

SPNS2 EXPORTS SPHINGOSINE-1-PHOSPHATE AND IMPORTS GLUCOSE

Corresponding Author: Professor Sarah Spiegel

This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications. Mentions of the previous journal have been redacted.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Weigel and colleagues provided adequate responses to my questions and concerns in the last version of their manuscript submitted to [REDACTED]. I have nevertheless reviewed changes and additions, and have a few comments.

Page 10, line 204. Although it is well accepted that activation of S1P synthesis by various stimuli can lead to S1P synthesis, export and S1PR activation, there is limited evidence for autocrine signaling. Based on current literature, I therefore consider it to be misleading to imply that there is an accepted consensus that that S1P activates S1PRs “on the same cell surface”.

The essential requirement for Spns2 for S1P-induced endothelial functions following this statement and shown in Figure 3 is intriguing, but difficult to reconcile with many key observations reported from in vivo models. For example, endothelial cell S1PR1 and erythrocyte-derived S1P are essential for developmental angiogenesis, but endothelial sphingosine kinases and Spns2 are not. Furthermore, S1P exported from hematopoietic cells and not endothelial cells is essential for LPS-induced S1PR1 signaling in S1PR1 Tango reporter mice, arguing against autocrine activation in sepsis. The reference to endothelial barrier dysfunction in Spns2 deficient mice is therefore very selective. Without providing in vivo evidence to show that physiological coupling is essential for S1PR signaling, the authors should be careful not to exaggerate the implications of their observations and the extent to which autocrine signaling explains physiological functions previously attributed to S1P signaling. The manuscript would benefit from a more nuanced discussion highlighting limitations and not only novelty and potential far-reaching implications of the study.

ED Figure 7B: A t-test is not appropriate to address significance in this experiment, which is also not adequately powered to state that S1PRs do not influence Spns2-dependent glucose transport. The data presented suggest that JTE-013 may reduce uptake. However, given the lack of specificity of JTE-013 for S1PR2, I would recommend that the dataset is replaced or excluded.

Please improve resolution of images in ED Fig 1, 9C.

Reviewer #2

(Remarks to the Author)

Weigel et al. thoroughly addressed my concerns in their revised manuscript and review reply. They supplemented the work with complementary experiments—mouse models, cellular assays, and in vitro cell-free binding/transport assays—to validate their core finding: SPNS2 (a major facilitator superfamily member) acts as an antiporter, exporting S1P while importing glucose.

They further identified key SPNS2 residues mediating glucose binding/import, and clarified that cytosolic S1P drives SPNS2 conformational changes to enable extracellular glucose uptake. This uncovers a novel link between sphingolipid signaling and glucose homeostasis.

These additions strongly reinforce their model, resolving my prior questions. I am satisfied with this improved manuscript and fully support its publication in Nature Communications.

Reviewer #3

(Remarks to the Author)

Review: 673773_0

The manuscript submitted by Weigel and coworkers, presents evidence that the sphingosine-1-phosphate (S1P) transporter spinster-homolog 2 (SPNS2) functions in fact as an antiporter for S1P and glucose. While its role in the S1P transport has been shown previously and has been considered the major function of SPNS2, observations on influencing glucose transport were attributed to S1P signaling and other glucose transporters. The results provided in the manuscript clearly demonstrate that deletion of SPNS2 directly influences blood glucose levels in animals and cellular uptake in vitro. Furthermore, reconstitution experiments with SPNS2 and labeled glucose confirm the binding of glucose to the transporter and confirm the role of predicted key residues.

What remains however unclear is the stoichiometry of the antiporter and a potential regulatory role of extracellular glucose on S1P export from cells. This definitely needs to be studied in more detail, but I agree that this can be analyzed in a subsequent study.

Taken together the manuscript is very well written and the results are very clearly presented. Thus, there are only a few minor points that I would like to address:

Minor points:

The relevance of SPNS2 for kidney transport is interesting. I am wondering if there is a co-localization with other key transporters in the proximal tubular cells.

Ex Data Fig 6: While the results can be interpreted as dependence on glucose uptake, the differences in mitochondrial activity may also be due to intracellular S1P levels; which are also influenced by SPNS2.

Ex Data Fig 8: B) only SPNS-OE1 cells are shown; it would be nice to have the normal cells side by side.

Reviewer #4

(Remarks to the Author)

In this work, Weigel, et al. propose that SPNS2, an MFS transporter, exhibits antiporter-like activity, coupling S1P export to glucose import. Using complementary evidence from mouse studies, cell-based assays, in vitro cell-free binding/transport assays, and MD simulations, they report that SPNS2 directly binds and transports glucose and identify key residues required for this engagement. Overall, the work is significant and the manuscript is well organized.

