nature portfolio

Peer Review File

Phosphoproteomics-directed manipulation reveals SEC22B as a hepatocellular signaling node governing metabolic actions of glucagon

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

NCOMMS-24-03555-T Wu et al, entitled “Phosphoproteomics-1 directed manipulation reveals SEC22B as a hepatocellular signaling 2 node governing metabolic actions of glucagon” is an original article. This study utilized liver phosphoproteomics to identify SEC22B protein as a crucial signaling node regulating glycogen, lipid, and amino acid metabolism, and mediating the metabolic effects of glucagon. The study also identified a few protein partners of SEC22B which were affected by glucagon. The rapid manipulation of intracellular protein action through protein phosphorylation affects metabolism, and utilizing phosphoproteomics can help address some of the unanswered questions. These findings provide valuable insights into the biological mechanisms of glucagon action including identifying a previously unappreciated player and may potentially lead to the development of new treatments for metabolic disorders. However, one weakness is that data collection is restricted to rodents in this study, so the translation of this work is unclear. Overall, this is an interesting well-executed study. However, a few lingering concerns remain.

Major comments

1. The authors began their study examining changes in rat liver proteome. Then they conducted a knock-out study with mice. However, since mice and rats have different genetic differences, it is unclear why they did not conduct an in-situ glucagon experiment with mice. They have conducted in situ time-resolved liver phosphoproteomics to reveal glucagon signaling nodes using rats and figured out that SEC22 Homolog B 49 (SEC22B) S137 phosphorylation is a top hit. Then they did a knock-out study with mice. Although both rats and mice can provide helpful information, it is important to note that they have genetic differences. It would be helpful to know why you chose to conduct the in-situ glucagon experiment on rats and not on mice before proceeding with the knock-out study on mice.

2. The data presented in Figure S3k, which confirms the knockout of SEC22B in mice, is significant in supporting your research findings. We propose moving this data to the main figure.

3. You mentioned that male Sprague-Dawley rats were used for the in-situ liver glucagon treatment studies. However, at the end of the paragraph in line 389, you mentioned 3-4 mice per group. So, I am a bit confused. Did you use both mice and rats for the in-situ experiment? If you didn't use mice for the in-situ glucagon treatment, could you please explain why? Also, could you clarify whether SEC22B is functionally and genetically conserved?

4. Please explain why you chose 2, 8, and 32 minutes for the in situ glucagon experiment.

5. According to available data, SEC22B is believed to be involved in autophagy and amino acid transport. Can you confirm this using your proteomics or Western blot data?

6. Hepatic SEC22B silencing resulted in a reduction of serum triglyceride and cholesterol levels while increasing the liver triglyceride levels. To confirm the changes in lipoprotein biogenesis and

maturation-related pathway analysis, further validation is needed as well as a brief discussion of the limitations of proteomics studies. It is particularly important to validate since you haven't seen proteomics data change CREB-S133 and there is change in CREB-S133 with Hepatic SEC22B silencing has been shown to have a positive effect on serum triglyceride and cholesterol levels, while at the same time increasing the liver triglyceride levels. In order to confirm these changes and to understand how they relate to lipoprotein biogenesis and maturation; a pathway analysis needs to be conducted. It is particularly important to validate this change because there was no change in the proteomics data for CREB-S133. However, there was a change in CREB-S133 as per the western blot analysis. (lines 328-335) Can you show glycogen synthesis pathway is affected in knock-out mice with western blot?

7. Could you please provide additional description in the figure legends for Fig Figure 5b, c, d? The information presented in Figure 5b is difficult to discern. The font is too small to read at 100% viewer or in a printed-out format. It's unclear from the figure and the legend what each square and the color mean on the right side of Figure 5b. It's also unclear what these changes in interaction ultimately mean. Is there a way to discern the meaning of these interactions? Could you provide some model to describe the changes?

8. Hepatic SEC22B silencing raised the serum levels of multiple 159 amino acid species selectively under acute glucagon treatment conditions (Fig. 4e-j, Fig. S4g-k). Would you be able to provide possible ways or literature to explain/support these changes?

9. Could you please provide the histology scoring for H&E and oil red O staining? (example: PMCID: PMC4275274).

10. It appears that the liver cells of the control group (SEC22b miR-NC) in Figure S3 have been damaged. Moreover, the H&E staining of GFP miR-NC does not resemble SEC22b miR-NC. Can this be quantified or examined by other methods looking at tissue damage (e.g. fibrosis)?

11. By analyzing the signal intensity, you should be able to determine the relative protein expression in Figure 3Sk. This will provide a better idea of the protein knockdown using Sec22b, a negative control (miR-NC), and/or AAV-Sec22b cDNA (Sec22b) or a control (GFP) in both fasted and refed states.

12. All males were used for this study. There needs to be some examination of sex differences. Does this system work the same way in females?

Reviewer #2 (Remarks to the Author):

In this manuscript, Wu and colleagues use phosphoproteomics to interrogate the mechanisms of glucagon signaling in liver in mice. Their data highlight phosphorylation of the vesicle trafficking protein SEC22 Homolog B (SEC22B) as a key target of glucagon action. The authors utilize SEC22 knockout and gain of function models in hepatocytes to validate the impact of this signaling in vitro.

As the mechanism of glucagon action is still being debated a century after its discovery, this study is important and generally well performed (though I am not an expert in phosphoproteomics, so I will not comment on that analysis). I do have several comments for the editor's consideration:

1. Glucagon concentrations in the perifusions appear high, considering that circulating plasma glucagon is in the pM range in rodents and humans under most conditions; this may be justifiable considering that portal vein hormone concentrations will be higher than circulating, but the authors need to explicitly explain/justify this dose.

2. The authors observe a surprising increase in lipid droplet concentrations in SEC22 KO hepatocytes (which is counter to what several other groups have observed, showing that glucagon action reduce liver triglyceride content). They speculate about several mechanisms that could explain this; however, in my view, this is a key fundamental, mechanistic point. For example, if the differences arise from differences between in vitro and in vivo programs, this renders their large amount of in vitro data of questionable relevance.

3. The data showing that liver triglycerides are increased in the SEC22 KO knockdown mice contrast with the effect that several groups have observed with glucagon agonism lowering liver triglycerides. This discrepancy is what it is, but should be explored in more detail experimentally (rather that merely textually).

4. I commend the authors on their very clear and streamlined graphical abstract/summary figure; this is an underappreciated aspect of many manuscripts and, in this case, is a great aid to the readers.

