted the text (**page 18, lines 387-390**):

"*Similarly*, we saw an increased sorting of Ypq1^{Y217D}-GFP to the vacuole lumen when it was co-expressed *with Ssh4^{S52R} under lysine starvation conditions*. *Sorting increased further upon overexpression of Ssh4^{S52R} in both lysine-replete and lysine-starved conditions* (Fig. 5D-E)."

5. Page 17, "the occurence of charge complementation....." is contradictory to the observation that under overexpression conditions, complementation was also seen in the presence of lysine.

Agreed. We changed "occurrence" to "increase" (**page 19, line 403**):

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From the authors:

We are grateful to the reviewers for their time and sharing their valuable critiques and insights to improve our manuscript. With the additional experimental data, models, and optimized text, we hope that the revised manuscript merits acceptance to JCB. We look forward to hearing from you.

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October 21, 2020

RE: JCB Manuscript #202001116R

Dr. Ming Li
University of Michigan-Ann Arbor
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Rm 3214, Biological Sciences Building
1105 N. University Ave.
Ann Arbor, MI 48109

Dear Dr. Li:

Thank you for submitting your revised manuscript entitled "A selective transmembrane recognition mechanism by a membrane-anchored ubiquitin ligase adaptor". Your paper has been seen again by the original reviewers who now recommend acceptance. Therefore, we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

As you will see, reviewer #1 has noted that the data provided in the response-to-reviewers should be included in the manuscript and we agree with this. Please include this data in the revised manuscript. We realize that you already have 5 supplementary figures but, given the circumstances, we will allow you to have more than 5 such figures, if needed, so that you may include this data. Please also address reviewer #1's other minor issue and be sure to provide a final point-by-point rebuttal with your revised manuscript.

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

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Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

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2) Figures limits: Articles and Tools may have up to 10 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly

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5) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

6) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies.

7) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

8) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

9) Supplemental materials: There are normally strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental figures. As mentioned above, we will allow you to exceed this limit, if needed, in order to include the extra data mentioned by reviewer #1. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

10) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. It should begin with "First author name(s) et al..." to match our preferred style.

11) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the

following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

12) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

B. FINAL FILES:

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Sincerely,

William Prinz, PhD
Monitoring Editor
Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology

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

The authors have been responsive to reviewer comments from the previous submission and have provided new data and made modifications to the text to moderate claims in the text that previously raised concerns about over-reaching. The manuscript provides convincing evidence in support of a model wherein conformational changes in ypq1 (a transporter responsible for the uptake of lysine into the yeast vacuole) that support lysine transport also influence opportunities for interactions between ssh4 and ypq1. It is furthermore shown that interactions between ypq1 and ssh4 occur via their transmembrane domains. This is strikingly supported by charge complementation assays focused on TMD5 of ypq1 and the single transmembrane domain of ssh4. Collectively, this research provides interesting new ideas about how the transport activity of ypq1 is coupled to its degradation.

Although my overall impression is positive, I have one important concern about data interpretation (see below). I also have a concern about presentation of new data only in the response to reviewers. If the data was needed to convince the reviewers then it should be made available to all readers. I therefore suggest that such data be included in the supplemental material.

1. Lines 505-509: It is stated the PQ motif mutations stabilize the inward facing conformation of ypq1. Yet lines 426-428, Figure 6 and Figure 7A imply that the prolines in the PQ motifs are important for a kink in their respective transmembrane domains when the transporter is in the inward facing conformation. Some clarification is required on how mutations to these prolines influence the conformatipon of ypq1. This has implications for speculation that ssh4 prefers to bind to the inward facing conformation of ypq1.

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

The authors have carefully addressed all my criticisms. This is a beautiful piece of work. Congratulations!

2nd Revision - Authors' Response to Reviewers: October 25, 2020

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

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Although my overall impression is positive, I have one important concern about data interpretation (see below). I also have a concern about presentation of new data only in the response to reviewers. If the data was needed to convince the reviewers then it should be made available to all readers. I therefore suggest that such data be included in the supplemental material.