However, I have reservations regarding the interpretation of the MD simulations. The authors combine unbiased MD, ensemble docking, and seeded MD to examine whether glucose can be imported by SPNS2, and use biased MD to estimate the free energy profile for S1P movement to a binding site. As currently presented, the MD results do not directly demonstrate that S1P facilitates glucose translocation. Rather, they support the idea that S1P presence may stabilize glucose binding at specific sites and/or alter vestibule accessibility. It would be helpful to clearly distinguish stable binding and transport. While the experimental data supports transport, the MD simulations do not show glucose crossing to the intracellular side. I therefore suggest that the authors either (i) revise the MD-related text to reflect "enhanced glucose binding/engagement in the IF state" rather than "facilitating import," or (ii) provide additional quantitative simulation evidence that is directly linked to translocation.

Specific points:

1) The authors state that upward movement/reorientation of S1P causes "notable structural changes and enlargement of the extracellular vestibule." From Fig. 4C,D, this is difficult to assess visually. Could the authors quantify the vestibule enlargement and report how consistently it is observed across independent replicas? What are those structural changes? Which TMs move?

2) The PMF calculations for upward S1P translocation are presented but not discussed. The PMF profile appears to show a barrier of around 2 kcal/mol for movement from site A to B/C. The authors should comment on the biological relevance of this barrier height.

3) According to Supplementary Table 2, conclusions regarding the SPNS2::S1P::glucose complex (with the glucose starting at site 3) rely on two 500 ns replicas. Given the stochastic nature of ligand entry and rearrangement, are two replicas sufficient to support reproducibility? Did the authors observe glucose movement toward S1P in both replicas? Please

discuss consistency across replicas and increase sampling if feasible.

4) It is unclear to me what specific question the OF-state glucose simulations (without S1P) are intended to address. If the goal is to support glucose import, OF simulations primarily inform extracellular recognition/initial binding rather than translocation, especially given the short simulation time (300-350 ns) (for which membrane-crossing events are not expected). What do the authors mean by stating that glucose was "unable to pass through the central cavity"? Please clarify the intended interpretation of this result in the context of S1P export, glucose import.

5) The simulations use a complex membrane composition, with charged lipids such as PIP2. Did the authors observe specific lipid-protein interactions, and do these interactions influence the stability of the "open" state described?

Minor point:

I suggest the authors include the actual simulation time for the MD movies provided.

Version 1:

Reviewer comments:

Reviewer #4

(Remarks to the Author)

Weigel et al. have addressed my concerns in their revised manuscript and detailed response to the reviewers. Their work represents a significant contribution to our understanding of how SPNS2 functions as an antiporter, exporting S1P while importing glucose.

The authors have clarified my concerns regarding the interpretation of the MD simulations and have provided the necessary additional details. I am satisfied with the revisions, and in my opinion, the manuscript is now suitable for publication in its current form.

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RESPONSES TO THE REVIEWERS' COMMENTS

Reviewer #1

Weigel and colleagues provided adequate responses to my questions and concerns in the last version of their manuscript submitted to [REDACTED]. I have nevertheless reviewed changes and additions and have a few comments.

Page 10, line 204. Although it is well accepted that activation of S1P synthesis by various stimuli can lead to S1P synthesis, export and S1PR activation, there is limited evidence for autocrine signaling. Based on current literature, I therefore consider it to be misleading to imply that there is an accepted consensus that S1P activates S1PRs “on the same cell surface”. The essential requirement for Spns2 for S1P-induced endothelial functions following this statement and shown in Figure 3 is intriguing, but difficult to reconcile with many key observations reported from *in vivo* models. For example, endothelial cell S1PR1 and erythrocyte-derived S1P are essential for developmental angiogenesis, but endothelial sphingosine kinases and Spns2 are not. Furthermore, S1P exported from hematopoietic cells and not endothelial cells is essential for LPS-induced S1PR1 signaling in S1PR1 Tango reporter mice, arguing against autocrine activation in sepsis. The reference to endothelial barrier dysfunction in Spns2 deficient mice is therefore very selective. Without providing *in vivo* evidence to show that physiological coupling is essential for S1PR signaling, the authors should be careful not to exaggerate the implications of their observations and the extent to which autocrine signaling explains physiological functions previously attributed to S1P signaling. The manuscript would benefit from a more nuanced discussion highlighting limitations and not only novelty and potential far-reaching implications of the study.