Reviewer #4 (Remarks to the Author):

In the manuscript by Wu et al., authors demonstrate that phosphorylation of SEC22B is a hepatocellular signaling node mediating specific metabolic actions of glucagon. They also discuss novel pathways and processes activated by glucagon, and predict the kinases involved. The experiments are well-designed and performed on qualified mouse models. The phosphoproteomic analysis appears sound as well. I have a few comments and questions:

1. The reader would benefit from a more detailed phosphoproteomic workflow, rather than a mere citation for another paper, and especially since phosphorylation is the central theme of the manuscript. The cited paper gives a choice between TiO2 and IMAC, it is not clear which of the two (or both) were used in this study. A paragraph describing the phosphoenrichment steps briefly, would be a great addition to the paper.

2. What was the enrichment specificity for phosphopeptides?

3. What was the confidence cut off (%) for a phospho-site being accepted as true?

4. What was the peptide amount loaded on the columns, for both global and phosphoproteomic samples? Line 466 says 1uL peptide but not the amount in ug, and nothing is mentioned for the phospho samples.

5. What was the reason for acquiring proteomics data in DIA mode, and phosphoproteomics data in DDA mode?

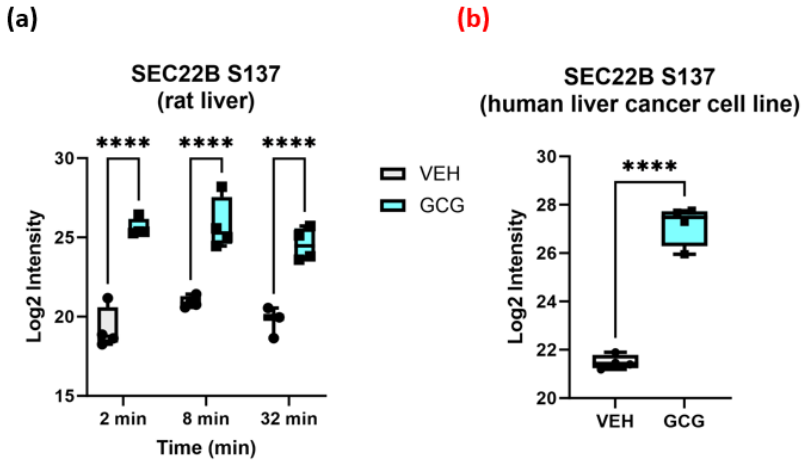
Reviewer Comments:

Reviewer #1 (Remarks to the Author)

NCOMMS-24-03555-T Wu et al, entitled “Phosphoproteomics-1 directed manipulation reveals SEC22B as a hepatocellular signaling 2 node governing metabolic actions of glucagon” is an original article. This study utilized liver phosphoproteomics to identify SEC22B protein as a crucial signaling node regulating glycogen, lipid, and amino acid metabolism, and mediating the metabolic effects of glucagon. The study also identified a few protein partners of SEC22B which were affected by glucagon. The rapid manipulation of intracellular protein action through protein phosphorylation affects metabolism, and utilizing phosphoproteomics can help address some of the unanswered questions. These findings provide valuable insights into the biological mechanisms of glucagon action including identifying a previously unappreciated player and may potentially lead to the development of new treatments for metabolic disorders. However, one weakness is that data collection is restricted to rodents in this study, so the translation of this work is unclear. Overall, this is an interesting well-executed study. However, a few lingering concerns remain.

Reply:
We thank this reviewer for taking the time to critically appraise our manuscript and appreciate acknowledgement of the discoveries that we have made. To address the point about relevance to humans, we have conducted further glucagon-phosphoproteomics studies in a human hepatocyte cell line, and can indeed show that, similar to rats, SEC22B-S137 phosphorylation is robustly (>30-fold) upregulated by glucagon. These new data are incorporated into Fig. 2b.

The results section now includes this statement: “Treating human SNU398 hepatoma cells overexpressing the glucagon receptor (GCGR) with 1 nM glucagon for 30 minutes robustly increases p-PKA motif expression and CREB-S133 levels (Fig. S1e-g), indicating the sensitivity of glucagon signaling in SNU398-GCGR cells. We then assessed these human cell samples using phosphoproteomic analysis. Although there was very little overlap between the phosphoproteomic profiles of the human hepatoma cell and rat liver samples (Fig. 2d-e), glucagon substantially increased SEC22B S137 phosphorylation (Fig. 2b), consistent with the findings in the rat liver study.”



In addition, clinical studies have implicated alterations in SEC22B S137 phosphorylation in liver disease. Specifically, lower levels of SEC22B S137 phosphorylation were observed in

patients with simple steatosis (SS) and non-alcoholic steatohepatitis (NASH), suggesting that SEC22B phosphorylation may play a crucial role in the development of these liver conditions (PMID: 28258704).

Major comments

1. The authors began their study examining changes in rat liver proteome. Then they conducted a knock-out study with mice. However, since mice and rats have different genetic differences, it is unclear why they did not conduct an in-situ glucagon experiment with mice. They have conducted in situ time-resolved liver phosphoproteomics to reveal glucagon signaling nodes using rats and figured out that SEC22 Homolog B 49 (SEC22B) S137 phosphorylation is a top hit. Then they did a knock-out study with mice. Although both rats and mice can provide helpful information, it is important to note that they have genetic differences. It would be helpful to know why you chose to conduct the in-situ glucagon experiment on rats and not on mice before proceeding with the knock-out study on mice.

Reply:

Firstly, as we note in the manuscript, similar to glucagon's metabolic effects (PMID: 21129328), the SEC22B phosphosite is conserved in all mammals and is regulated by nutritional status in mice.

