

Structural basis of bile acid lateral diffusion facilitated by the OST α / β transporter

Corresponding Author: Professor Wen-Tao Hou

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)

Summary Xu et al. report the structural basis and transport mechanism of the human bile acid transporter OST α / β . The authors determined high-resolution cryo-EM structures of the unique heteromeric OST α / β complex in both apo- and substrate-bound states. They reveal that the complex adopts a "dimer of heterodimers" assembly and a GPCR-like fold that is distinct from other SLC transporters, including other bile acid transporters. By integrating structural biology with functional assays and MD simulations, the authors propose a "facilitated lateral diffusion" mechanism for bile acid transport. The manuscript is well-written, and the topic is biologically relevant. The structures reveal several intriguing features of the OST α / β complex. However, I have several comments and questions I would like the authors to address to strengthen their conclusions.

1. Cholesterol and Substrate Recognition: The central cavity is described as lipid-filled, containing densities modeled as phosphatidic acid and cholesterol. Given the structural similarity between cholesterol and bile acids, how does the cholesterol naturally present in the membrane avoid inhibiting substrate recognition? A discussion on how the presence of membrane cholesterol might affect or compete with substrate binding would be insightful.

2. Oligomeric State: The authors observe that OST α / β forms a dimer of heterodimers stabilized by the central lipid-filled cavity. Is this tetrameric assembly obligate for function? I suggest the authors attempt to disrupt dimer formation by mutating residues at the OST α -OST α interface. Determining whether the dimer is functionally required for transport, or if the heterodimer alone suffices, is critical for the proposed model.

3. Role of Palmitoylation: The manuscript notes that the deletion of the palmitoylated segment of Loop TM3-4 significantly reduced transport activity. Could the authors clarify the specific contribution of the palmitoylation modification? Specifically, regarding the mutagenesis: how many cysteines were mutated? Did the authors compare single versus double or triple cysteine mutants? This detail is important to understand how this modification regulates transport activity.

4. Substrate Orientation and Flippase Activity: The orientation of the bound taurocholate appears to be inverted relative to the cholesterol density observed in the apo state. Does this suggest that the protein could act as a flippase during translocation across the membrane?

5. Disease-Related Mutations: Are there known human mutations in the OST α / β complex associated with cholestatic liver disease? If so, mapping these mutations onto the new structures would be valuable. Discussing whether they cluster at the dimer interface, the bile acid binding site, or the translocation pathway would significantly enhance the physiological relevance of the structural findings.

6. Structural Homology Search: As the authors note that OST α / β possesses a GPCR-like 7-TM architecture, have they performed a structural search using DALI or Foldseek to identify proteins with similar folds? This comparison could place the unique fold in a broader evolutionary context.

7. OST β Interaction Interface: While the necessity of OST β is established, the specific residues mediating the interaction are identified here (e.g., Arg29 of OST β with Gly280 of OST α). It would be valuable to see if point mutations disrupting this specific interface (e.g., R29A on OST β) abolish complex formation or transport. This would confirm the structural role of the β -subunit beyond the known requirement for co-expression.

Reviewer #2

(Remarks to the Author)

Summary

The authors present a cryo-EM structure of the human OST α /beta bound to taurocholate structure. The authors also measured K_d values using SPR of several bile acids. The study addresses an important question regarding the function of the OST α /beta transporter, which is the primary basolateral efflux mechanism for bile acids in ileal enterocytes. Because OST α /beta is essential for returning absorbed bile acids to the portal circulation and maintaining efficient enterohepatic recirculation, clarifying its regulation and activity has the potential to make a meaningful contribution to the field. Although, there seem to be some overlap with previous published literature (<https://www.nature.com/articles/s41586-025-10029-7#Sec10>), this group presents additional data (e.g., SPR K_d values and OST α /b TCA structure). However, there are missing details that limit the manuscript's impact and clarity.

Major Comments

- The authors should further clarify how their work advances the field relative to prior published studies and add some text to the discussion
- How about the role of OST α /beta in the liver? Could the authors add some text to the introduction
- Could the transport activity of mutants be impacted by protein expression levels at the membrane?
- Were uptake studies performed at 10 min? Is this still in the linear range? Not clear how long TCA was exposed to cells in Fig. 3f.
- Why did the authors use cell suspension studies instead of adherent cells for uptake studies?
- Can the author be more specific e.g., line 35 ...efflux of bile acids and steroids into blood circulation? Published studies and also the authors show that TCA can be transported both ways. What is/are the driving forces for substrate transport? It seems that TCA would first interact with the groove near the palmitoyl on the intracellular side and then move to the extracellular space.
- Several figures would benefit from clearer labeling, a more thorough description of replicates. Please specify n and the meaning of error bars. Also, can the authors add where applicable software that was used to generate data/images