Among the 9 figures in the rebuttal, we did not include 3 in the manuscript. Due to Supplementary Figure limits, we did not include Supporting Figure 2 (Ssh4-Ypq1 IP in rsp5 mutant background). Supporting Figure 4 (Ypq1 oligomerization IP) was not included for reasons that will be discussed below. Supporting Figure 8 (crystal structures of SemiSWEET templates) was not included because these data are already published and were not produced in our lab.

We thank the editors for allowing us to have more Supplementary Figures. With this, we included Supporting Figure 2 now as **Fig. S3**. This is accompanied by the following statements in the main text **(page 11, lines 238-241)**:

"*In one condition, we expressed Ypq1-GFP and Ssh4 in a hypomorphic Rsp5 (G747E) mutant to potentially reduce Ypq1-GFP degradation (Fig. S3A). No co-IP was observed even after overexpressing Ssh4 (Fig. S3B).*"

We respectfully disagree about adding Supporting Figure 4, and we would like to appeal to not include this figure for the following reasons:

1) It is outside the scope of this study

Our study focuses on Ypq1-Ssh4 interaction, and not Ypq1-Ypq1 interaction which is communicated by this data. Adding this piece of data would add marginal and tangential value to the current paper, but it would have more value elsewhere in conjunction with further investigation. Following both reviewers' advice, we believe that the current revised story is strong as it is, and adding more data would not necessarily translate to a better story. In fact, it may add confusion as Ypq1 oligomerization may represent another layer of regulation that needs to be studied more carefully.

2) We have addressed Reviewer 2's question about oligomerization through other means

Reviewer 2 asked about the oligomeric state of Ypq1 due to our confusing statements about SemiSWEET being a dimer. We have addressed this issue by clarifying it in Supporting

Figure 3 (now Fig. 6A). We also did our due diligence to perform this experiment and found that Ypq1 oligomerization is not affected by lysine starvation and so does not affect our conclusions.

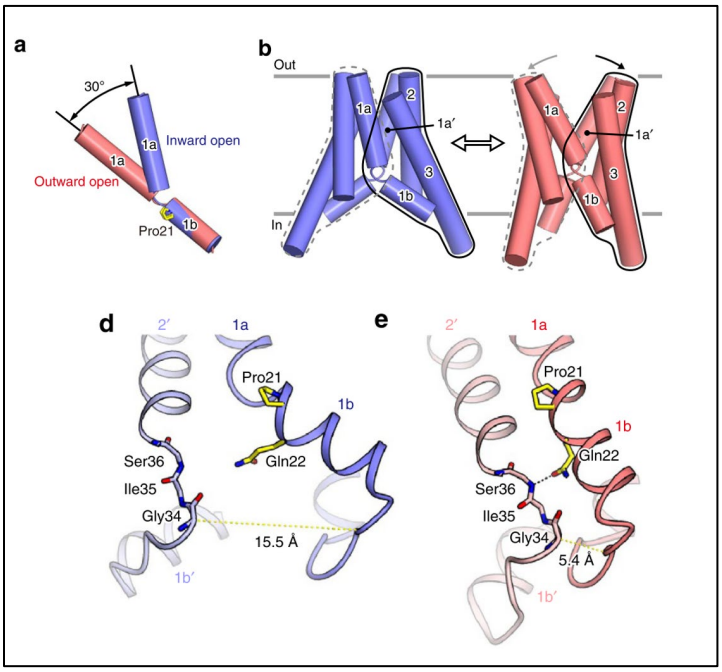
3) We want to avoid dual publication

By choosing not to publish this figure in this paper, we would avoid ethical concerns regarding dual publication of this data. We have presented this data to the reviewers in the interest of scientific discussion, but we intend to use it for publication of an ongoing study.