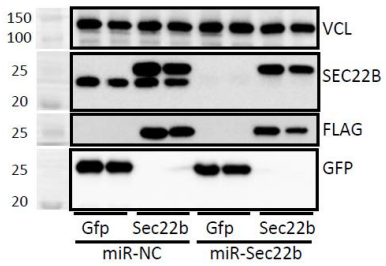
In addition, we chose to conduct our studies in rats initially as they are well known to respond to glucagon (PMID: 5909499). In addition, as detailed in the methods section, we chose to use a perfused liver model. We chose this model as it is well known that primary hepatocytes are less-responsive to glucagon as compared to perfused rat liver and are dedifferentiated on moving to an artificial media and plating (PMID: 6363185). Plus, the perfused liver model allows provision of the stimuli (in this case glucagon) in a natural anatomical manner, that is, via the portal vein and flowing though the liver sinusoid in a natural manner. As compared to mice, which are a lot smaller (25g mouse vs. 250g rat), the perfusion model is thus a lot bigger leading to far less complications and problems and thus more robust data. Furthermore, the methods for the perfused rat liver are far more validated than that of mouse. Lastly, it was also a matter of convenience, as Prof. Greg Smith, is an expert in this technique and has an interest in glucagon biology, and we knew that together we could use this valid model for our investigations. While we acknowledge that there are genetic differences in rat vs. mouse, they are at least from the same family of rodents, i.e. Muridae, thus minimising the risk that we would follow an epiphenomenon of individual species. We continued with our AAV/genetic studies of mice for reasons of economics (less agistment costs and require use of less AAV) and familiarity with this animal model.

2. The data presented in Figure S3k, which confirms the knockout of SEC22B in mice, is significant in supporting your research findings. We propose moving this data to the main figure.

Reply:

This is a good suggestion and we have moved this into the main figure as Fig. 3o.

(o)



3. You mentioned that male Sprague-Dawley rats were used for the in-situ liver glucagon treatment studies. However, at the end of the paragraph in line 389, you mentioned 3-4 mice per group. So, I am a bit confused. Did you use both mice and rats for the in-situ experiment? If you didn't use mice for the in-situ glucagon treatment, could you please explain why? Also, could you clarify whether SEC22B is functionally and genetically conserved?

Reply:
This was a mistake and we thank the reviewer for picking this up. We only used rats for in-situ experiment. We have corrected "mice" to "rats" in manuscript. This mistake has been corrected and the section now reads "3-4 rats per group". As shown in Fig. 2c, and new Fig 2b, Sec22B expression and S137 phosphorylation is conserved from mouse, to rat and human. Concerning, the whole sequence as compared to mouse, rat (100%) and human (98.1%) are highly conserved (from UniProt database).

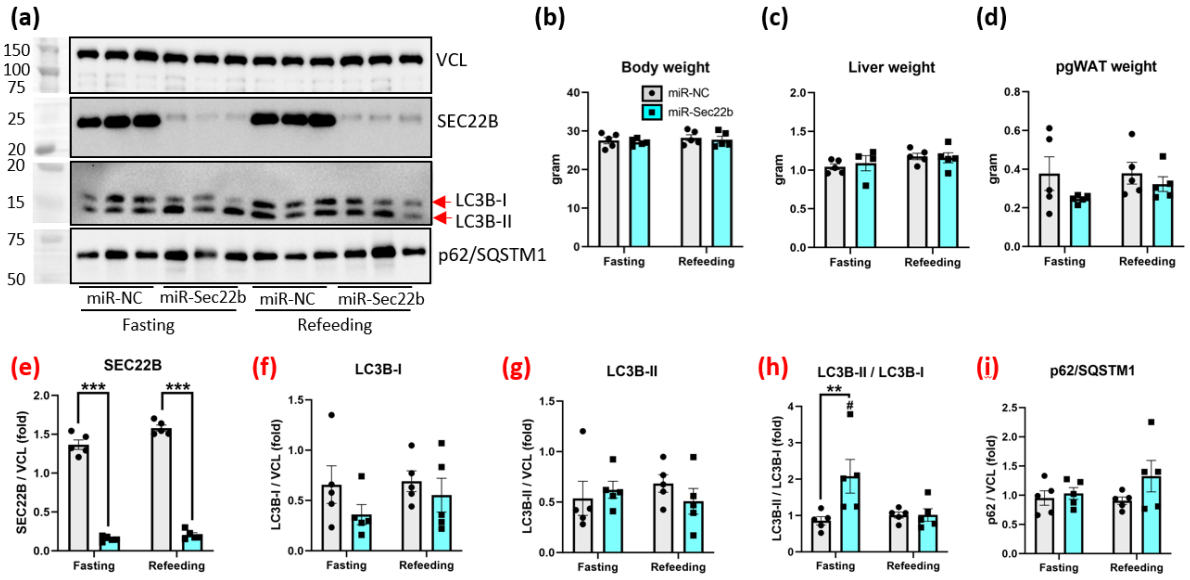
4. Please explain why you chose 2, 8, and 32 minutes for the in situ glucagon experiment.

Reply:
These times were chosen as glucagon is known to exert rapid and sustained changes in metabolism (PMID: 28275047). We chose 2 min and multiples of 4 of this time point with 3 time points in order to resolve potential kinetics of glucagon signalling, particularly as metabolic effects of glucagon (i.e. glycogen depletion) may have feedback effects on signalling that may be either restrictive or enhancing/permissive (PMID: 33792899). Indeed, as shown in Fig. 1f, we show that PKA and PKG signalling is rapidly and sustainably induced whereas PAK signalling is more gradually induced.
The methods section has been altered to reflect the reason behind this decision and now reads: "A rapid time-course design was chosen as GPCR signaling is known to be rapid and temporal dynamics are important for physiology (PMID: 29074251), and these aspects are yet to be explored for glucagon receptor signalling in the liver."

5. According to available data, SEC22B is believed to be involved in autophagy and amino acid transport. Can you confirm this using your proteomics *or* Western blot data?

Reply:
The relationship between SEC22B and autophagy is complex and not entirely clear. SEC22B plays a critical role in autophagy, as evidenced by studies showing that its knockdown leads to reduced LC3 lipidation (PMID: 25432021). Paradoxically, SEC22B depletion inhibits the trafficking of lysosomal proteases to the lysosome, resulting in impaired autophagosome LC3-II degradation and increased autophagosome numbers (PMID: 21242315). All of these studies have been conducted in vitro, highlighting the need for further investigation to fully understand SEC22B's role in autophagy in vivo.

We did western blot for classical autophagy related proteins, including microtubule-associated protein 1 light chain 3 (LC3) and p62/SQSTM1. There's no statistical difference in p62, LC3B-I or LC3B-II, but SEC22B KD group showed higher LC3B-II / LC3B-I ratio in fasting state.
The results section now includes this statement: "As SEC22B manipulation affects amino acid metabolism, we assessed several classical autophagy-related proteins, including microtubule-associated protein 1 light chain 3 (LC3) and p62/SQSTM1, in the fasting and refeeding study. While there were no significant differences in p62, LC3B-I, or LC3B-II levels, SEC22B silencing resulted in a higher LC3B-II/LC3B-I ratio during the fasting state (Fig. S3a, S3f-i)."