Minor comments:

- Is bromosulfophtalein still clinically used?
- Could the author be consistent with the use of the gene or protein name?
- Use abbreviations first time they are mentioned and be consistent in using them (also in figure legend)
- Typo: line 43 transporters
- Main, line 93 where affinities are listed, refer to Figure 1
- Add software version information used in the method section

Reviewer #3

(Remarks to the Author)

In this manuscript, the authors report the cryo-electron microscopy structure of human OST α /beta (a bile-acid transporter) revealing a GPCR-like 7-TM architecture distinct from all established folds in the solute carrier superfamily. By also solving the structure of the transporter bound to the bile acid taurocholate, and with the support of structure-guided mutagenesis, functional assays, and molecular dynamics simulations, they propose that OST α /beta translocates taurocholate along a surface groove through a facilitated lateral diffusion mechanism distinct from known solute carriers.

The finding that the structure of OST α /beta resembles that of GPCRs is, in my opinion, fascinating. This is however only shown in Supplementary Figure 1d. A reader familiar with GPCRs will appreciate the similarity, but I think the authors should substantiate this with additional data (an additional different view in the figure, some quantitative similarity data, e.g. RMSD in the transmembrane bundle, ...). Also, this similarity needs some additional discussion. How remarkable is the fact that the fold of OST α /beta is so different to the rest of solute carriers? I presume this was expected from phylogeny? Also, do AI-based structure predictions models correctly predict the structure of OST α /beta? GPCRs have been shown to function as lipid scramblases; any similarities with the lateral diffusion mechanism of OST α /beta? I am not familiar with this field, and I cannot appreciate the impact, but in any case, I believe this point deserves more in-depth discussion.

I have significant concerns regarding the molecular dynamics simulations. The methods employed — steered molecular dynamics and well-tempered metadynamics — are complex enhanced-sampling techniques that require careful validation and thorough reporting. Yet the results section dedicates only a few lines to their description, and the manuscript lacks essential details such as the number of replicas, convergence criteria, or any assessment of the reliability of the calculated energies. The authors present a movie and a couple of figures, but provide little explanation of how the simulation outcomes were analyzed or interpreted. This level of detail is insufficient for a publication that relies on these methods to support its central mechanistic conclusions. Without more rigorous reporting and analysis, the simulations do not, in my view, adequately support the proposed lateral diffusion mechanism.

In Supplementary Figures 3b and 4b, the authors show electrostatic surface representations of the protein. The only reference to how this was calculated seems to be: "The surface of OST α /beta is colored by the electrostatic potential calculated in PyMOL" This is an insufficient description of the methodology.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The revised manuscript by Xu et al. has been strengthened by the additional data, including analyses of the OST α –OST α and OST α –OST β interface mutants, palmitoylation-site mutagenesis, discussion of the cholesterol competition, flippase/orientation model, and homology analysis. The authors have adequately addressed the concerns I raised during the first round of review. In my opinion, the manuscript is now suitable for publication in Nature Communications. I have only a few minor comments for the authors.

Minor comments:

1. The text states that K191A “markedly reduced transport activity” (lines 214-215) and describes Lys191 as a key residue whose role was “confirmed by our assays” (lines 297-298). However, in Fig. 3f, K191A (as well as N222A, T229A, and Q260A) appears to be labeled as NS. Please confirm that the statistical significance annotations are correct and support the conclusions stated in the text.
2. The legend for Fig. 3f does not specify the number of biological replicates (n) or explain the meaning of the error bars, unlike the legends for Fig. 2f/2g. Please include this information for consistency.
3. The authors states that “the side chain of Arg241 rotates by 4.5 Å relative to its position in apo structure” (line 205). However, the legend for Fig. 3c reports a displacement of ~4.9 Å.
4. The Methods section refers to the cryo-EM processing workflow for the TC-bound OST $\alpha\beta$ complex as Fig. S3 (line 500), whereas the main text cites the same workflow as Supplementary Fig. 5 (line 188). Please verify the correct figure reference. In addition, please use a consistent format for figure citations throughout the manuscript (e.g., “Supplementary Fig.” vs. “Fig. S”).