Response: We thank the reviewer for this thoughtful and detailed critique. We agree that numerous studies demonstrate essential roles for endothelial S1PR1 and erythrocyte derived S1P in developmental angiogenesis independently of endothelial sphingosine kinases and SPNS2 and that S1P exported from hematopoietic cells is required for LPS induced S1PR1 signaling in systemic inflammation. Our intention was not to challenge these well-supported *in vivo* paradigms, nor to imply that endothelial autocrine S1P signaling universally explains physiological S1P functions. Rather, our findings demonstrate that, in cultured cells, certain biological processes that are known to require SPNS2 and S1P autocrine actions are also dependent on the presence of glucose. We emphasize in the revised manuscript that this requirement likely reflects context-specific coupling that may not be dominant in all *in vivo* settings, particularly where circulating or hematopoietic-derived S1P is abundant and is expected to be the primary driver of S1PR1 activation, such as during developmental angiogenesis, lymphocyte trafficking and sepsis. We also clarified that the reference to endothelial barrier dysfunction in Spns2-deficient mice represents a selective example and does not establish a general requirement for endothelial SPNS2 in all S1P-mediated physiological responses.

In response to the reviewer's comments, we have revised the manuscript to more explicitly acknowledge these limitations, clarify the distinction between autocrine and paracrine S1P actions, and temper statements that might be interpreted as overextending the implications of autocrine signaling. We now emphasize that our data support a model in which SPNS2-dependent S1P export can contribute to specific cellular processes and contexts, rather than serving as a universal mechanism underlying all S1P-dependent vascular functions. We believe this more nuanced

discussion better aligns our conclusions with existing *in vivo* literature while preserving the novelty of our mechanistic observations.

ED Figure 7B: A t-test is not appropriate to address significance in this experiment, which is also not adequately powered to state that S1PRs do not influence Spns2-dependent glucose transport. The data presented suggest that JTE-013 may reduce uptake. However, given the lack of specificity of JTE-013 for S1PR2, I would recommend that the dataset is replaced or excluded.

Response: We would like to clarify that the statistical analysis for ED Figure 7B was performed using a one-way analysis of variance followed by Dunnett's multiple comparisons test, rather than a t-test. Under these conditions, agonists had no significant effects on control cells, and antagonists had no significant effects on SPNS2 overexpressing cells. While JTE-013 showed a trend toward reduced glucose uptake it is not statistically significant. We acknowledge its limited specificity for S1PR2 and have revised the text to note this limitation.

Please improve resolution of images in ED Fig 1, 9C.

Response: We have improved the resolution of the images in these figures to enhance visual quality.

Reviewer #2

Weigel et al. thoroughly addressed my concerns in their revised manuscript and review reply. They supplemented the work with complementary experiments—mouse models, cellular assays, and in vitro cell-free binding/transport assays—to validate their core finding: SPNS2 (a major facilitator superfamily member) acts as an antiporter, exporting S1P while importing glucose.

They further identified key SPNS2 residues mediating glucose binding/import and clarified that cytosolic S1P drives SPNS2 conformational changes to enable extracellular glucose uptake. This uncovers a novel link between sphingolipid signaling and glucose homeostasis.

These additions strongly reinforce their model, resolving my prior questions. I am satisfied with this improved manuscript and fully support its publication in Nature Communications.

Response: We are pleased that the additional experiments and clarifications have addressed the concerns and strengthened the mechanistic model and appreciate the reviewer's support for publication of our revised manuscript in Nature Communications.

Reviewer #3

The manuscript submitted by Weigel and coworkers, presents evidence that the sphingosine-1-phosphate (S1P) transporter spinster-homolog 2 (SPNS2) functions in fact as an antiporter for S1P and glucose. While its role in the S1P transport has been shown previously and has been considered the major function of SPNS2, observations on influencing glucose transport were attributed to S1P signaling and other glucose transporters. The results provided in the manuscript clearly demonstrate that deletion of SPNS2 directly influences blood glucose levels in animals and cellular uptake in vitro.

Furthermore, reconstitution experiments with SPNS2 and labeled glucose confirm the binding of glucose to the transporter and confirm the role of predicted key residues. What remains however unclear is the stoichiometry of the antiporter and a potential regulatory role of extracellular glucose on S1P export from cells. This definitely needs to be studied in more detail, but I agree that this can be analyzed in a subsequent study.

Taken together the manuscript is very well written and the results are very clearly presented. Thus, there are only a few minor points that I would like to address:

Response: We thank the reviewer for the positive evaluation of our manuscript and noting that the manuscript is very well written and the results are very clearly presented. We agree that the stoichiometry of the SPNS2 antiport mechanism and the potential regulatory role of extracellular glucose on S1P export are questions to be analyzed in subsequent studies.