The co-immunoprecipitation proteomics analysis revealed that glucagon treatment induced an interaction between SEC22B and the ubiquitin ligase RNF5. In breast cancer cells, RNF5 has been shown to stimulate the ubiquitination and subsequent degradation of the L-glutamine carrier proteins SLC1A5 and SLC38A2, leading to decreased glutamine uptake (PMID: 25759021). Consequently, it is plausible that the glucagon-induced interaction between SEC22B and RNF5 might impact amino acid transporters, thereby potentially influencing amino acid metabolism. This has mentioned in the Discussion section of our manuscript.

6. Hepatic SEC22B silencing resulted in a reduction of serum triglyceride and cholesterol levels while increasing the liver triglyceride levels. To confirm the changes in lipoprotein biogenesis and maturation-related pathway analysis, further validation is needed as well as a brief discussion of the limitations of proteomics studies.
It is particularly important to validate since you haven't seen proteomics data change CREB-S133 and there is change in CREB-S133 with Hepatic SEC22B silencing has been shown to have a positive effect on serum triglyceride and cholesterol levels, while at the same time increasing the liver triglyceride levels. In order to confirm these changes and to understand how they relate to lipoprotein biogenesis and maturation; a pathway analysis needs to be conducted. It is particularly important to validate this change because there was no change in the proteomics data for CREB-S133. However, there was a change in CREB-S133 as per the western blot analysis. (lines 328-335)

Reply:
Regarding the limitations of proteomics, we have added these sentences to the limitation part of the discussion: "There are some limitations of our phosphoproteomics workflow which could

lead to biased information yielded. In particular, the tryptic digestion used could introduce bias in phosphosite identification, as well as with the TiO_2 based enrichment Tyr-phosphorylations being underrepresented in comparison to Thr- and Ser-phosphorylations, just based on the fact that there are less frequent. Also, despite the enrichment, very low abundant phosphosites were probably not detected if they fell below the sensitivity threshold of the mass spectrometer."

There was actually no change in rat liver CREB-S133 phosphorylation by glucagon (Fig. S1c-d), despite that we were able to observe higher CREB-S133 levels in glucagon treated hepatoma cells (Fig. S1e, S1g) using the same antibody.

We agree that further validation is required to show that hepatocyte SEC22B and SEC22B phosphorylation potentially affect lipoprotein biogenesis/maturation, but we believe this is currently outside the scope of the present manuscript. Also, it has been reported that SEC22B is the crucial component of VLDL-transport vesicles (VTV) (PMID: 20450495, PMID: 22449872), and blocking VLDL secretion causes hepatic steatosis (PMID: 18515909). Therefore, hypolipidemia and liver steatosis in the SEC22B KD group suggest that SEC22B may be the key protein involved in VLDL secretion from the liver into the bloodstream.

In any case, we have performed additional analysis of the affinity-proteomics data. Our hepatocyte SEC22B interactome studies demonstrates that many proteins involved in the ER-Golgi interface were enriched (Fig. 5; Table S2), which is a cellular site of lipoprotein biogenesis and maturation (PMID: 22517366), thereby suggesting that this may be the cellular site where SEC22B affects these hepatic lipoprotein metabolisms. In addition, several proteins involved in glycogen metabolism were found to interact with SEC22B, including glycogen synthase (GYS2) and starch-binding-domain-containing protein 1 (STBD1). STBD1 is crucial in shuttling glycogen to liver lysosomes (PMID: 27358407). It binds to glycogen, positioning it on membranes and guiding its transport to lysosomes, possibly orchestrating a "glycophagy" pathway for glycogen breakdown (PMID: 20810658; PMID: 21893048). Hence, the reduced liver glycogen levels observed in the SEC22B silencing group might be due to the disrupted interaction between SEC22B and STBD1. This has mentioned in the Discussion section of our manuscript. Additional research could be conducted to experimentally test this hypothesis, but this is outside of the scope of the present manuscript.

7. Could you please provide additional description in the figure legends for Fig Figure 5b, c, d? The information presented in Figure 5b is difficult to discern. The font is too small to read at 100% viewer or in a printed-out format. It's unclear from the figure and the legend what each square and the color mean on the right side of Figure 5b. It's also unclear what these changes in interaction ultimately mean. Is there a way to discern the meaning of these interactions? Could you provide some model to describe the changes?

Reply:
Regarding figures 5b-d, the crucial information lies in the Term name and its corresponding -log10(P-adj) value. The colored boxes were used solely to highlight the presence of proteins within each term, providing supplementary details. Therefore, we removed the color boxes to enhance clarity and enlarge the text.

GO:CC		stats	
Term name	Term ID	p_{adj}	$-\log_{10}(p_{adj})$ 0 ≤16
endoplasmic reticulum membrane	GO:0005789	6.228×10^{-18}	
endoplasmic reticulum subcompartment	GO:0098827	6.895×10^{-18}	
nuclear outer membrane-endoplasmic reticulum membrane...	GO:0042175	9.587×10^{-18}	
organelle subcompartment	GO:0031984	3.162×10^{-16}	
endoplasmic reticulum	GO:0005783	2.819×10^{-14}	
organelle membrane	GO:0031090	3.187×10^{-10}	
endomembrane system	GO:0012505	4.537×10^{-10}	
membrane	GO:0016020	4.253×10^{-5}	
cytoplasm	GO:0005737	3.817×10^{-4}	
intracellular membrane-bounded organelle	GO:0043231	1.728×10^{-2}	
membrane-bounded organelle	GO:0043227	3.074×10^{-2}	

Within the discussion, we have made the point that the S137 phosphosite is within the coiled-coil domain of SEC22B which thus could have effects on the SNARE function, as phosphorylation within this domain has effects on the function of other SNARE proteins (PMID: 35972760). This discussion section reads: " However, the mechanism by which hepatic SEC22B and SEC22B-S137 phosphorylation mediates the metabolic effects of glucagon is unclear. The S137 site of SEC22B is located within the coiled-coil domain (AA 134-194), which is a critical region for facilitating interactions among SNARE proteins 46*. Furthermore, phosphorylation within the coiled-coil domain of SNARE proteins is known to regulate SNARE function (PMID: 35972760). This indicates that phosphorylation at SEC22B-S137 might influence protein interactions, potentially affecting the functionality or stability of the SNARE complex. To investigate the potential mechanisms behind the differential effects of the SEC22B S137A mutant, an unbiased assessment of the liver hepatocyte-SEC22B interactome was investigated by co-immunoprecipitation and mass spectrometry assays. This technology enabled the identification of direct protein interactions of SEC22B, aiding to the understanding of intricate protein assembly into complexes and network formation (PMID: 27975227)."*

In any case, we have conducted further analysis of our IP-proteomics data using mathematical models. The scatter plots display Log2(Sec22b-WT or Sec22b-S137A / control), with the x-axis representing GCG and the y-axis representing VEH. Proteins that shift away from the diagonal towards the x-axis suggest glucagon-induced interactions, which can be quantified using statistical analysis (intercept).