Reviewer #2

(Remarks to the Author)

Thank you for the opportunity to review the revised manuscript. The authors have addressed the majority of the comments and concerns raised in the previous review, and the revisions have substantially improved the manuscript. I have a few remaining minor comments:

- The authors demonstrated by Western blot that OST α protein levels are reduced in the mutants when analyzed in whole cell lysates. However, this observation is not discussed in the Results section and is only mentioned in the Supporting Information. The authors conclude that the reduced activity is due to the amino acid substitutions. Should the transport activity be normalized to the protein expression levels measured by Western blot? How do the authors distinguish between reduced transport activity caused by altered substrate binding vs from lower protein abundance? If space allows, could the author add a sentence about the protein expression measured in the results.
- In their response to the reviewers, the authors state that they analyzed protein expression at the membrane. However, according to the Methods section, the Western blots were performed using whole cell lysates rather than enriched membrane fractions. Whole-cell lysates contain proteins from all cellular compartments.

Reviewer #3

(Remarks to the Author)

I thank the authors for their detailed response. Some points are resolved; regarding the lipid scramblase analogy, I would only suggest citing the specific GPCR scramblase studies rather than a general reference. However, there are two points that, in my opinion, are not yet adequately addressed.

“GPCR-like” architecture

I am not satisfied with the continued description of the fold as “GPCR-like,” and I think the authors' own data argue against it. The closest structural homolog they report is TMEM184C (a non-GPCR); among retinal proteins, the best match is a microbial (type-1) rhodopsin (KR2), which superposes better than bovine rhodopsin, the actual GPCR. Microbial and animal (GPCR) rhodopsins are a textbook case of convergent 7-TM evolution: they share no sequence homology and differ in helix–loop topology and retinal geometry. A shared 7-TM bundle is therefore not a GPCR signature, and calling OST α “GPCR-like” on the basis of a hit to a microbial opsin is misleading.

I ask the authors to describe the fold as a 7-TM architecture throughout the manuscript, and to remove the “GPCR-like” framing. If a comparison to GPCRs is retained, it should be presented explicitly as a convergent structural resemblance of the 7-TM bundle.

Molecular dynamics

I thank the authors for the substantially expanded SMD reporting. My concern about whether the simulations support the central mechanistic claim, however, is not resolved.

First, it is not clear which method underlies the energetics. The Methods and main text describe steered MD only, yet Supplementary Fig. 7f presents a “One-dimensional free-energy profile ... obtained from the full WT-MetaD campaign ... reconstructed from the accumulated HILLS file.” Well-tempered metadynamics is not mentioned anywhere else (not in the main text, not in the Methods) and none of its parameters are reported (e.g., collective variable and its rationale, bias factor, Gaussian height and width, total sampling time, number of walkers/replicas, convergence assessment, or error estimate). This is the only panel in the paper that legitimately reports a free energy, and it is undocumented. The authors must either fully describe this metadynamics protocol and its convergence, or remove the panel.

Second, the energetic conclusion in the main text ("the energy barrier along the central channel is substantially higher than those of the lateral pathways") is drawn from SMD quantities that are not free energies. The SMD restraint potential (Fig. 7a) is the harmonic spring energy, and the pulling force (Fig. 7b) integrates to work. Neither is a free-energy barrier. Finally, three non-equilibrium pulls per route cannot support a quantitative barrier comparison. The confidence bands in Fig. 7a,b are very large and there is no work-distribution or Jarzynski/Crooks analysis, so no converged ΔF or error is available. To support the proposed mechanism energetically, the authors should compute a proper potential of mean force for both candidate routes along an appropriate pathway coordinate, e.g., by umbrella sampling with WHAM, or by the metadynamics they apparently already ran, with a stated convergence criterion (e.g. time evolution of the free-energy surface) and error estimate, and to report all method parameters. Absent this, the SMD results should be presented as qualitative evidence for the translocation pathway and the substrate–protein contacts, and the free-energy and barrier language should be removed from the main text.

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REVIEWER COMMENTS

Reviewer1:

Summary: Xu et al. report the structural basis and transport mechanism of the human bile acid transporter OST α/β . The authors determined high-resolution cryo-EM structures of the unique heteromeric OST α/β complex in both apo- and substrate-bound states. They reveal that the complex adopts a "dimer of heterodimers" assembly and a GPCR-like fold that is distinct from other SLC transporters, including other bile acid transporters. By integrating structural biology with functional assays and MD simulations, the authors propose a "facilitated lateral diffusion" mechanism for bile acid transport.

The manuscript is well-written, and the topic is biologically relevant. The structures reveal several intriguing features of the OST α/β complex. However, I have several comments and questions I would like the authors to address to strengthen their conclusions.

1. Cholesterol and Substrate Recognition: The central cavity is described as lipid-filled, containing densities modeled as phosphatidic acid and cholesterol. Given the structural similarity between cholesterol and bile acids, how does the cholesterol naturally present in the membrane avoid inhibiting substrate recognition? A discussion on how the presence of membrane cholesterol might affect or compete with substrate binding would be insightful.

