AAGAB is an assembly chaperone regulating AP1 and AP2 clathrin adaptors

Chun Wan, Lauren Crisman, Bing Wang, Yuan Tian, Shifeng Wang, Rui Yang, Ishara Datta, Toshifumi Nomura, Suzhao Li, Haijia Yu, Qian Yin and Jingshi Shen
DOI: 10.1242/jcs.258587

Editor: Jennifer Lippincott-Schwartz

Review timeline

Original submission:	1 March 2021
Editorial decision:	29 April 2021
First revision received:	4 June 2021
Editorial decision:	24 June 2021
Second revision received:	19 July 2021
Editorial decision:	23 August 2021
Third revision received:	23 August 2021
Accepted:	31 August 2021

Original submission

First decision letter

MS ID#: JOCES/2021/258587

MS TITLE: AAGAB is an assembly chaperone regulating AP1 and AP2 clathrin adaptors

AUTHORS: Chun Wan, Lauren Crisman, Bing Wang, Yuan Tian, Shifeng Wang, Ishara Datta, Toshifumi Nomura, Suzhao Li, Haijia Yu, Qian Yin, and Jingshi Shen
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.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. 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.

Reviewer 1

Advance summary and potential significance to field

In this manuscript, the authors continue to study the function of AAGAB as an assembly chaperone for adaptor protein complexes. In a previous study, they found that AAGAB serves to regulate the assembly of the AP-2 adaptor complex, a key complex involved in the recruitment of clathrin and receptors to form coated pits and facilitate receptor-mediated endocytosis. The advance in this study is that the authors show, perhaps not surprisingly, that in addition to its role catalyzing AP-2 assembly, AAGAB also regulates the assembly of the homologous AP-1. They find that the assembly is catalyzed by the binding of AAGAB to the gamma subunit of AP-1, allowing recruitment of the sigma subunit, and formation of a ternary hemicomplex. This then allows assembly of beta and mu subunits and AAGAB is released. Accordingly interference with AAGAB expression leads to trafficking defects that are manifested by a combination of impaired AP-2 and AP-1 expression.

Comments for the author

While the manuscript builds on the previous published paper showing that AAGAB is required for AP-2 assembly, and shows that AP-1 assembly is dependent on AAGAB, the advances in the current manuscript are not unanticipated. It is not unexpected that AP-2 and AP-1 would be assembled in a similar fashion, and indeed this was proposed in the previous paper. One question that would be interesting and significant and move beyond documenting that AAGAB assembles AP-1 in a manner similar to AP-2 is why AAGAB impacts both AP-1 and AP-2 assembly but not AP-3 assembly. A molecular delineation of this sort would definitely provide key insight into AAGAB function, and provide worthwhile information to the scientific community.
Another question of interest that should be discussed is why AAGAB, as such a crucial assembly chaperone for both AP-1 and AP-2, appears to have such a narrow impact when heterozygous mutations are present (causing primarily hyperkeratosis in a subset of derma). Is it due to the heterozygosity? Or is there marked compensation in most other tissues?

Specific concerns:
Can the authors demonstrate that the endogenous AAGAB and AP-1 gamma interact in cells, and not only over-expressed proteins? It is clear that the proteins can interact directly from the experiments using purified proteins, but it would be important to demonstrate that they interact in cells when expressed at endogenous levels.
While the proteomic comparison of receptor levels on the cell surface in AAGAB, AP-1 and AP-2 KD is interesting, this reviewer would advise caution in interpreting a lack of change for AAGAB as necessarily being due to conflicting functions of AP-1 vs AP-2 being affected. In the absence of clear data to determine if AAGAB is more important for assembly of one complex over the other, such interpretations are difficult. In addition, AP-2 and AP-1 do not necessarily function "antagonistically."
The authors show AAGAB KO in Fig. 1 and AP1G1 KO in Fig. 3, but then show functional experiments that include AP2S1 KO. However, there is no data provided showing the efficacy of the AP-2 KO, and this needs to be provided for each experiment where such cells are used.
Similarly, in Fig. 4, the authors maintain that the more significant increase of TfR observed on the PM upon AAGAB KD compared to mild increases for AP-1 or AP-2 KD results from AAGAB's combined effect on both AP-1 and AP-2 simultaneously. Key controls are missing, such as knockdown of both AP-1 and AP-1 in the same cell line and demonstrating a similar boost in TfR cell surface levels on the PM to that observed in AAGAB KO cells.

Reviewer 2

Advance summary and potential significance to field

If the manuscript were appropriately modified, it would document an important role of AAGAB in regulating the stability and likely the assembly of AP-1, and document a requirement for AAGAB in regulating the cell surface proteome. These would be novel conclusions, and introduce a novel regulator of the assembly of a key multisubunit protein complex.

Comments for the author

In this manuscript, Wan et al show that alpha and gamma adaptin binding protein (AAGAB) facilitates AP-1 assembly and maintains the cell surface proteome of cultured cells beyond its role in assembling AP-2. The work follows from an exciting 2019 publication in which the authors showed that AAGAB promotes assembly of AP-2 and is necessary for AP-2/ clathrin-dependent endocytosis. A supplemental figure of that paper showed that the gamma chain of AP-1 was destabilized in AAGAB knockout cells. Here they show that all AP-1 subunits, but not subunits of AP-3, COPI, or COPII coats, are depleted from AAGAB knockout HeLa cells.

Coimmunoprecipitations, size exclusion chromatography, and in vitro assembly experiments suggest that AAGAB binds to and stabilizes the gamma chain and its interaction with sigma1 subunits. Cell surface proteomics analyses show that a large number of proteins are up- or down regulated by knockout of AP-1, AP-2 or AAGAB, with AAGAB causing the largest change in both the number of proteins affected and the extent to which they are affected. Upregulation of surface levels of the transferrin receptor is validated. Together the data suggest that key features of genetic disruption of AAGAB might result from defects in the function of AP-1 function and not just AP-2.

The paper addresses an interesting and new aspect of cell biology. The data supporting a physical interaction of AAGAB with AP-1 gamma adaptin go beyond the observations in the 2019 paper and are intriguing, as are the proteomics data on the impact of AAGAB depletion relative to AP-1 or AP-2 depletion. However, as is the conclusions drawn are incompletely supported by the data and the paper does not present a complete story. The biochemical experiments lack key controls and provide no insight into whether AAGAB merely stabilizes the gamma chain or prepares it for interacting with sigma1, or even whether it is capable of binding to sigma1 alone. Controls showing a lack of interaction with chains of non-interacting complexes (e.g. AP-3delta) are lacking. While the proteomics data on the impact of protein cell surface expression are interesting, there are no mechanistic studies to explain the impact of AAGAB on the levels of proteins such as the transferrin receptor, which is not thought to bind to AP-1. Does knockout of AAGAB or AP-1 impact the total cellular levels of affected proteins? Of levels of their mRNA? Is the impact of AAGAB depletion on increased cell surface expression antagonized by blockade of recycling (e.g. by treatment with primaquine or by knockdown of RAB11)? Some mechanistic insight into how AP-1 might impact the surface expression of selected hits in the screen need to be provided in order for this paper to have an impact on the field. These and additional concerns are detailed below.