As shown in Figure a, glucagon exerts a discernible influence on protein binding preferences for the SEC22B-WT protein. Highlighted in red, glucagon treatment induces numerous shifts of proteins away from the diagonal (black reference line, x=y). However, many of these protein shifts are not observed in the SEC22B-S137A mutant protein upon glucagon treatment (Figure b).

Additionally, we analyzed the 20 proteins that selectively interact with SEC22B-WT (Figure 5b). To quantify the binding preference induced by glucagon, we calculated the intercepts of these 20 proteins. As depicted in Figure c, all these proteins exhibited significantly greater shifting distances for SEC22B-WT compared to SEC22B-S137A.

We can include these figures in our manuscript if you believe they are necessary.

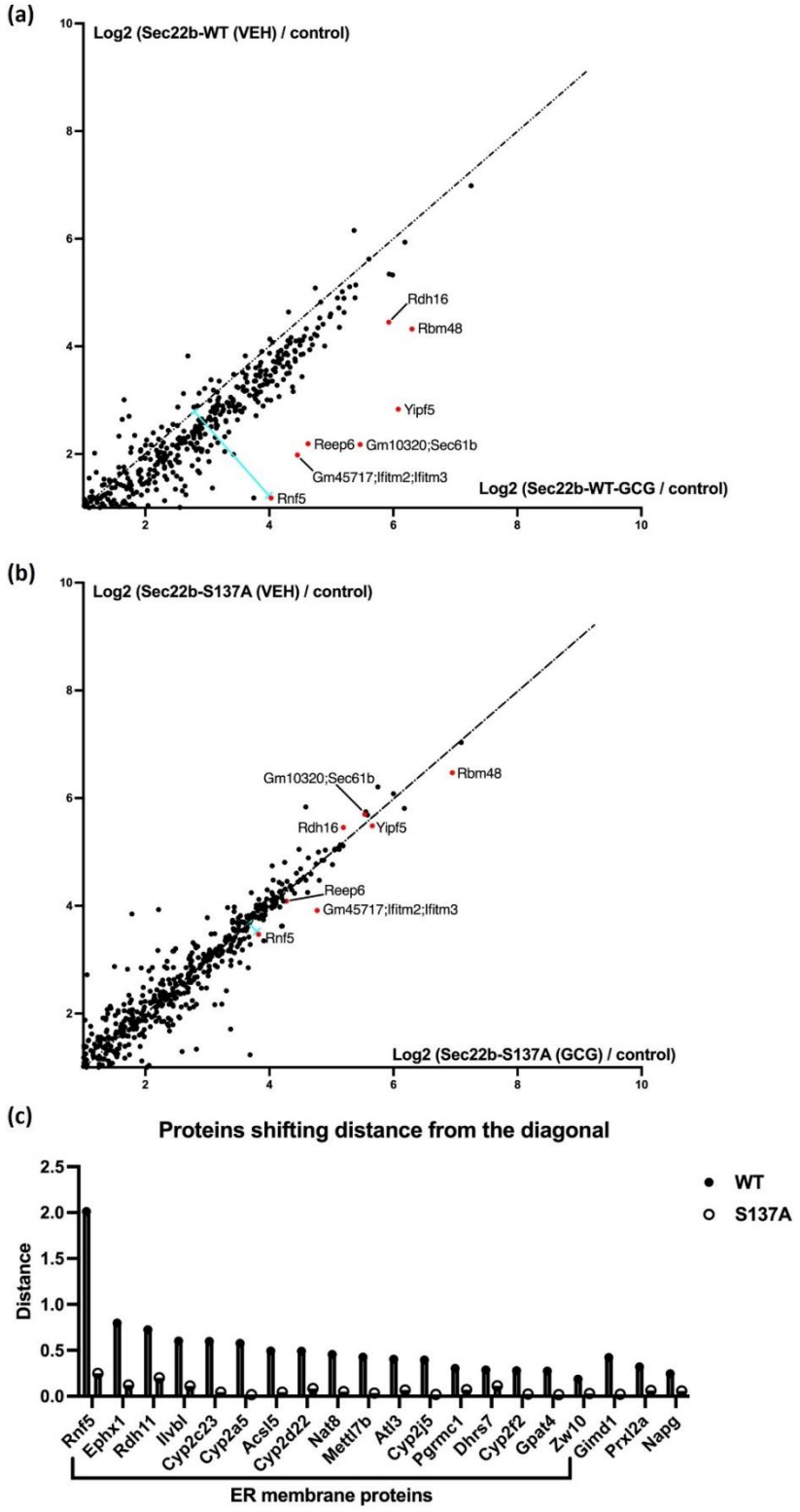


Figure. *(a-b) Scatter plot of proteins bounded with SEC22B WT and SEC22B S137A in VEH and GCG conditions (Log2>1). The blue line is the intercept, which depicts proteins shifting distance from the diagonal (black dot line, x=y). (c) Calculated the intercept of 20 proteins that exclusively bounded with SEC22B (in Sec22b-WT + Glucagon group).*

However, we currently can't provide a model to describe/explain the change of SEC22B interaction partners affected by glucagon and/or S137 phosphorylation. This phosphosite is within the coiled-coiled domain of SEC22B, which ultimately may affect SNARE function and 3D structure and/or localisation and thus interaction partners. Without structural biology experiments and detailed live-cell microscopy this is currently vague and future experiments should address this but is outside the scope of the present manuscript.

8. Hepatic SEC22B silencing raised the serum levels of multiple 159 amino acid species selectively under acute glucagon treatment conditions (Fig. 4e-j, Fig. S4g-k). Would you be able to provide possible ways or literature to explain/support these changes?