A: Thank you for this insightful comment. We agree that cholesterol and bile acids share substantial structural similarity, as bile acids are biosynthetically derived from cholesterol. Indeed, in our apo-form structure, densities at the lateral groove can be fitted as cholesterol molecules, suggesting that this hydrophobic surface pocket is naturally occupied by membrane cholesterol in the absence of substrate.

However, in the TC-bound structure, these cholesterol densities are replaced by taurocholate molecules. This observation indicates that bile acids can directly displace cholesterol and occupy the substrate-binding site. Although both molecules share the

steroid nucleus, bile acids contain additional hydroxyl groups and, in the case of conjugated bile acids such as TC, a negatively charged sulfonate-containing side chain. These chemical features, which are absent in cholesterol, enable specific polar interactions with residues lining the groove, including Arg241, Arg244, and Gln260. Moreover, despite the high abundance of cholesterol in biological membranes, OST α/β exhibits robust bile acid transport activity in both cellular and reconstituted systems, indicating that membrane cholesterol does not substantially impede substrate recognition under physiological conditions.

Therefore, we speculate that cholesterol may transiently occupy the groove under resting conditions, whereas bile acids are preferentially recognized through a combination of hydrophobic and specific polar interactions at the amphipathic groove, allowing them to outcompete membrane cholesterol during transport. We have added a discussion of this point in the revised manuscript.

2. Oligomeric State: The authors observe that OST α/β forms a dimer of heterodimers stabilized by the central lipid-filled cavity. Is this tetrameric assembly obligate for function? I suggest the authors attempt to disrupt dimer formation by mutating residues at the OST α -OST α interface. Determining whether the dimer is functionally required for transport, or if the heterodimer alone suffices, is critical for the proposed model.

A: Thank you for this valuable suggestion. To evaluate the functional importance of the OST α -OST α dimer interface, we generated several interface mutants predicted to disrupt protomer-protomer interactions. Specifically, residues Trp90 and Tyr128, which are located at the core of the dimer interface, were substituted with alanine. Vesicle-based transport assays showed that the W90A and Y128A mutants exhibited approximately 40% and 50% reductions in transport activity, respectively, compared with the wild-type complex. In contrast, mutations of residues located at the periphery of the interface, including L114A and L199A, did not cause a significant decrease in transport activity (Fig. 2f). These results indicate that residues forming the central OST α -OST α interface contribute more critically to transporter function than those

positioned at the interface boundary.

We have incorporated these new data and discussion into the revised manuscript.

3. Role of Palmitoylation: The manuscript notes that the deletion of the palmitoylated segment of Loop TM3-4 significantly reduced transport activity. Could the authors clarify the specific contribution of the palmitoylation modification? Specifically, regarding the mutagenesis: how many cysteines were mutated? Did the authors compare single versus double or triple cysteine mutants? This detail is important to understand how this modification regulates transport activity.

A: To investigate the functional role of the palmitoylated TM3-4 loop, we generated a series of mutants targeting the palmitoylation sites. Single mutations of Cys160A or Cys162A, as well as the double mutant C160A/C162A, resulted in approximately 30–40% reductions in transport activity compared with the wild-type transporter. In addition, mutations of other palmitoylated cysteines that are not directly involved in substrate coordination also caused measurable decreases in activity (approximately 10–20%).

Furthermore, truncation of the palmitoylated segment of the TM3-4 loop led to an approximately 40% reduction in transport activity. The consistent functional defects observed across multiple independent mutations support an important contribution of this palmitoylated region to OST α/β -mediated transport (Fig. 2g).

Structurally, the palmitoylated loop forms part of the semi-open substrate-binding pocket and lies adjacent to the TC-binding groove. Although the palmitoyl groups do not directly coordinate the substrate, their position suggests that they may help shape the local membrane environment surrounding the transport pathway. Together with previous studies¹ demonstrating roles of protein palmitoylation in membrane stabilization and protein-lipid interactions, as well as our molecular dynamics simulations, we speculate that palmitoylation contributes both to stabilizing OST α/β within the membrane and to facilitating substrate recruitment into the lateral binding groove. We have added these new experimental data and discussion to the revised manuscript.

4. Substrate Orientation and Flippase Activity: The orientation of the bound taurocholate appears to be inverted relative to the cholesterol density observed in the apo state. Does this suggest that the protein could act as a flippase during translocation across the membrane?