1. In describing Figure 1, the authors should indicate that their prior paper (Gulbranson et al., 2019) already showed that AP-1 gamma chain was reduced in abundance in AAGAB knockout cells. The data in Figure 1 are distinct, but it should be clear that this extends data already published.

2. Figure 2: Figure 2A/B is difficult to interpret without separate SEC profiles of AP-1gamma alone and AAGAB alone. These should be shown. In addition, in this experiment, what is the composition of the smaller peak? Is this the included volume of the column? The y-axis should be better labeled or described in the legend (is this A280?). How was the stoichiometry estimated to be 1:1? Was this based on the SEC profile alone? The labeling for panel 2E appears to be missing the absence or presence of 3x-FLAG-AAGAB. I assume the lanes are the same as in panel D? This needs to be clearly labeled on the figure; it might be more helpful to have this panel included within panel D. Does 3x-FLAG-AAGAB coprecipitate with HA-sigma1 in the absence of AP-1gamma? Does it stabilize the sigma1 chain alone? There are no data to test whether AAGAB interacts first with sigma1 and then with gamma. Finally, there are no controls to indicate that the binding correlates with stabilization of AP-1 chains. For example, does AAGAB not pull down the AP-3 delta chain or the corresponding subunit of COPI?

3. The authors state on page 6-7 that AP1-dependent cargo proteins are poorly defined, but this is not true. Robinson and colleagues have published several papers on cargo proteins that are depleted from clathrin-coated vesicle preparation in cells depleted of AP-1 (e.g. see Hirst et al., 2012, Curr. Biol.). It would be helpful for the authors to compare their proteomic hits with those of Hirst et al. In addition, the authors state that surface expression of proteins relies on clathrin-mediated transport, but this is the opposite of the truth – surface expression is antagonized by clathrin-dependent endocytosis and by post-endocytic sorting by AP-1 and AP-3. This needs to be clarified, and the expectations of the consequences (i.e., rationale) of AP-1 depletion clarified as well – particularly given that most sorting signals that engage AP-1 also engage AP-2. How would you expect depletion of AP-1 to result in increased cell surface expression? How would depletion of AP-1 or AP-2 result in decreased cell surface expression?

4. In Table 2, what does relative expression level indicate – WT relative to knockout or knockout relative to WT? This must be indicated on the Table. The same concern applies to Table 3. Data in Figure 3 comparing AP1G1 KO, AP2S1 KO, and AAGAB KO should be analyzed statistically (e.g. by one-way ANOVA).

5. In Figure 4A, how were surface levels quantified in the flow cytometry experiments? Do the data represent normalized mean, median, or geometric mean fluorescent intensity levels? This needs to be indicated in the figure. Note, Students' t-test is not an appropriate statistical test given that multiple samples are being analyzed; statistical significance should be assessed using a one-way ANOVA test.

6. Is it surprising that only ~200 proteins were upregulated by AP-2 knockdown whereas 700 were upregulated by AP-1 knockdown? How do the authors explain increased surface expression of some proteins upon AP-2 knockdown? The results are interesting, but there are no mechanistic data to address how knockdown influenced surface expression. In addition, some mechanistic insight needs to be provided. For example, given that TfR is not thought to be an AP-1 cargo, and that in HeLa cells it is thought that about 70% of TfR is intracellular at any given time, it is difficult to understand why surface levels of TfR would rise ~10-fold in AAGAB KO cells and also rise substantially in AP1G1 KO cells. Were total cellular levels of TfR (and ECE1, TMEM206, and APLP2, if possible to measure) also increased in AAGAB knockout cells? What is the ratio of intracellular vs. extracellular TfR in these cells (e.g. by comparing total levels to biotinylated TfR in blots)? Is the mRNA of these proteins affected? These questions need to be answered before concluding the AP-1 and/or AAGAB impact trafficking of these proteins directly.

7. In Figures 6B and 6D, can soluble GST-gamma subunit be pulled down in the presence of the sigma1 subunit and the absence of AAGAB? Can sigma1 subunits be stabilized and associate with His6-SUMO-AAGAB in the absence of GST-gamma?

8. The model in Figure 7 is not completely supported by the data in the paper, as there are no data to exclude the possibility that AAGAB binds to the sigma chain first and then recruits gamma1. Is there any evidence that either chain is made in excess of the other? The model also lacks any potential explanation for how loss of AAGAB expression would result in the upregulation or downregulation of transmembrane proteins.

Reviewer 3

Advance summary and potential significance to field

In this manuscript the authors describe a role for the alpha and gamma adaptin binding protein (AAGAB) in the assembly of the AP1 clathrin adaptor. In a previous paper, Gulbranson et al., they already established that AAGAB played a role in AP2 assembly.

In this manuscript Wan and Crisman et al. showed that (1) AP1 and AP2 subunits are selectively downregulated in AAGAB KO cells. (2) They showed that AAGAB directly interacted with gamma-adaptin and that a tertiary complex of AAGAB*gamma-adaptin*sigma-adaptin could form. (3) Surface proteomics showed that whereas there were moderate effects on protein surface levels in

both gamma-adaptin and sigma2-adaptin KO cells, the effect was much enhanced in AAGAB KO cells. (4) The results from the surface proteomics was confirmed by studying surface labeling of transferrin receptor, whose intracellular trafficking depends both on AP1 and AP2. (5) Despite altered levels of surface receptors, neither AAGAB KO cells nor gamma-adaptin KO or sigma2-adaptin KO cells showed proliferative effects. And (6) AAGAB co-expression in E. coli stabilized gamma-adaptin and sigma adaptin.

From these studies, they conclude, that AAGAB first binds to gamma-adaptin followed by recruitment of the sigma1 subunit and subsequent full assembly into a heteromeric complex consisting of gamma-adaptin beta1-adaptin, mu1-adaptin and sigma1-adaptin. They also allude that the enhanced effect on surface receptor levels observed in the AAGAB KO cells was because of a combined effect due to loss of both AP1 and AP2 function. Further, they would like to conclude that AAGAB is essential for AP1 and AP2 assembly and that neither complex may assemble spontaneously.

Taken together, these studies advance the field and should be a broad interest to cell biologist working on membrane trafficking and beyond. For the most part, the data are clean and the experimental execution is of high quality. However, a few areas need additional considerations as detailed in the comments section.