Reply:
With any metabolite within the blood compartment, the levels are always a balance between the rates of uptake/removal and the rates of appearance/production. A net increase in a metabolite level in an acute setting could simply mean that the rate of appearance exceeds the rate of removal. As the liver is a chief site of amino acid catabolism and glucagon stimulates this catabolism, we predict that the rate up uptake/catabolism is blunted by SEC22B deletion, hence causing the accumulation with glucagon.

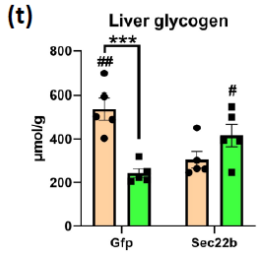
SEC22B is known to participate in autophagy (PMID: 27932448), which could potentially impact amino acid metabolism (PMID: 26453774). Also, a previous study has demonstrated that the inactivation of the COPII protein SEC24C can facilitate the degradation of the amino acid transporter SLC6A14 (PMID: 30445147). In breast cancer cells, the ubiquitin ligase RNF5 has the capability to stimulate ubiquitination and subsequent degradation of the L-glutamine carrier proteins SLC1A5 and SLC38A2, which would decrease glutamine uptake (PMID: 25759021). Our interactome data indicate that glucagon selectively induces the interaction of SEC22B-WT with RNF5. This suggests that the glucagon-induced interaction between SEC22B and RNF5 could potentially affect amino acid transporters, thereby influencing amino acid metabolism. This has mentioned in the Discussion section of our manuscript.

9. Could you please provide the histology scoring for H&E and oil red O staining? (example: PMCID: PMC4275274).

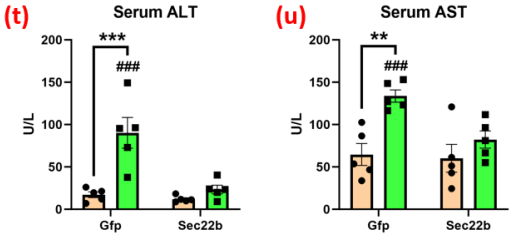
Reply:
Thanks for your suggestion. We collaborated with a Clinician Scientist Dr. med. Mohammad Rahbari to perform unbiased histological scoring of the images, which revealed no significant changes in the NAFLD Activity Score, including steatosis, lobular inflammation, or hepatocyte ballooning (Fig. S4d-g). This lack of significant changes may be attributed to the short duration of the SEC22B silencing study (18 days).

10. It appears that the liver cells of the control group (SEC22b miR-NC) in Figure S3 have been damaged. Moreover, the H&E staining of GFP miR-NC does not resemble SEC22b miR-NC. Can this be quantified or examined by other methods looking at tissue damage (e.g. fibrosis)?

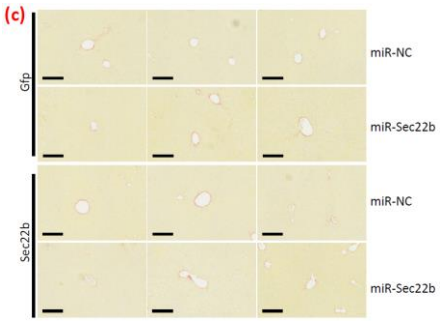
Reply:
Fig. S3s (now Fig S4a) is not an H&E stain, but is a PAS stain. PAS staining stains for starch and thus glycogen in the liver cells (i.e. hepatocytes). We agree that the staining appears different in SEC22B cDNA/miR-NC versus GFP cDNA/miR-NC and this is validated by the biochemical assay of glycogen in Fig. S3t.



In any case, we have conducted serum liver damage marker analyses and indeed we can show that both ALT and AST levels are higher with SEC22B silencing, which is reversed by re-expression of exogenous SEC22B as shown below (new Fig. 3t-u).

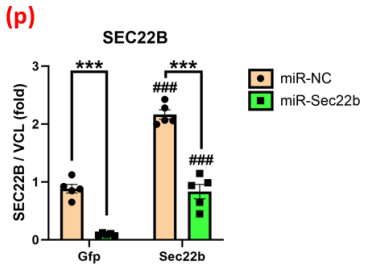


Additionally, we performed Picrosirius Red (PSR) staining and did not detect any fibrosis development during the short duration of the SEC22B silencing study (Fig. S4c).



11. By analyzing the signal intensity, you should be able to determine the relative protein expression in Figure 3Sk. This will provide a better idea of the protein knockdown using Sec22b, a negative control (miR-NC), and/or AAV-Sec22b cDNA (Sec22b) or a control (GFP) in both fasted and refed states.

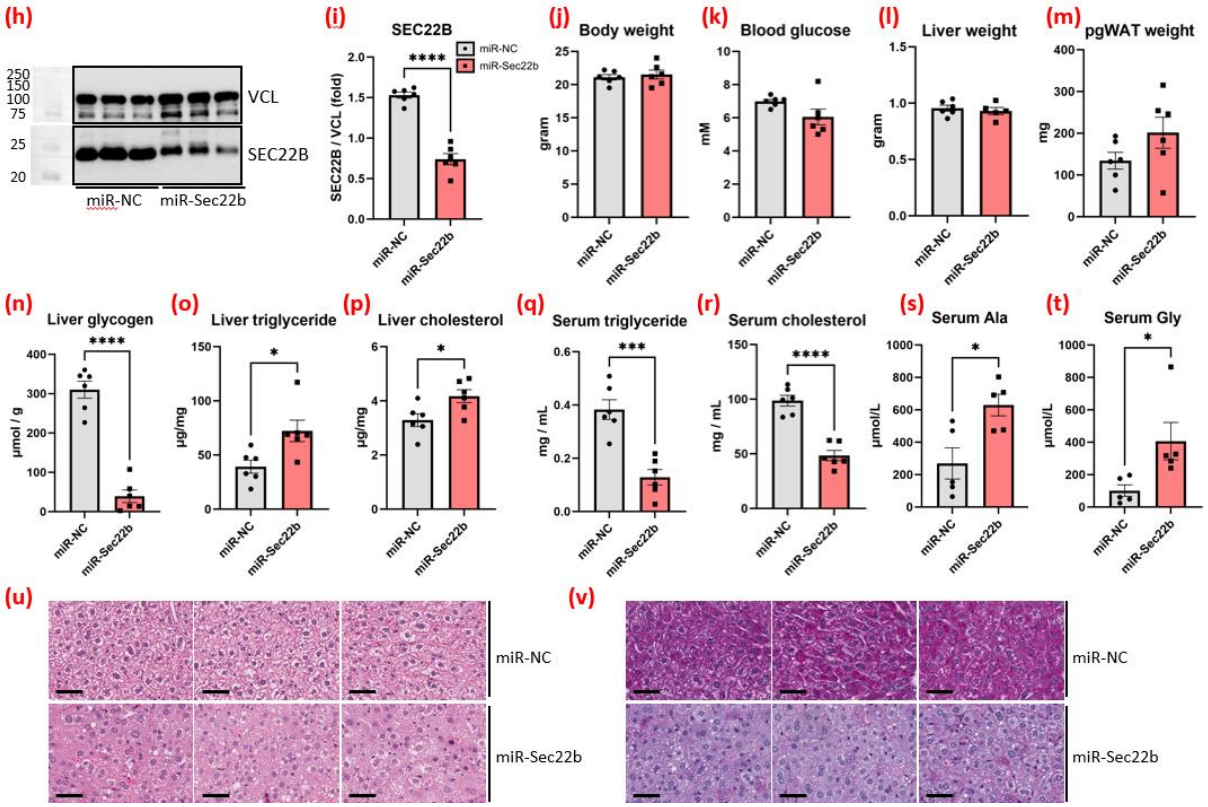
Reply:
This is a good suggestion. We have done this (new Fig. 3p) and show that we can an excellent silencing (~10% of endogenous expression) and a comparable re-expression (~95% of endogenous expression).