A: Thank you for this insightful comments. The orientation of TC in the bound structure is in fact opposite to that of the cholesterol molecules observed in the apo state. This may be because that OST α / β facilitates the recognition of the amphipathic bile acid molecule between the two membrane leaflets by providing a favorable environment for its polar headgroup. However, because our current structures represent static snapshots and do not capture intermediate states of substrate translocation, we cannot directly demonstrate a complete flip-flop mechanism. Therefore, according to our data, we can only propose a facilitating lateral diffusion mechanism.

5. Disease-Related Mutations: Are there known human mutations in the OST α / β complex associated with cholestatic liver disease? If so, mapping these mutations onto the new structures would be valuable. Discussing whether they cluster at the dimer interface, the bile acid binding site, or the translocation pathway would significantly enhance the physiological relevance of the structural findings.

A: To our knowledge, only two pathogenic variants of OST α / β have been reported to date, and neither directly maps to the substrate-binding groove, translocation pathway, or dimerization interface identified in our structures. One reported variant is a nonsense mutation in OST α (Q186), which results in a substantial reduction in transport activity (Fig. 2f). The second is a frameshift mutation in OST β occurring after F27, leading to premature termination of translation and complete loss of transporter function (Fig. 2f).

Because both pathogenic variants are truncation mutations that are expected to broadly impair protein folding, assembly, or expression. At present, no disease-associated missense mutations have been reported in the dimer interface, substrate-binding site, or the proposed translocation pathway. Therefore, currently available

clinical genetic data are not yet sufficient to directly validate the mechanistic model proposed here.

6. Structural Homology Search: As the authors note that OST α/β possesses a GPCR-like 7-TM architecture, have they performed a structural search using DALI or Foldseek to identify proteins with similar folds? This comparison could place the unique fold in a broader evolutionary context.

A: Thank you for the constructive suggestion. A sodium-pumping rhodopsin KR2 from *Krokinobacter eikastus* was retrieved using Foldseek with comparable folding architecture, showing an RMSD of 5.706 Å over 226 C α atoms (Supplementary Fig. 4). KR2 belongs to the microbial rhodopsin superfamily and possesses a GPCR-like 7-TM architecture^{2,3}. In addition, TMEM184 family is identified the closest structural homolog of OST α/β , consistent with a previous phylogenetic analysis reported in 2022⁴. Although members of the TMEM184 family have been implicated in diverse biological processes, including cell signaling and neuronal function⁵, no experimental structures have been reported to date.

We have added the information in the revised manuscript.

7. OST β Interaction Interface: While the necessity of OST β is established, the specific residues mediating the interaction are identified here (e.g., Arg29 of OST β with Gly280 of OST α). It would be valuable to see if point mutations disrupting this specific interface (e.g., R29A on OST β) abolish complex formation or transport. This would confirm the structural role of the β -subunit beyond the known requirement for co-expression.

A: Thank you for this valuable suggestion. Based on our structure, we generated several point mutations targeting the OST α -OST β interface. Mutations of interface residues in OST β , including W26A, R29A, and W36A, each resulted in approximately 30–40% reductions in transport activity (Fig. 2f). Furthermore, Western blot analysis showed

that the membrane expression levels of the W26A, R29A, and W36A mutants were markedly reduced compared with the wild-type complex, suggesting that disruption of the OST α –OST β interface compromises membrane stability of the transporter (Supplementary Fig. 6). These results support the functional importance of the OST α –OST β interface and further demonstrate that the β -subunit is required for maintaining the functional integrity of the transporter complex.

Reviewer2:

Summary

The authors present a cryo-EM structure of the human OST α /beta bound to taurocholate structure. The authors also measured K_d values using SPR of several bile acids. The study addresses an important question regarding the function of the OST α /β transporter, which is the primary basolateral efflux mechanism for bile acids in ileal enterocytes. Because OST α /β is essential for returning absorbed bile acids to the portal circulation and maintaining efficient enterohepatic recirculation, clarifying its regulation and activity has the potential to make a meaningful contribution to the field. Although, there seem to be some overlap with previous published literature (<https://www.nature.com/articles/s41586-025-10029-7#Sec10>), this group presents additional data (e.g., SPR K_d values and OST α /b TCA structure). However, there are missing details that limit the manuscript's impact and clarity.

Major Comments

1. The authors should further clarify how their work advances the field relative to prior published studies and add some text to the discussion.

A: Thank you for the constructive suggestion. In addition to determining the apo structure, our work provides several complementary mechanistic insights. First, we systematically characterized the binding affinities of a panel of physiologically relevant bile acids toward OST α /β, revealing differential substrate recognition properties.