Comments for the author

Major comments:
1) I understand that in the previous work by Gulbranson et al. controls were done to show that AAGAB did not directly bind to sigma2-adaptin. Why not show the same for sigma1? Without directly showing that sigma1 only pulls down with AAGAB in the presence of gamma-adaptin, it cannot be concluded that this is the case just because this is the case for AP2 assembly.

2) Although the level of both AP1 and AP2 subunits are greatly reduced in AAGAB KO cells, I am wondering if the remaining subunits may exist as unassembled subunits or if there is some low level of spontaneous assembly happening in the absence of AAGAB. This could easily be tested by anti-gamma co-immunoprecipitations.

3) It was concluded that the effect of AAGAB KO on surface levels is a combined effect of AP1 and AP2 downregulation. If this were true, a gamma-adaptin and sigma2-adaptin double KO should have the same effect on transferrin receptor surface levels as does AAGAB KO. This seems easy enough to test and would take away speculations.

Minor comment:
1) On the top of page 8 it reads … (Fig. 3E and Table 2): I believe it should read ‘Table S2’. Please correct.

First revision

Author response to reviewers' comments

Reviewer 1

“One question that would be interesting and significant and move beyond documenting that AAGAB assembles AP-1 in a manner similar to AP-2 is why AAGAB impacts both AP-1 and AP- 2 assembly but not AP-3 assembly. A molecular delineation of this sort would definitely provide key insight into AAGAB function, and provide worthwhile information to the scientific community.”

Response: The Reviewer raised an important question. Our findings suggest that AAGAB selectively interacts with AP-1 and AP-2 subunits but not AP-3. Interestingly, a recent large- scale interactome study confirmed that AAGAB-interacting proteins included AP-1 and AP-2 subunits but not AP-3

subunits (Luck et al. 2020, Nature PMID: 32296183). We revised the Discussion section of the manuscript to further discuss this point.

"Another question of interest that should be discussed is why AAGAB, as such a crucial assembly chaperone for both AP-1 and AP-2, appears to have such a narrow impact when heterozygous mutations are present (causing primarily hyperkeratosis in a subset of derma). Is it due to the heterozygosity? Or is there marked compensation in most other tissues?"

Response: This is another intriguing question. Patients bearing heterozygous *AAGAB* mutations are largely normal except the skin condition. We did not find an AAGAB-like protein encoded in the human genome that might compensate for the loss of AAGAB. Thus, we anticipate that partial reduction of AAGAB expression caused by heterozygous mutations only subtly impacts clathrin-mediated trafficking such that their effects are restricted to a small subset of cargo proteins in selected tissues (e.g., the skin). This point is further discussed in the Discussion section of the revised manuscript.

"Can the authors demonstrate that the endogenous AAGAB and AP-1 gamma interact in cells, and not only over-expressed proteins? It is clear that the proteins can interact directly from the experiments using purified proteins, but it would be important to demonstrate that they interact in cells when expressed at endogenous levels."

Response: The Reviewer correctly pointed out that it is critical to measure interactions at endogenous levels. In our co-IP experiments, we tested AAGAB rescue gene expression at various levels including the endogenous level. Identical results were obtained from these experiments. Importantly, these co-IP data are further supported by the results of our biochemical experiments using recombinant proteins, which definitively demonstrated that AAGAB directly interacts with AP-1.

"While the proteomic comparison of receptor levels on the cell surface in AAGAB, AP-1 and AP- 2 KD is interesting, this reviewer would advise caution in interpreting a lack of change for AAGAB as necessarily being due to conflicting functions of AP-1 vs AP-2 being affected. In the absence of clear data to determine if AAGAB is more important for assembly of one complex over the other, such interpretations are difficult. In addition, AP-2 and AP-1 do not necessarily function "antagonistically."

Response: We fully agree with the Reviewer that a phenotype of *AAGAB* KO cells might not be the simple addition of AP-1 and AP-2 deficiency. It is possible that surface levels of a cargo protein are indirectly controlled by AP-1 and/or AP-2 (i.e., through intermediate regulators) rather than being a direct target. We revised the Discussion section of the manuscript to explicitly discuss this point. We thank the Reviewer for improving the clarity of the manuscript in this regard.

"The authors show AAGAB KO in Fig. 1 and AP1G1 KO in Fig. 3, but then show functional experiments that include AP2S1 KO. However, there is no data provided showing the efficacy of the AP-2 KO, and this needs to be provided for each experiment where such cells are used."

Response: The *AP2S1* KO cell line was validated using two complementary approaches. First, since none of the anti-AP2S1 antibodies we tested could detect endogenous sigma proteins using immunoblotting (likely due to its small size), we confirmed the loss of AP2 adaptor using anti-AP2M1 antibodies (e.g., data shown in Figure 4A). AP2 subunits are dependent on each other such that loss of one subunit invariably leads to degradation of other subunits. Second, our high-resolution whole-cell proteomic analysis confirmed a complete loss of sigma proteins in the *AP2S1* KO cell line.

"Similarly, in Fig. 4, the authors maintain that the more significant increase of TfR observed on the PM upon AAGAB KD compared to mild increases for AP-1 or AP-2 KD results from AAGAB's combined effect on both AP-1 and AP-2 simultaneously. Key controls are missing, such as knockdown of both AP-1 and AP-1 in the same cell line and demonstrating a similar boost in TfR cell surface levels on the PM to that observed in AAGAB KO cells."

Response: Based on the Reviewer's suggestion, we performed the double KO experiment. We

observed that surface TfR levels were strongly upregulated in *AP1G1/AP2S1* double KO cells, comparable to their levels in *AAGAB* KO cells. These findings further suggest that the phenotype of *AAGAB* KO cells reflects the combinatorial effects of AP1 and AP2 deficiency. We added these new data to Figure 4 of the revised manuscript and thank the Reviewer for suggesting this important experiment.

Reviewer 2

"1. In describing Figure 1, the authors should indicate that their prior paper (Gulbranson et al., 2019) already showed that AP-1 gamma chain was reduced in abundance in AAGAB knockout cells. The data in Figure 1 are distinct, but it should be clear that this extends data already published."

Response: Based on the Reviewer's suggestion, we revised the text to clarify the point.

"2. Figure 2: Figure 2A/B is difficult to interpret without separate SEC profiles of AP-1gamma alone and AAGAB alone. These should be shown. In addition, in this experiment, what is the composition of the smaller peak? Is this the included volume of the column? The y-axis should be better labeled or described in the legend (is this A280?). How was the stoichiometry estimated to be 1:1? Was this based on the SEC profile alone? The labeling for panel 2E appears to be missing the absence or presence of 3x-FLAG-AAGAB. I assume the lanes are the same as in panel D? This needs to be clearly labeled on the figure; it might be more helpful to have this panel included within panel D. Does 3x-FLAG-AAGAB coprecipitate with HA-sigma1 in the absence of AP-1gamma? Does it stabilize the sigma1 chain alone? There are no data to test whether AAGAB interacts first with sigma1 and then with gamma. Finally, there are no controls to indicate that the binding correlates with stabilization of AP-1 chains. For example, does AAGAB not pull down the AP-3 delta chain or the corresponding subunit of COPI?"