Minor points:

The relevance of SPNS2 for kidney transport is interesting. I am wondering if there is a co-localization with other key transporters in the proximal tubular cells.

Response: We appreciate this suggestion. While co-localization of SPNS2 with other proximal tubular transporters would indeed be of interest, this analysis is beyond the scope of the current study. Nevertheless, we tried to co-image SPNS2 with SGLT1, SGLT2 and GLUT2 by immunofluorescent microscopy in mouse kidney tissue. However, the staining appeared rather unspecific and, therefore, we cannot make an informed statement on this. We have noted this as an important direction for future work.

Ex Data Fig 6: While the results can be interpreted as dependence on glucose uptake, the differences in mitochondrial activity may also be due to intracellular S1P levels; which are also influenced by SPNS2.

Response: We thank the reviewer for this important point. We agree that altered mitochondrial activity may reflect, at least in part, changes in intracellular S1P levels in addition to effects on glucose uptake. This possibility has now been acknowledged in the revised manuscript.

Ex Data Fig 8: B) only SPNS-OE1 cells are shown; it would be nice to have the normal cells side by side.

Response: We appreciate this suggestion. The subcellular localizations of GLUT1 and GLUT3 in control cells are very similar to those we observed in SPNS2-overexpressing cells. Because the purpose of this experiment was to visualize GLUT1 and GLUT3 localization specifically in SPNS2-overexpressing cells, we did not include control images in this panel. However, in Supplementary Fig. 8d,e, we did show that GLUT1 is not detectable at the cell surface in control cells and is not altered by SPNS2 overexpression.

Reviewer #4

In this work, Weigel, et al. propose that SPNS2, an MFS transporter, exhibits antiporter-like activity, coupling S1P export to glucose import. Using complementary evidence from mouse studies, cell-based assays, in vitro cell-free binding/transport assays, and MD simulations, they report that SPNS2 directly binds and transports glucose and identify key residues required for this engagement. Overall, the work is significant, and the manuscript is well organized.

However, I have reservations regarding the interpretation of the MD simulations. The authors combine unbiased MD, ensemble docking, and seeded MD to examine whether glucose can be imported by SPNS2 and use biased MD to estimate the free energy profile for S1P movement to a binding site. As currently presented, the MD results do not directly demonstrate that S1P facilitates glucose translocation. Rather, they support the idea that S1P presence may stabilize glucose binding at specific sites and/or alter vestibule accessibility. It would be helpful to clearly distinguish stable binding and transport. While the experimental data supports transport, the MD simulations do not show glucose crossing to the intracellular side. I therefore suggest that the authors either (i) revise the MD-related text to reflect "enhanced glucose binding/engagement in the IF state" rather than "facilitating import," or (ii) provide additional quantitative simulation evidence that is directly linked to translocation.

Response: We thank the reviewer for the thoughtful and detailed evaluation of our work and for recognizing its overall significance and organization. We particularly appreciate the reviewer's careful assessment of the MD simulations and agree that clarity is essential when distinguishing between ligand binding, engagement, and full translocation. In response, we have revised the manuscript to more precisely align the MD interpretations with what is directly supported by the simulations, and we address each specific point below. We agree with the reviewer that the MD simulations do not directly capture full glucose translocation to the intracellular side. Accordingly, as suggested, we revised the MD-related text to reflect enhanced glucose engagement in the IF state rather than facilitating import. The other experimental data provide the functional evidence for glucose import.

Specific points:

1) The authors state that upward movement/reorientation of S1P causes "notable structural changes and enlargement of the extracellular vestibule." From Fig. 4C,D, this is difficult to assess visually. Could the authors quantify the vestibule enlargement and report how consistently it is observed across independent replicas? What are those structural changes? Which TMs move?

Response 1: As suggested, we added quantitative analyses to measure changes in the extracellular vestibule enlargement (New Supplementary Fig. 10a). To quantify expansion of the vestibule upon upward movement of S1P (shown in Fig. 4c), four C α atoms of residues 124, 250, 337, and 363 were chosen in TM1, TM5, TM7, and TM8, respectively, and a best fit circle was drawn to represent the opening at that plane. During the simulation, the initial radius of the opening increases as the S1P moves upward (a representative trajectory is shown in Supplementary Fig. 10a). Across 3 independent runs, the vestibule radius increases by 1.60 ± 0.07 fold, demonstrating consistent extracellular vestibule enlargement upon upward movement of S1P. This expansion is accompanied by specific structural rearrangements of surrounding TM helices. Upon S1P reorientation from a staggered to a more vertical conformation of its hydrocarbon chain, C α -C α distances between representative residues 124-337 (TM1-TM7), 250-363 (TM5-TM8), 337-363 (TM7-TM8) increase by approximately 2 Å, 3 Å, and 8 Å, respectively.