Second, we determined a taurocholate (TC)-bound structure, which directly visualizes substrate engagement within the lateral groove and provides structural evidence for the proposed transport pathway. Third, comparison of the apo and TC-bound structures revealed a distinct conformation of the key substrate-coordinating residue Arg241, suggesting a critical role in substrate recruitment and gating during transport.

Following your suggestion, we have expanded the Discussion section to highlight these findings and to better place our results in the context of the recent literature.

2. How about the role of OSTalpha/beta in the liver? Could the authors add some text to the introduction.

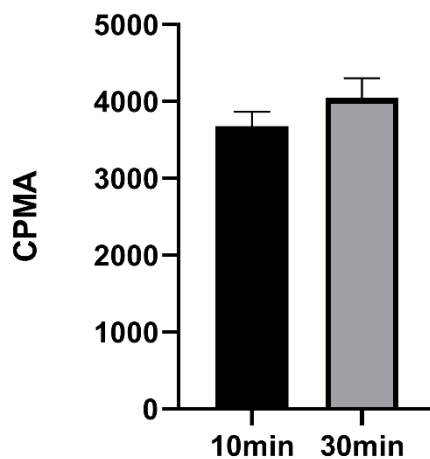
A: Thank you for the advice. We have now added related contents in the introduction.

3. Could the transport activity of mutants be impacted by protein expression levels at the membrane?

A: Yes, it would. According to your suggestion, we examined the protein expression levels at the membrane using Western Blot, and found that several mutants suffer from a lower expression level in the membrane compared with WT, which resulted in the reduction of transport activity. We have incorporated these results in the revised manuscript and shown in the new Supplementary Fig. 6.

4. Were uptake studies performed at 10 min? Is this still in the linear range? Not clear how long TCA was exposed to cells in Fig. 3f.

A: Yes, the uptake studies were all performed for 10 min and then recorded. Centrifugation and other manipulations are required to terminate the reactions during experiments, which introduces substantial experimental errors if the incubation duration is too short. Our experimental data demonstrate that transport activity reaches saturation within the 10-30 min incubation window (shown as the figure below), meaning that the uptake of substrates has reached maximum after 10 min.



Supplementary Fig. X1 Transport uptake nearly reaches saturation after 10 minutes.

5. Why did the authors use cell suspension studies instead of adherent cells for uptake studies?

A: We used suspension cells to express the proteins, thus we used the same cell line HEK293F for transport assays for consistency.

6. Can the author be more specific e.g., line 35 ...efflux of bile acids and steroids into blood circulation? Published studies and also the authors show that TCA can be transported both ways. What is/are the driving forces for substrate transport? It seems that TCA would first interact with the groove near the palmitoyl on the intracellular side and then move to the extracellular space.

A: We believe that the driving force for OST α/β -mediated transport is the transmembrane concentration gradient of bile acids across the basolateral membrane of enterocytes. OST α/β functions as a facilitated diffusion transporter and therefore does not require ATP hydrolysis or coupling to ion gradients. Instead, substrate movement is expected to occur passively down its electrochemical gradient. In the physiological context of intestinal bile acid absorption, bile acids are first taken up from the intestinal lumen by ASBT at the apical membrane and subsequently trafficked intracellularly with the assistance of IBABP. This process results in elevated intracellular bile acid

concentrations relative to portal blood, thereby favoring basolateral efflux through OST α / β ⁶. Conversely, when the gradient is reversed, OST α / β is also capable of mediating transport in the opposite direction, consistent with its bidirectional transport properties observed in our assays.

7. Several figures would benefit from clearer labeling, a more thorough description of replicates. Please specify n and the meaning of error bars. Also, can the authors add where applicable software that was used to generate data/images

A: We are sorry for the lack of clarity. We have supplemented the information you have asked in the revised manuscript. We thank you for the considerate the suggestion.

Minor comments:

8. Is bromosulfophtalein still clinically used?

A: No, bromosulfophtalein has been ceased to use clinically. Thank you for the reminder, we have deleted the contents.

9. Could the author be consistent with the use of the gene or protein name?

A: The gene names for OST α and OST β are *SLC51A* and *SLC51B*, respectively, and we have revised the manuscript for consistency.

10. Use abbreviations first time they are mentioned and be consistent in using them (also in figure legend)

A: Revised.

11. Typo: line 43 transporters

A: Corrected.

12. Main, line 93 where affinities are listed, refer to Figure 1

A: Listed.

13. Add software version information used in the method section

A: Added.