Response: Based on the Reviewer's suggestion, we added the SEC profile of AAGAB alone to Figure 2A and relabeled the y axis. AP1 gamma alone, on the other hand, cannot be expressed in *E. coli* in the absence of AAGAB. Please note that our unpublished data show that standalone AAGAB exists entirely as a tetramer in solution such that it elutes earlier than the AP1-gamma/AAGAB binary complex. The earlier small peak with an elution volume of 8 mL is close to the excluded volume for this SEC column, likely representing protein aggregates. The second small peak with an elution volume of 18 mL represents impurities unrelated to AAGAB or AP1 proteins. After SEC purification, however, the impurities were removed. The binding stoichiometry was calculated based on both SEC profiles and Coomassie blue staining of the purified proteins. Next, in response to the Reviewer's suggestion, we relabeled the lanes of Figure 2E and explicitly stated in the figure legend that the whole cell lysates were related to the co-IP samples. Our extensive studies of AP1 and AP2 assembly demonstrated that AAGAB interacts with the alpha/gamma subunit to form binary complexes, which then recruit the sigma subunit to form ternary complexes. Our preliminary experiments suggest that without the alpha/gamma subunit, the binding of the sigma subunit to AAGAB likely represents an unproductive intermediate (please see our response to Point 8 for further explanations). Our findings suggest that AAGAB selectively interacts with AP1 and AP2 subunits but not AP3 or COPI. Interestingly, a recent large-scale interactome study confirmed that AAGAB-interacting proteins included AP1 and AP2 subunits but not AP3 or COPI subunits (Luck et al. 2020, Nature PMID: 32296183). We revised the Results and Discussion sections of the manuscript to further discuss these points. We thank the Reviewer for these excellent suggestions, which substantially improved the quality of the manuscript.

"3. The authors state on page 6-7 that AP1-dependent cargo proteins are poorly defined, but this is not true. Robinson and colleagues have published several papers on cargo proteins that are depleted from clathrin-coated vesicle preparation in cells depleted of AP-1 (e.g. see Hirst et al., 2012, Curr. Biol.). It would be helpful for the authors to compare their proteomic hits with those of Hirst et al. In addition, the authors state that surface expression of proteins relies on clathrin-mediated transport, but this is the opposite of the truth – surface expression is antagonized by clathrin-dependent endocytosis and by post-endocytic sorting by AP-1 and AP-3. This needs to be clarified, and the expectations of the consequences (i.e., rationale) of AP-1 depletion clarified as well – particularly given that most sorting signals that engage AP-1 also engage AP-2. How would you expect depletion of AP-1 to result in increased cell surface expression? How would depletion

of AP-1 or AP-2 result in decreased cell surface expression?"

Response: We cited the Hirst et al. paper in our revised manuscript and thank the Reviewer for bringing it to our attention. The two studies used completely different approaches: the Hirst et al. paper relied on AP1 mislocalization (knocksideways) while our work used gene KO. Thus, it is difficult to directly compare the results. Nevertheless, key findings of the Hirst et al. study are consistent with the conclusions of this work. For example, a group of membrane proteins such as SORT1 and ROR1 were downregulated in both studies while other membrane proteins such as FOLR1 were upregulated in both studies. We revised the Results section of the manuscript to discuss the findings of the Hirst el al. paper in the context of this work. While the main goal of this study is to establish a role of AAGAB in AP1 assembly and AP1-mediated trafficking, we agree with the Reviewer that future research is needed to define how cargo trafficking is impacted by mutations of AP1, AP2 or AAGAB. We note that, while some cargoes are directly regulated by AP1 and/or AP2, surface levels of other cargoes might be indirectly controlled by AP-1 and/or AP-2 (i.e., through intermediate regulators). For instance, mutations of AP2 may inhibit the retrieval and reuse of an exocytic regulator, resulting in downregulation of surface proteins dependent on the exocytic regulator for surface delivery. We revised the Results and Discussion sections of the manuscript to further discuss these points.

"4. In Table 2, what does relative expression level indicate – WT relative to knockout or knockout relative to WT? This must be indicated on the Table. The same concern applies to Table 3. Data in Figure 3 comparing AP1G1 KO, AP2S1 KO, and AAGAB KO should be analyzed statistically (e.g. by one-way ANOVA)."

Response: The relative expression level indicates KO relative to WT in Tables S2-S4 of the revised manuscript (the original Table S2 was divided into Tables S2-S4 based on the Editor's suggestion). We used the reads in the passage control relative to those in the initial plasmid library to calculate fold changes in Table S5 of the revised manuscript (previously Table S3). Moreover, we analyzed the statistical significance of Figure 3E using one-way ANOVA and updated the figure accordingly.

"5. In Figure 4A, how were surface levels quantified in the flow cytometry experiments? Do the data represent normalized mean, median, or geometric mean fluorescent intensity levels? This needs to be indicated in the figure. Note, Students' t-test is not an appropriate statistical test given that multiple samples are being analyzed; statistical significance should be assessed using a one-way ANOVA test."

Response: The data represent mean fluorescent intensity normalized to WT levels. We analyzed statistical significance using one-way ANOVA and thank the Reviewer for the suggestion.

"6. Is it surprising that only ~200 proteins were upregulated by AP-2 knockdown whereas 700 were upregulated by AP-1 knockdown? How do the authors explain increased surface expression of some proteins upon AP-2 knockdown? The results are interesting, but there are no mechanistic data to address how knockdown influenced surface expression. In addition, some mechanistic insight needs to be provided. For example, given that TfR is not thought to be an AP-1 cargo, and that in HeLa cells it is thought that about 70% of TfR is intracellular at any given time, it is difficult to understand why surface levels of TfR would rise ~10-fold in AAGAB KO cells and also rise substantially in AP1G1 KO cells. Were total cellular levels of TfR (and ECE1, TMEM206, and APLP2, if possible to measure) also increased in AAGAB knockout cells? What is the ratio of intracellular vs. extracellular TfR in these cells (e.g. by comparing total levels to biotinylated TfR in blots)? Is the mRNA of these proteins affected? These questions need to be answered before concluding the AP-1 and/or AAGAB impact trafficking of these proteins directly."