12. All males were used for this study. There needs to be some examination of sex differences. Does this system work the same way in females?

Reply:

To determine whether SEC22B has sex-specific effects, we repeated the refeeding experiment with female mice. Consistent with the results observed in male mice, SEC22B silencing (Fig. S4h-i) did not affect blood glucose, body weight, liver weight, or adipose tissue weight (Fig. S4j-m). Importantly, SEC22B silencing depleted liver glycogen, resulted in higher liver triglyceride and liver cholesterol levels and lower serum triglyceride and serum cholesterol levels (Fig. S4n-r; Fig. S4u-v). Furthermore, SEC22B silencing induced higher serum alanine and glycine levels (Fig. S4s-t). These results indicate the metabolic effects of SEC22B are conserved in both males and females. We have included these results to our manuscript.



Reviewer #2 (Remarks to the Author)

In this manuscript, Wu and colleagues use phosphoproteomics to interrogate the mechanisms of glucagon signaling in liver in mice. Their data highlight phosphorylation of the vesicle trafficking protein SEC22 Homolog B (SEC22B) as a key target of glucagon action. The authors utilize SEC22 knockout and gain of function models in hepatocytes to validate the impact of this signaling in vitro. As the mechanism of glucagon action is still being debated a century after its discovery, this study is important and generally well performed (though I am not an expert in phosphoproteomics, so I will not comment on that analysis).

Reply:
We thank this reviewer for taking the time to review our manuscript and provide critical and thoughtful appraisal.

I do have several comments for the editor's consideration:

1. Glucagon concentrations in the perifusions appear high, considering that circulating plasma glucagon is in the pM range in rodents and humans under most conditions; this may be justifiable considering that portal vein hormone concentrations will be higher than circulating, but the authors need to explicitly explain/justify this dose.

Reply:
This is a valid point. There are very little data on hepatic portal vein concentrations of glucagon and no data interstitial glucagon concentrations in liver. Recent work from the Campbell group has shown that portal vein glucagon concentrations in the mouse can reach 400 pM (PMID: 31335319). In addition, our own unpublished data has shown hepatic portal vein concentrations in the mouse reach and average of ~600pM (some mice were at ~800 pM) during high protein diet feeding. Both of these studies have used the next generation of glucagon ELISA (PMID: 27245336). Given that we used 1.15 nM (1150 pM) this is only slightly above that observed during physiological manipulations and thus we feel that it is representative of a physiological response. Nevertheless, the choice of a higher range of glucagon to ensure full activation of all hepatocytes, as all hepatocytes across the lobule express the glucagon receptor (PMID: 29555772).

We have adjusted the methods section to justify the choice of this concentration and it now reads: "The concentration of 1.15nM was chosen as a slightly supra-physiological concentration (concentrations can reach 0.4nM in the portal vein (PMID: 31335319) in order to stimulate all glucagon receptors maximally."

2. The authors observe a surprising increase in lipid droplet concentrations in SEC22 KO hepatocytes (which is counter to what several other groups have observed, showing that glucagon action reduce liver triglyceride content). They speculate about several mechanisms that could explain this; however, in my view, this is a key fundamental, mechanistic point. For example, if the differences arise from differences between in vitro and in vivo programs, this renders their large amount of in vitro data of questionable relevance.

Reply:
Glucagon has multiple effects on liver lipid metabolism (PMID: 28275047). It simultaneously stimulates FA oxidation and lipolysis but blocks VLDL export (PMID: 28275047). The net effect of glucagon action is indeed to reduce liver triglyceride levels, particularly in obese mice with fatty liver (PMID: 25485909). Given that our in vivo data indicate that glucagon action operates via effects on SEC22B (see Fig. 4), the data that SEC22B silencing causes accumulation of lipid droplets is actually in line with the prior knowledge that glucagon lowers hepatocellular

triglyceride levels (PMID: 32132708). We do however agree that in vitro data do not always reflect the in vivo state (a point in case are the differences between phosphoproteomics in rat liver (in situ) vs. the human cell line (in vitro), which is why we conducted our studies in live mice.

3. The data showing that liver triglycerides are increased in the SEC22 KO knockdown mice contrast with the effect that several groups have observed with glucagon agonism lowering liver triglycerides. This discrepancy is what it is, but should be explored in more detail experimentally (rather that merely textually).

Reply:
Given that our in vivo data indicate that glucagon action operates via effects on SEC22B, the data that SEC22B silencing causes accumulation of lipid droplets is actually in line with the prior knowledge that glucagon lowers hepatocellular triglyceride levels (PMID: 32132708).

4. I commend the authors on their very clear and streamlined graphical abstract/summary figure; this is an underappreciated aspect of many manuscripts and, in this case, is a great aid to the readers.

Reply:
Thank you for this comment. We worked for some time on making the graphical abstract an accurate visual representation of our studies and we are glad that this is appreciated.