As previously suggested by Referee #3, to limit emphasis on MD simulations in the main text, these additional data are presented and discussed in the Supplementary information.

2) The PMF calculations for upward S1P translocation are presented but not discussed. The PMF biological relevance of this profile appears to show a barrier of around 2 kcal/mol

for movement from site A to B/C. The authors should comment on the barrier height.

Response 2: We thank the reviewer for highlighting this point. We have now added discussion of the PMF results, noting that a barrier of ~2 kcal/mol is relatively modest and consistent with thermally accessible ligand rearrangements on biologically relevant timescales. It suggests a rapid and efficient transport process under physiological conditions. We clarify that this PMF reflects local repositioning of S1P within the transporter, rather than full translocation, and supports the feasibility of S1P-driven conformational coupling.

3) According to Supplementary Table 2, conclusions regarding the SPNS2::S1P::glucose complex (with the glucose starting at site 3) rely on two 500 ns replicas. Given the stochastic nature of ligand entry and rearrangement, are two replicas sufficient to support reproducibility? Did the authors observe glucose movement toward S1P in both replicas? Please discuss consistency across replicas and increase sampling if feasible.

Response 3: We acknowledge the reviewer's concern regarding reproducibility and sampling and for noting an error in the previous version of Supplementary Table 2 related to the SPNS2::S1P::glucose simulations. We have corrected this table in the revised manuscript and clarified the sampling strategy and limitations. In the revision, we expanded the sampling for the SPNS2::S1P::glucose complex by additional run extending the simulations to a total of three independent replicas: one replica run for 600 ns (terminated after glucose translocated into the solution) and two replicas run for 1000 ns each. Across all three simulations, glucose consistently moved out of its initial binding cavity and interacted with S1P, demonstrating reproducible qualitative behavior. In two of the three replicas, glucose successfully passed within 600 ns whereas in the third replica glucose remained still interacting with S1P up to 1000 ns. We clarified that while the precise timing of glucose movement varies across replicas as expected for a stochastic process the qualitative behavior, namely glucose movement toward and interaction with S1P, is consistently observed.

4) It is unclear to me what specific question the OF-state glucose simulations (without S1P) are intended to address. If the goal is to support glucose import, OF simulations primarily inform extracellular recognition/initial binding rather than translocation, especially given the short simulation time (300-350 ns) (for which membrane-crossing events are not expected). What do the authors mean by stating that glucose was "unable to pass through the central cavity"? Please clarify the intended interpretation of this result in the context of S1P export, glucose import.

Response 4: We appreciate this request for clarification. As suggested, we have now extended the simulations to 600 ns for three replicas. The OF-state simulations were intended to assess extracellular glucose entry into the central cavity, not full translocation. We have revised the text to clarify that the statement "unable to pass through the central cavity" refers to the absence of spontaneous deep penetration or inward movement toward the intracellular side. We now explicitly frame these simulations as supporting the idea that S1P-dependent conformational changes are required for productive glucose engagement exiting its initial binding cavity.

5) The simulations use a complex membrane composition, with charged lipids such as PIP2. Did the authors observe specific lipid-protein interactions, and do these interactions influence the stability of the "open" state described?

Response 5: Our use of a complex membrane composition was intended to provide a physiologically relevant environment, rather than to explicitly interrogate lipid-specific effects. While our simulations show several PI(4,5)P₂ interactions with basic residues, interrogation of their effects on the stability of various conformational states will require more controlled studies (e.g. varying lipid composition and concentration) and therefore we chose not to comment on these. We note, however, in our manuscript that lipid regulation of SPNS2 has been examined previously. In particular, Tang et al. (Mol Cell, 2023) demonstrated that SPNS2 recruits PI(4,5)P₂, which synergistically regulates S1P transport. While our simulations were not designed to probe this mechanism directly, these prior findings are consistent with the possibility that specific lipid-lipid-protein interactions may modulate SPNS2 function and conformational stability.

Minor point:

I suggest the authors include the actual simulation time for the MD movies provided.

Response: As suggested, we have updated the latest version of the MD simulation movies to include the actual simulation time visualized on the screen for each trajectory.