Response: In response to the Reviewer's suggestion, we quantified the surface and total levels of TfR using immunoblotting and flow cytometry. Consistent with previous observations, about 70-80% of TfR molecules were found in intracellular compartments in WT cells. In *AAGAB* KO cells, however, virtually all TfR molecules were found on the cell surface. These new data are included in Figure S1 of the revised manuscript. AAGAB regulates its targets at the protein level without affecting their mRNA levels (Gulbranson et al. 2019, Dev Cell; PMID: 31353312).
While our proteomic analysis supports the roles of AAGAB in AP1- and AP2-dependent trafficking, we acknowledge that the underlying mechanisms could differ across cargo proteins. While some

cargoes are directly regulated by AP1 and/or AP2, surface levels of other cargoes might be independently controlled by AP-1 and/or AP-2 (i.e., through intermediate regulators). We revised the Discussion section of the manuscript to explicitly discuss these points.

"7. In Figures 6B and 6D, can soluble GST-gamma subunit be pulled down in the presence of the sigma1 subunit and the absence of AAGAB? Can sigma1 subunits be stabilized and associate with His6-SUMO-AAGAB in the absence of GST-gamma?"
Response: In the absence of AAGAB, neither gamma nor sigma can be expressed in *E. coli*. Thus, we cannot directly test whether gamma and sigma interact with each other in the absence of AAGAB. Our extensive studies of AP1 and AP2 assembly demonstrated that AAGAB interacts with the alpha/gamma subunit to form binary complex, which then recruits the sigma subunit to form a ternary complex. In our preliminary studies, we observed that, without the alpha/gamma subunit, the binding of the sigma subunit to AAGAB likely represents an unproductive intermediate (please see our response to Point 8 for further explanations).

"8. The model in Figure 7 is not completely supported by the data in the paper, as there are no data to exclude the possibility that AAGAB binds to the sigma chain first and then recruits gamma1. Is there any evidence that either chain is made in excess of the other? The model also lacks any potential explanation for how loss of AAGAB expression would result in the upregulation or downregulation of transmembrane proteins."

Response: Our studies of AP1 and AP2 assembly indicate that the alpha/gamma subunit binds to AAGAB to form a binary complex, which then recruits the sigma subunit to form a ternary complex. Our preliminary experiments suggest that the binding of the sigma subunit to AAGAB (without the alpha/gamma subunit) is prone to dissociation and likely represents an unproductive intermediate. Nevertheless, we agree with the Reviewer that additional research is needed to definitively answer the question. In a separate project, we are collaborating with structural biologists to determine the atomic structures of the binary and ternary complexes and measure their binding affinities. Guided by the structures, we expect to design biochemical and genetic experiments to precisely define the assembly pathways. We revised the Discussion section and Figure 7 to further discuss these points and thank the Reviewer for this important suggestion.

Reviewer 3

"1)I understand that in the previous work by Gulbranson et al. controls were done to show that AAGAB did not directly bind to sigma2-adaptin. Why not show the same for sigma1? Without directly showing that sigma1 only pulls down with AAGAB in the presence of gamma-adaptin, it cannot be concluded that this is the case just because this is the case for AP2 assembly."

Response: Our studies of AAGAB-regulated AP1/AP2 assembly demonstrated that the alpha/gamma subunit binds to AAGAB to form a binary complex, which then recruits the sigma subunit to form a ternary complex. Our preliminary experiments suggest that the binding of sigma subunit to AAGAB (without alpha or gamma subunit) is prone to dissociation and likely represents an unproductive intermediate. Nevertheless, we fully agree with the Reviewer that additional research is required to definitively answer this question. In a separate project, we are collaborating with structural biologists to determine the atomic structures of the binary and ternary complexes and to measure their binding affinities. Guided by the structures, we expect to design biochemical and genetic experiments to precisely define the sequence of the assembly pathways. We revised the Discussion section and Figure 7 to more accurately depict our model. We thank the Reviewer for the important advice.

"2) Although the level of both AP1 and AP2 subunits are greatly reduced in AAGAB KO cells, I am wondering if the remaining subunits may exist as unassembled subunits or if there is some low level of spontaneous assembly happening in the absence of AAGAB. This could easily be tested by anti-gamma co-immunoprecipitations."

Response: The Reviewer raised an important point. We observed that surface levels of TfR in *AAGAB* KO cells are similar to those in *AP1G1/AP2S1* double KO cells (please see our response to Point 3 for further explanations). Moreover, Tf uptake is abrogated in *AAGAB* KO cells, similar to

AP2S1 KO cells. These data indicate that KO of *AAGAB* results in a complete loss of functional AP1 and AP2 adaptors. The residual signals detected by mass spectrometry likely represent nonfunctional polypeptides such as protein aggregates or partial degradation products.

"3) It was concluded that the effect of AAGAB KO on surface levels is a combined effect of AP1 and AP2 downregulation. If this were true, a gamma-adaptin and sigma2-adaptin double KO should have the same effect on transferrin receptor surface levels as does AAGAB KO. This seems easy enough to test and would take away speculations."

Response: Based on the Reviewer's suggestion, we performed the double KO experiment. We observed that surface TfR levels were strongly upregulated in *AP1G1/AP2S1* double KO cells, comparable to those in *AAGAB* KO cells. These findings further suggest that the phenotype of *AAGAB* KO cells reflects the combinatorial effects of AP1 and AP2 deficiency. These new data are included in Figure 4 of the revised manuscript.

"Minor comment: 1) On the top of page 8 it reads … (Fig. 3E and Table 2): I believe it should read 'Table S2'. Please correct."

Response: We corrected the labeling and thank the Reviewer for pointing it out.

Second decision letter

MS ID#: JOCES/2021/258587

MS TITLE: AAGAB is an assembly chaperone regulating AP1 and AP2 clathrin adaptors

AUTHORS: Chun Wan, Lauren Crisman, Bing Wang, Yuan Tian, Shifeng Wang, Ishara Datta, Toshifumi Nomura, Suzhao Li, Haijia Yu, Qian Yin, and Jingshi Shen
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 gave favourable reports but raised some critical 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, depending on further comments from reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. 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.

Reviewer 1

Advance summary and potential significance to field

In this revised manuscript, AAGAB is implicated as an assembly chaperone for adaptor protein complexes. In a previous study, they found that AAGAB serves to regulate the assembly of the AP-2 adaptor complex, a key complex involved in the recruitment of clathrin and receptors to form coated pits and facilitate receptor-mediated endocytosis. The advance in this study is that the authors show, perhaps not surprisingly, that in addition to its role catalyzing AP-2 assembly, AAGAB also regulates the assembly of the homologous AP-1. They find that the assembly is catalyzed by the binding of AAGAB to the gamma subunit of AP-1, allowing recruitment of the sigma subunit, and formation of a ternary hemicomplex. This then allows assembly of beta and mu subunits and AAGAB is released. Accordingly interference with AAGAB expression leads to trafficking defects that are manifested by a combination of impaired AP-2 and AP-1 expression.