Reviewer #4 (Remarks to the Author):

In the manuscript by Wu et al., authors demonstrate that phosphorylation of SEC22B is a hepatocellular signaling node mediating specific metabolic actions of glucagon. They also discuss novel pathways and processes activated by glucagon, and predict the kinases involved. The experiments are well-designed and performed on qualified mouse models. The phosphoproteomic analysis appears sound as well. I have a few comments and questions:

1. The reader would benefit from a more detailed phosphoproteomic workflow, rather than a mere citation for another paper, and especially since phosphorylation is the central theme of the manuscript. The cited paper gives a choice between TiO2 and IMAC, it is not clear which of the two (or both) were used in this study. A paragraph describing the phosphoenrichment steps briefly, would be a great addition to the paper.

Reply:
The reviewer makes a valid point regarding the specification of TiO2 instead of IMAC. While the paper we cite does provide a detailed protocol, I have added a brief paragraph to our manuscript for clarity.

2. What was the enrichment specificity for phosphopeptides?

Reply:
The enrichment specificity is 88.37% when calculating the number of phosphopeptides (as shown in the phospho(STY) file) versus the number of non-phosphopeptides. While we believe this detail does not necessarily need to be included in the manuscript, we are open to adding this information if you think it is necessary.

3. What was the confidence cut off (%) for a phospho-site being accepted as true?

This is already explained in line 510, but we have tried to make it clearer by adding "(phospho)" in front to "peptides" to indicate that this FDR applies to both peptides and phosphopeptides. The phosphorylation localisation probability score filter has also been added to the text.

4. What was the peptide amount loaded on the columns, for both global and phosphoproteomic samples? Line 466 says 1uL peptide but not the amount in ug, and nothing is mentioned for the phospho samples.

This is an immunoprecipitation (IP) proteomics study, so we do not have a set amount of protein (in µg) that is loaded onto the mass spectrometer (MS). At that stage in the preparation, we do not know the exact concentration of our sample post-IP. We expect variable concentrations of peptides per sample, particularly in the control, where only background proteins are likely to adhere to the beads, while our samples are enriched for the target proteins. Normalizing the peptide concentrations at this stage would likely result in an artificial increase in background signal. Instead, we resuspend the dried peptides in the same volume and load 1 µL per sample to ensure an equivalent volume is loaded for each sample. During acquisition, we monitor the signal to ensure it is sufficient.
For the phosphoproteomic part of the study, we have added the note "(1 µg on column for each sample)" to line 466 to clarify the peptide amount loaded for these samples.

5. What was the reason for acquiring proteomics data in DIA mode, and phosphoproteomics data in DDA mode?

There was no specific reason for choosing DDA over DIA. At the time, we opted to acquire the phosphoproteomics data using Data-Dependent Acquisition (DDA) because we believed it would yield better results. However, we could have also acquired the phosphoproteomics data using Data-Independent Acquisition (DIA).

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript NCOMMS-24-03555-T Wu et al, entitled “Phosphoproteomics-1 directed manipulation reveals SEC22B as a hepatocellular signaling 2 node governing metabolic actions of glucagon” shows that glucagon stimulation triggers various liver signaling events, as evidenced by time-resolved phosphoproteomics. The study identified SEC22B S137 as a phospho target of glucagon signaling and several protein partners which were due to glucagon stimulation. It was revealed that SEC22B protein plays a crucial role in lipid, amino acid, and glycogen metabolism. The study found that SEC22B is conserved in humans, mice, and rats and extensively regulates lipid metabolism in male and female rodents. Additionally, the study addressed the limitations of proteomics and provided more detailed data on lipid metabolism. This reviewer thanks the authors for their great efforts to address the comments provide from the subsequent review. Overall, the paper demonstrates a comprehensive approach and effectively fulfills its objectives. I only have a few remaining minor comments which might be address in the discussion.

1) The researchers detailed the effects of liver glucagon-SEC22B on triglyceride-rich lipoprotein metabolism, as well as its impacts on HDL/LDL-cholesterol and VLDL production rates. They also discussed SEC22B's effects on glycogen metabolism, amino acid transporters, and amino acid metabolism. However, it is suggested that the authors should further explain how glucagon affects amino acid metabolism.

2) Based on these studies and previous observations in extreme cases of glucagon deficiency or excess secretion, the physiological role of glucagon has expanded to include hepatic protein metabolism. A paper by Wewer Albrechtsen NJ, Holst JJ, Cherrington AD, et al. ("100 years of glucagon and 100 more," Diabetologia, 2023) covered how changes in glucagon secretion affect diabetes, and the author can use this paper to explain how SEC22B protein phosphorylation may play a role in disease conditions and glucagon resistance.

Reviewer #2 (Remarks to the Author):

The authors have addressed my concerns and I congratulate them on their work.

Reviewer #4 (Remarks to the Author):

Thank you for addressing my comments. I have no further questions.

REVIEWERS' COMMENTS

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This manuscript NCOMMS-24-03555-T Wu et al, entitled “Phosphoproteomics-1 directed manipulation reveals SEC22B as a hepatocellular signaling 2 node governing metabolic actions of glucagon” shows that glucagon stimulation triggers various liver signaling events, as evidenced by time-resolved phosphoproteomics. The study identified SEC22B S137 as a phospho target of glucagon signaling and several protein partners which were due to glucagon stimulation. It was revealed that SEC22B protein plays a crucial role in lipid, amino acid, and glycogen metabolism. The study found that SEC22B is conserved in humans, mice, and rats and extensively regulates lipid metabolism in male and female rodents. Additionally, the study addressed the limitations of proteomics and provided more detailed data on lipid metabolism. This reviewer thanks the authors for their great efforts to address the comments provide from the subsequent review. Overall, the paper demonstrates a comprehensive approach and effectively fulfills its objectives. I only have a few remaining minor comments which might be address in the discussion.

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We thank this reviewer for the appreciation of our work and their time and attention. With regard to point 1, at present it is not clear exactly how glucagon affects amino acid metabolism but probably does so through the steps of amino acid uptake and intracellular metabolism in a coordinated fashion. Whether SEC22B somehow affects intracellular amino acid metabolism is presently unknown and will be a direction for future investigations. Thus, we decided not to discuss this further.

With regards to point 2, we are currently working on the role of glucagon and SEC22B in models of type 2 diabetes and the role of glucagon resistance. Although clearly important, since none of the studies in the present manuscript addressed this angle, we didn’t feel that we need to discuss this in the present manuscript.

Reviewer #2 (Remarks to the Author):

The authors have addressed my concerns and I congratulate them on their work.

Reviewer #4 (Remarks to the Author):

Thank you for addressing my comments. I have no further questions.

We appreciate the time and efforts of reviewers 2 and 4, and thank them for their helpful and constructive feedback.