Reviewer3:

In this manuscript, the authors report the cryo-electron microscopy structure of human OST α/β (a bile-acid transporter) revealing a GPCR-like 7-TM architecture distinct from all established folds in the solute carrier superfamily. By also solving the structure of the transporter bound to the bile acid taurocholate, and with the support of structure-guided mutagenesis, functional assays, and molecular dynamics simulations, they propose that OST α/β translocates taurocholate along a surface groove through a facilitated lateral diffusion mechanism distinct from known solute carriers.

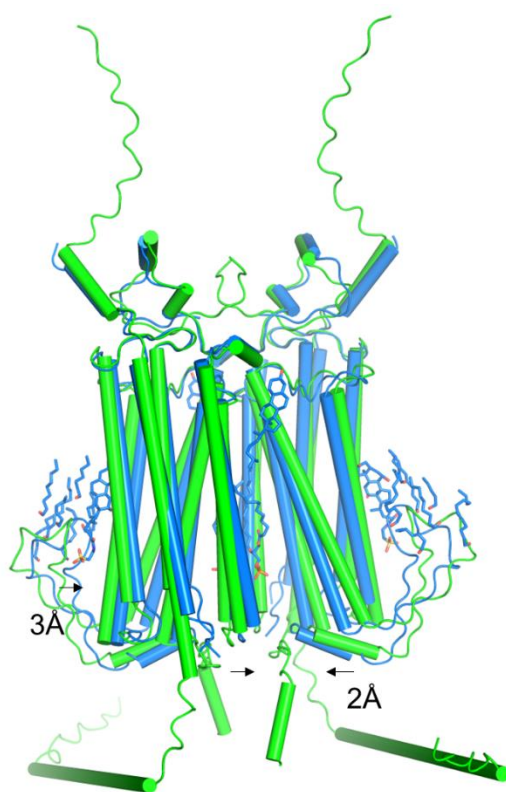
1. The finding that the structure of OST α/β resembles that of GPCRs is, in my opinion, fascinating. This is however only shown in Supplementary Figure 1d. A reader familiar with GPCRs will appreciate the similarity, but I think the authors should substantiate this with additional data (an additional different view in the figure, some quantitative similarity data, e.g. RMSD in the transmembrane bundle, ...). Also, this similarity needs some additional discussion. How remarkable is the fact that the fold of OST α/β is so different to the rest of solute carriers? I presume this was expected from phylogeny?

Also, do AI-based structure predictions models correctly predict the structure of OST α/β ?

A: We performed a structural search with Foldseek, and a sodium-pumping rhodopsin KR2 from *Krokinobacter eikastus* was retrieved with comparable folding architecture, showing an RMSD of 5.706 Å over 226 Ca atoms (Supplementary Fig. 4b). KR2 belongs to the microbial rhodopsin superfamily and possesses a GPCR-like 7-TM architecture^{2,3}. Subsequently, we performed structural alignments against bovine rhodopsin, who also adopts a comparable 7-TM helical fold, with the RMSD values of 6.439 Å (Supplementary Fig. 4a). In addition, structural similarity analysis using Foldseek identified the TMEM184 family as the closest structural homolog of OST α/β ,

consistent with a previous phylogenetic analysis reported in 2022⁴. Although members of the TMEM184 family have been implicated in diverse biological processes, including cell signaling and neuronal function⁵, no experimental structures have been reported to date. The structural similarity between OST α and TMEM184 proteins suggests that they may share common architectural features, providing a basis for future structural and functional investigations.

In addition, the AI-based structure predictions model *AlphaFold3* was utilized to generate the complete tetrameric structure. The RMSD between this predicted model and the experimentally determined structure was 2.071 Å (as shown below). However, the presence of central cavity phospholipids draws the intramembrane leaflet helices of the two OST α monomers inward by approximately 2 Å. Furthermore, AlphaFold3 cannot predict palmitoylation modification of cysteines on the loop. Compared with the predicted structure, this loop shifts approximately 3 Å toward TM4 in the cryo-EM structure, thereby forming a substrate groove together with TM4 and TM5. Meanwhile, the amino acid residues at the substrate-interacting sites undergo deflections of varying magnitudes.



Supplementary Fig. X2 Superposition of OST α / β complex with AlphaFold 3 predicted structure. Experimental structure colored in blue and AlphaFold 3 predicted structure colored in green.

2. GPCRs have been shown to function as lipid scramblases; any similarities with the lateral diffusion mechanism of OST α / β ? I am not familiar with this field, and I cannot appreciate the impact, but in any case, I believe this point deserves more in-depth discussion.