Comments for the author

A number of the concerns outlined in the initial reviews have been addressed in this revised version of the manuscript, but there are several key issues that remain unresolved. First, with regard to the overall advance provided in the manuscript, as noted in the previous reviews the finding that AAGAB catalyzes AP-1 assembly similar to that of AP-2 is not entirely unexpected, and a more significant advance would come from a molecular understanding of why AP-3 is capable of assembling without AAGAB, unlike AP-1 and AP-2. The revision does not contain any new experimental data or molecular delineation that would further promote this understanding. Second, it is still unclear why the authors have not demonstrated interactions between the endogenous AAGAB and AP-1, especially since there are effective antibodies available? Even if the transfected proteins are expressed, as noted by the authors "at endogenous levels," why not demonstrate interactions by the actual endogenous proteins? The most convincing demonstration of a protein-protein interaction in cells comes from endogenous rather than exogenous proteins.

Reviewer 2

Advance summary and potential significance to field

If the authors had addressed the concerns raised previously, the paper might have supported a role for a common assembly factor, AAGAP, in the assembly of the multisubunit protein complexes adaptor protein-1 (AP-1) and AP-2, but not other related protein complexes. It might have shown that loss of expression of this assembly factor has an additive effect on cargoes of both AP-1- and AP-2-dependent trafficking. As is, however, the paper lacks suitable controls and does not assess the impact of knockdown of either AP-1 or AAGAP on known AP-1-dependent cargoes.

Comments for the author

In the revised manuscript and response to the reviewers, Wan et al have addressed a few of the concerns raised in the review of the original manuscript. However, the responses largely failed to adequately address the major concerns. In some cases, the authors addressed concerns in the response to reviewers but did not accordingly change the paper. In other rather critical cases, they simply did not address the concern. I have reiterated the concerns here. Unless these concerns are addressed adequately, the conclusions in the paper are not justified.

1. In the response to reviewers, the authors nicely answered questions raised in the initial review about Figure 2, but only addressed (partially) the characterization of AAGAB alone in the paper. In panel A, the positions of included and excluded volumes in the elution volume should be noted on the figure or in the figure legend. In panel B, the SDS-PAGE profile of AAGAB should also be shown to validate distinct peaks for the complex vs. the AAGAB tetramer. The text should indicate how the 1:1 stoichiometry was estimated; to this reviewer's eye, the Coomassie Blue labeling does not support the conclusion and I do not understand how the elution profile would support it either.

2. I do not understand the authors' response to the original concern that they have not demonstrated whether AAGAB binds to sigma1 alone. Figure 2 panels D and E should include a lane in which AAGAB was mixed together with sigma1 alone and tested for co-IP with AAGAB. There is also no negative control in the experiment, which is why I suggested testing for co-IP with AP-3 delta chain (or the corresponding COPI subunit). The authors might have believable data, but they are not showing these data in the paper. Without proper controls, the conclusions are simply not supported.

3. The authors did not quite grasp the concern raised about the lack of consideration of data in the Hirst et al 2012 paper on the "AP-1-ome". This paper identified transmembrane proteins that are isolated in AP-1-coated vesicles, and thus that are likely direct AP-1-dependent cargoes. While the authors added a citation to this paper in their manuscript, the authors failed to note whether any of the hits in their proteomic analyses of proteins affected at the plasma membrane corresponded to any of those identified in the Hirst et al paper as an AP-1 cargo. As I indicated previously, transferrin receptor does not bind AP-1 and thus is not a direct AP-1 cargo; thus, focusing on transferrin receptor response to AP-1 and AAGAP knockdown, while convenient, does not tell the reader whether the results support the conclusions drawn (although see point #5 below). Were ECE1, TMEM206, APLP2, or ITGA3 identified in the Hirst et al paper? If they or other hits were, it would raise some confidence that the effects observed here might be direct.

4. It would be helpful in Figure 4B to change the title of the y-axis in the figure to "Normalized surface levels of TfR (MFI)" reflect that mean fluorescence intensity (MFI) was used to quantify the data.

5. It is disappointing that the authors did not address the mechanism of TfR surface expression adequately in their AP-1 and AAGAB knockouts as suggested in the initial review. The authors state in their response that AAGAB does not regulate its targets at the mRNA level and cite their previous paper, but then they show here in a new Figure S1 that, as I suspected, total cellular levels of TfR are increased about 2.5X in cells knocked down for AAGAB. First, I see no data in either paper to support the conclusion that TfR mRNA levels are not altered by AAGAB depletion. Second, the data shown support the conclusion that the major impact of AAGAB loss of expression is on total TfR expression; while it is true that there is a notable shift in the total pool towards surface expression, the total increase in surface expression is a factor of both this shift and the dramatically increased TfR expression. This needs to be acknowledged. Moreover, it would be helpful to understand if the increased expression is due to transcriptional feedback or the well-known translational control of TfR levels by iron depletion. The latter could reflect changes in iron handling inside the cell, e.g., by impacting DMT1/SLC11A2 recycling or rescue from lysosomal degradation. This might lead the authors to a mechanism for their TfR results, given that TfR does not bind to AP-1.

6. The authors maintain that sigma does not bind to AAGAB, but do not show the data. The authors maintain that gamma and sigma cannot be expressed together without AAGAB, but do not show the data. These are important points to understand how AAGAB impacts AP-1 assembly and important controls for the specificity of AAGAB for gamma and alpha chain interactions; thus, the data must be shown. Lastly, how the stoichiometry was estimated 1:1:1 for AAGAB:gamma:sigma must be indicated in the manuscript. I see a much weaker band for sigma than for gamma and AAGAB in the Coomassie stain in panel B.

7. One minor point in addition to these previously raised concerns. In the abstract and again in the 2nd paragraph of the introduction, perhaps the authors previously assumed that AP-2 assembles spontaneously, but anyone studying assembly of multisubunit protein complexes would not have made the same assumption. I strongly suggest striking this phrase from both places.

Reviewer 3

Advance summary and potential significance to field

This is a revised manuscript.

Comments for the author

The authors successfully addressed most of my concerns. I have only a few minor comments on the revised manuscript that should be addressed.
1) Fig 6D: If I am not mistaken, sigma 1 is pulled down by AAGAB in the absence of gamma-adaptin. This should be mentioned in the text. Even an unproductive binding would indicate that there are indeed binding sites for sigma 1 on AAGAB as one might expect for a gamma-sigma assembly chaperone.

2) Sup Fig. 1: The ratio of surface to total TfR should be calculated and shown. Also, this data indicates that KO of AAGAB most likely affects lysosomal degradation of TfR. Please clarify this point in the manuscript.

Second revision

Author response to reviewers' comments

Reviewer 1

"First, with regard to the overall advance provided in the manuscript, as noted in the previous reviews the finding that AAGAB catalyzes AP-1 assembly similar to that of AP-2 is not entirely unexpected, and a more significant advance would come from a molecular understanding of why AP-3 is capable of assembling without AAGAB, unlike AP-1 and AP-2. The revision does not contain any new experimental data or molecular delineation that would further promote this understanding."