In our previous papers submitted to JCB and other journals, we were not asked to include all data presented in the rebuttal. We hope the editors and reviewers would agree that authors have the right not to include some rebuttal figures within reasonable grounds.

1. Lines 505-509: It is stated the PQ motif mutations stabilize the inward facing conformation of ypq1. Yet lines 426-428, Figure 6 and Figure 7A imply that the prolines in the PQ motifs are important for a kink in their respective transmembrane domains when the transporter is in the inward facing conformation. Some clarification is required on how mutations to these prolines influence the conformatipon of ypq1. This has implications for speculation that ssh4 prefers to bind to the inward facing conformation of ypq1.

Thank you for pointing this out. Previous studies suggest that the PQ motif serves as a hinge to enable transport. The role of proline is more obvious, and that is to introduce a kink for transitioning from one conformation to another. The role of glutamine is not as well understood, especially since some eukaryotic SWEETs do not have glutamine[1]. However, Lee et al.[2] showed in *E. coli* SemiSWEET that this glutamine may function in stabilizing the outward-open conformation by forming cross-protomer hydrogen bonds with residues at the cytosolic end of TM2 (**please see figure below**). Since TM1 would naturally bend at proline, it would need additional electrostatic forces to stabilize it in a straightened conformation. This is reflected further in *L. biflexa* SemiSWEET, which when mutated at glutamine of the PQ motif caused it to crystallize in the inward-open conformation[3].



The PQ glutamine forms cross-protomer hydrogen bonds during the outward-open conformation. *Taken from Lee et al., 2015.* A-B) TM1 and SemiSWEET structures in inward-open (blue) and outward-open (pink) conformations. Pro21 of the PQ motif is shown in stick representation. D-E) Close-up views of the PQ motif, shown in stick models in the inward-open (D) and outward-open (E) conformations. In (E), Gln22 of TM1 forms hydrogen bonds with Ser36 of TM2 of the other protomer in the outward-open conformation.

At the moment, the field does not have structures of PQ-loop proteins that have a mutation on the proline. Therefore, based on the available data, we can only speculate that mutating the PQ motif would cause Ypq1 to be stabilized in an inward-open conformation. Based on our degradation data, PQ motif mutants had decreased interactions with Ssh4 and were spared from degradation. From this we speculated that inward-open Ypq1 is *not* recognized by Ssh4. Conversely, outward-open Ypq1 could be recognized.

To clarify the role of the PQ glutamine, we have made the following changes to the text:

Results (page 20, lines 425-432)

Discussion (page 23, lines 510-512):

"In *L. biflexa* SemiSWEET, mutating *glutamine of* the PQ motif stabilized the protein in the inward-open conformation... *No structural data are available for proline or double mutants.*"

Because there is too much uncertainty, we found it fit not to favor a conformation in our Discussion. This change does not affect our model, which argues that there is one conformation that will be preferred by Ssh4. We have changed our text as follows:

Discussion (page 24, lines 517-520):

"*At the moment, we can only propose that Ssh4 recognizes Ypq1 in one conformation, and ignores it in the opposite conformations. Biophysical evidence and further experimentation are necessary to determine the identity of these conformations.*"

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

The authors have carefully addressed all my criticisms. This is a beautiful piece of work. Congratulations!

Thank you very much!

References:

1. Newstead, S. & Barr, F. Molecular basis for KDEL-mediated retrieval of escaped ER-resident proteins - SWEET talking the COPs. *Journal of cell science* vol. 133 (2020).
2. Lee, Y., Nishizawa, T., Yamashita, K., Ishitani, R. & Nureki, O. Structural basis for the facilitative diffusion mechanism by SemiSWEET transporter. *Nat. Commun.* **6**, 6112 (2015).
3. Latorraca, N. R. *et al.* Mechanism of Substrate Translocation in an Alternating Access Transporter. *Cell* **169**, 96-107.e12 (2017).