Response: The Reviewer inquired about AP3 assembly, a point also raised by Reviewer 2. After consulting the Editor, we carried out new coIP experiments to examine whether AAGAB binds AP3. We found that AAGAB does not interact with AP3 subunits (Figure S1), consistent with our conclusion that AAGAB selectively regulates AP1 and AP2 but not AP3. We anticipate that AP3 assembly also requires a specific assembly chaperone, analogous to the role of AAGAB in AP1/2 assembly. We revised the Discussion section of the manuscript to further discuss this point.

"Second, it is still unclear why the authors have not demonstrated interactions between the endogenous AAGAB and AP-1, especially since there are effective antibodies available? Even if the transfected proteins are expressed, as noted by the authors "at endogenous levels," why not demonstrate interactions by the actual endogenous proteins? The most convincing demonstration of a protein-protein interaction in cells comes from endogenous rather than exogenous proteins."

Response: Based on the Reviewer's suggestion, we performed new coIP experiments to characterize endogenous protein-protein interactions. Using antibodies recognizing endogenous AAGAB or AP1 subunits, we confirmed that AAGAB interacts with AP1. Included in Figure S1 of the revised manuscript, these new data further support the conclusion we made.

Reviewer 2

"1. In the response to reviewers, the authors nicely answered questions raised in the initial review about Figure 2, but only addressed (partially) the characterization of AAGAB alone in the paper. In panel A, the positions of included and excluded volumes in the elution volume should be noted on the figure or in the figure legend. In panel B, the SDS-PAGE profile of AAGAB should also be shown to validate distinct peaks for the complex vs. the AAGAB tetramer. The text should indicate how the 1:1 stoichiometry was estimated; to this reviewer's eye, the Coomassie Blue labeling does not support the conclusion and I do not understand how the elution profile would support it either."

Response: In response to the Reviewer's suggestion, we added information of included and excluded volumes to the legend of Figure 2A. We also added coomassie blue staining data of AAGAB alone to Figure 2B. While SEC and coomassie blue data are consistent with a 1:1 ratio, *we acknowledge that the data do not definitively establish the stoichiometry. Thus, we removed the stoichiometry estimate from the text.*

"2. I do not understand the authors' response to the original concern that they have not demonstrated whether AAGAB binds to sigma1 alone. Figure 2 panels D and E should include a lane in which AAGAB was mixed together with sigma1 alone and tested for co-IP with AAGAB. There is also no negative control in the experiment, which is why I suggested testing for co-IP with AP-3 delta chain (or the corresponding COPI subunit). The authors might have believable data, but they are not showing these data in the paper. Without proper controls, the conclusions are simply not supported."

Response: We should have stated more clearly to avoid confusion. In this work, we focus on the AAGAB:*γ* to AAGAB:*γ*:σ assembly route because it is similar to AAGAB-guided AP2 assembly and thus likely represents a conserved assembly pathway. Our initial plan was to further characterize sigma interactions in a future project. However, we agree with the Reviewer that the experiment also fits into the scope of this study and may yield new insights into AAGAB function. Based on discussions with the Editor, we performed new sets of coIP experiments and observed that AAGAB interacts with AP1 sigma alone, in contrast to AP2-AAGAB interactions. These new data are included in Figure 2E-F of the revised manuscript. We also performed coIP experiments to examine whether AP3 delta binds AAGAB. We observed that AP3 delta does not interact with AAGAB (Figure S1), supporting the specificity of AAGAB-AP1/2 interactions.
Since AP2 sigma does not bind to AAGAB, we posit that AAGAB:*γ* to AAGAB:*γ*:σ likely represents the main assembly pathway. However, we acknowledge that AAGAB:σ to AAGAB:*γ*:σ may represent an alternative assembly pathway for AP1 adaptor. We revised the text of the manuscript (highlighted in blue) to explicitly discuss these points.

"3. The authors did not quite grasp the concern raised about the lack of consideration of data in the Hirst et al 2012 paper on the "AP-1-ome". This paper identified transmembrane proteins that are isolated in AP-1-coated vesicles, and thus that are likely direct AP-1- dependent cargoes. While the authors added a citation to this paper in their manuscript, the authors failed to note whether any of the hits in their proteomic analyses of proteins affected at the plasma membrane corresponded to any of those identified in the Hirst et al paper as an AP-1 cargo. As I indicated previously, transferrin receptor does not bind AP-1 and thus is not a direct AP-1 cargo; thus, focusing on transferrin receptor response to AP-1 and AAGAP knockdown, while convenient, does not tell the reader whether the results support the conclusions drawn (although see point #5 below). Were ECE1, TMEM206, APLP2, or ITGA3 identified in the Hirst et al paper? If they or other hits were, it would raise some confidence that the effects observed here might be direct."

Response: The Hirst et al paper used knocksideways to acutely relocate AP1 to mitochondria and then examined changes in cargo proteins in CCVs. While the Hirst et al paper used a fundamentally different approach, a subset of cargoes including FOLR1, SORT1 and ROR1 were identified in both studies. After consulting the Editor, we revised the text of the manuscript to further discuss this point.

"4. It would be helpful in Figure 4B to change the title of the y-axis in the figure to "Normalized surface levels of TfR (MFI)" reflect that mean fluorescence intensity (MFI) was used to quantify the data."

Response: We changed the labeling based on the Reviewer's suggestion and thank the Reviewer for improving the clarity of figure in this regard.

5. It is disappointing that the authors did not address the mechanism of TfR surface expression adequately in their AP-1 and AAGAB knockouts as suggested in the initial review. The authors state in their response that AAGAB does not regulate its targets at the mRNA level and cite their previous paper, but then they show here in a new Figure S1 that, as I suspected, total cellular levels of TfR are increased about 2.5X in cells knocked down for AAGAB. First, I see no data in either paper to support the conclusion that TfR mRNA levels are not altered by AAGAB depletion.

Second, the data shown support the conclusion that the major impact of AAGAB loss of expression is on total TfR expression; while it is true that there is a notable shift in the total pool towards surface expression, the total increase in surface expression is a factor of both this shift and the dramatically increased TfR expression. This needs to be acknowledged. Moreover, it would be helpful to understand if the increased expression is due to transcriptional feedback or the wellknown translational control of TfR levels by iron depletion. The latter could reflect changes in iron handling inside the cell, e.g., by impacting DMT1/SLC11A2 recycling or rescue from lysosomal degradation. This might lead the authors to a mechanism for their TfR results, given that TfR does not bind to AP-1.

Response: We apologize for misunderstanding. We previously showed that AAGAB does not directly regulate AP2 mRNA levels, leading to the conclusion that AAGAB regulates its substrates at the protein level (Gulbranson et al., 2019. PMID: 31353312). Nevertheless, it is possible that AAGAB indirectly regulates gene transcription. We performed qRT-PCR experiments to examine TfR mRNA levels in WT and *AAGAB* KO cells. Indeed, we found that TfR mRNA was markedly elevated in *AAGAB* KO cells, suggesting that transcriptional regulation plays an important role in upregulation of TfR expression. These new data are added to Figure S2 of the revised manuscript. We also revised the text (shown in blue) to discuss the implications of the results.

6. The authors maintain that sigma does not bind to AAGAB, but do not show the data. The authors maintain that gamma and sigma cannot be expressed together without AAGAB, but do not show the data. These are important points to understand how AAGAB impacts AP-1 assembly and important controls for the specificity of AAGAB for gamma and alpha chain interactions; thus, the data must be shown. Lastly, how the stoichiometry was estimated 1:1:1 for AAGAB:gamma:sigma must be indicated in the manuscript. I see a much weaker band for sigma than for gamma and AAGAB in the Coomassie stain in panel B.

Response: Based on the Reviewer's advice, we co-expressed AP1 gamma and sigma subunits in E. coli with and without AAGAB. We found that gamma and sigma could not be expressed unless AAGAB was also co-expressed, further supporting the essential role of AAGAB in stabilizing AP1 subunits. These new data are included in Figure S3 of the revised manuscript. Moreover, we performed new coIP experiments to further characterize AAGAB binding to AP1 subunits and the data are included in Figures 2E-F and S1 of the revised manuscript (please see our response to Point 2). While the coomassie blue staining data are consistent with a 1:1:1 ratio (staining of sigma subunit is intrinsically weak due to its small size), we acknowledge that these data do not establish a precise stoichiometry. Thus, we removed the stoichiometry estimate.

7. One minor point in addition to these previously raised concerns. In the abstract and again in the 2nd paragraph of the introduction, perhaps the authors previously assumed that AP-2 assembles spontaneously, but anyone studying assembly of multisubunit protein complexes would not have made the same assumption. I strongly suggest striking this phrase from both places.

Response: We removed both sentences based on the Reviewer's suggestion.

Reviewer 3

"1)Fig 6D: If I am not mistaken, sigma 1 is pulled down by AAGAB in the absence of gamma-adaptin. This should be mentioned in the text. Even an unproductive binding would indicate that there are indeed binding sites for sigma 1 on AAGAB as one might expect for a gamma-sigma assembly chaperone."

Response: Based on the Reviewer's suggestion, we added AP1 sigma coIP data to Figure 2 and discussed the implications of the findings in the revised manuscript (changes are shown in blue). We thank the Reviewer for the suggestion.

"2)Sup Fig. 1: The ratio of surface to total TfR should be calculated and shown. Also, this data indicates that KO of AAGAB most likely affects lysosomal degradation of TfR. Please clarify this point in the manuscript."

Response: In response to the Reviewer's advice, we added the ratios of surface-to-total TfR levels to Figure S2 (previously Figure S1). We also revised the text to state that *AAGAB* KO may affect lysosomal degradation of TfR.

Third decision letter

MS ID#: JOCES/2021/258587

MS TITLE: AAGAB is an assembly chaperone regulating AP1 and AP2 clathrin adaptors

AUTHORS: Chun Wan, Lauren Crisman, Bing Wang, Yuan Tian, Shifeng Wang, Rui Yang, Ishara Datta, Toshifumi Nomura, Suzhao Li, Haijia Yu, Qian Yin, and Jingshi Shen
ARTICLE TYPE: Research Article

As you will see below, the reviewers gave favourable overall reports but reviewer #2 asks for you to make a small editorial change of clarification. Once you have done this, please send the paper back to our office and I will accept the paper.
I look forward to receiving your revised 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.)

Reviewer 1

Advance summary and potential significance to field

Overall the authors have made major attempts to improve the manuscript and address the concerns outlined in the first submission. In particular, evidence is now presented with regard to AP-3 (and its ability to assemble without AAGAB unlike AP-1 and AP-2), as well as demonstration that endogenous AAGAB and AP-1 interact. This reviewer would recommend that those new data be included in the body of the manuscript, rather than as supplemental data, because the endogenous interaction is significant.

Comments for the author

Comments addressed in the summary above.

Reviewer 2

Advance summary and potential significance to field

The paper documents that AAGAB, a protein previously identified by these authors to regulate the early assembly of the heterotetrameric AP-2 complex, also is required for the assembly of the related heterotetrameric complex AP-1, but not the similar AP-3 and COPI complexes. The authors show that depletion of AAGAB has similar effects on cell surface expression of target proteins as knockout of both AP-1 and AP-2, suggesting that it's major function is in the assembly of these two protein complexes. Supplemental data include proteomic analyses to document cargoes whose cell surface expression is impacted by AAGAB deficiency. This is important in documenting similarities and differences in the control of the assembly of heteromeric protein complexes in mammalian cells.

Comments for the author

In their revised manuscript, the authors have finally addressed the concerns over necessary controls, and the new data largely support their previous conclusions and add some new twists to the story. The paper is now complete and will be a solid contribution to the literature.
The new added text requires one very minor modification. On page 6 in the revised text, the phrase “AP3 delta subunit (Fig. S1) which is equivalent to AP1 gamma subunit and AP2 alpha subunit” needs to be rephrased. These subunits are not equivalent, i.e., cannot substitute for each other. Perhaps a more correct way to phrase it would be “AP3 delta subunit (Fig. S1), which is the structural and functional equivalent within AP3 to the gamma and alpha subunits within AP1 and AP2, respectively”.

Reviewer 3

Advance summary and potential significance to field

This is a review of a revised manuscript.
All my previous comments have been successfully addressed. Therefore, I support publication of this manuscript.

Comments for the author

I have no further comments.

Third revision

Author response to reviewers' comments

Reviewer 2

“The new added text requires one very minor modification. On page 6 in the revised text, the phrase “AP3 delta subunit (Fig. S1) which is equivalent to AP1 gamma subunit and AP2 alpha subunit” needs to be rephrased. These subunits are not equivalent, i.e., cannot substitute for each other. Perhaps a more correct way to phrase it would be “AP3 delta subunit (Fig. S1), which is the structural and functional equivalent within AP3 to the gamma and alpha subunits within AP1 and AP2, respectively”.”

Response: We revised the sentence based on the suggestion of the reviewer.

Fourth decision letter

MS ID#: JOCES/2021/258587

MS TITLE: AAGAB is an assembly chaperone regulating AP1 and AP2 clathrin adaptors

AUTHORS: Chun Wan, Lauren Crisman, Bing Wang, Yuan Tian, Shifeng Wang, Rui Yang, Ishara Datta, Toshifumi Nomura, Suzhao Li, Haijia Yu, Qian Yin, and Jingshi Shen
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.