A: Thank you for this insightful suggestion. The recently reported lipid scramblase activity of Class A GPCRs⁷ provides an interesting conceptual framework for considering the transport mechanism of OST α / β . Both systems appear to utilize a membrane-exposed groove that allows amphipathic molecules to remain partially embedded within the lipid bilayer during translocation, rather than transporting substrates through a fully enclosed aqueous pathway, as the alternating-access mechanisms used by classical SLC transporters. Nevertheless, GPCR scramblases catalyze the bidirectional redistribution of phospholipids between membrane leaflets, whereas OST α / β transports substrates across the membrane.

According to your advice, we have added more discussion in the Discussion section.

3. I have significant concerns regarding the molecular dynamics simulations. The methods employed — steered molecular dynamics and well-tempered metadynamics — are complex enhanced-sampling techniques that require careful validation and thorough reporting. Yet the results section dedicates only a few lines to their description, and the manuscript lacks essential details such as the number of replicas, convergence criteria, or any assessment of the reliability of the calculated energies. The authors present a movie and a couple of figures, but provide little explanation of how the simulation outcomes were analyzed or interpreted. This level of detail is insufficient for a publication that relies on these methods to support its central mechanistic conclusions. Without more rigorous reporting and analysis, the simulations do not, in my view, adequately support the proposed lateral diffusion mechanism.

A: To strengthen the reliability of our molecular dynamics simulations, we supplemented additional datasets and conducted multiple replicate simulations, which mainly include: the overall free energy profiles throughout the simulation trajectories, positional variations between the sulfur atom of the substrate and key amino acid residues, as well as RMSD and free energy changes of the central cavity and lateral groove during substrate translocation. Detailed supporting data have been incorporated into the Discussion and Method, along with newly-added Supplementary Fig. 7

4. In Supplementary Figures 3b and 4b, the authors show electrostatic surface representations of the protein. The only reference to how this was calculated seems to be: “The surface of OST α/β is colored by the electrostatic potential calculated in PyMOL” This is an insufficient description of the methodology.

A: We thank the reviewer for pointing out this incomplete methodological description. We have now supplemented the detailed calculation workflow for electrostatic potential surfaces in the revised Methods section and Supplementary Figure legends. The electrostatic surfaces were generated via APBS-PDB2PQR pipeline within PyMOL^{8,9}: hydrogen atoms and AMBER partial charges were assigned at pH 7.0, the Poisson-Boltzmann equation was solved under physiological salt conditions (0.15 M NaCl), and the resulting potential grid was projected onto the solvent-accessible surface with a potential scale of -68 to $+68$ kT/e. These complete technical parameters are now available in the Method.

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- carrier-like proteins from proteomes. *PLOS ONE* **17**, e0271062 (2022).
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 8. Baker, N.A., Sept, D., Joseph, S., Holst, M.J. & McCammon, J.A. Electrostatics of nanosystems: Application to microtubules and the ribosome. *Proceedings of the National Academy of Sciences* **98**, 10037–10041 (2001).
 9. Dolinsky, T.J., Nielsen, J.E., McCammon, J.A. & Baker, N.A. PDB2PQR: an automated pipeline for the setup of Poisson-Boltzmann electrostatics calculations. *Nucleic Acids Research* **32**, W665–W667 (2004).

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The revised manuscript by Xu et al. has been strengthened by the additional data, including analyses of the OST α –OST α and OST α –OST β interface mutants, palmitoylation-site mutagenesis, discussion of the cholesterol competition, flippase/orientation model, and homology analysis. The authors have adequately addressed the concerns I raised during the first round of review. In my opinion, the manuscript is now suitable for publication in Nature Communications. I have only a few minor comments for the authors.

Minor comments:

1. The text states that K191A “markedly reduced transport activity” (lines 214-215) and describes Lys191 as a key residue whose role was “confirmed by our assays” (lines 297-298). However, in Fig. 3f, K191A (as well as N222A, T229A, and Q260A) appears to be labeled as NS. Please confirm that the statistical significance annotations are correct and support the conclusions stated in the text.

A: Thank you for this insightful comment. In fact, in Fig. 3f, K191A is labeled as ***. Owing to an error during figure generation, the label was displaced leftward, which we have now revised.

2. The legend for Fig. 3f does not specify the number of biological replicates (n) or explain the meaning of the error bars, unlike the legends for Fig. 2f/2g. Please include this information for consistency.

A: Revised.

3. The authors states that “the side chain of Arg241 rotates by 4.5 Å relative to its position in apo structure” (line 205). However, the legend for Fig. 3c reports a displacement of ~4.9 Å.

A: Revised.

4. The Methods section refers to the cryo-EM processing workflow for the TC-bound OST α/β complex as Fig. S3 (line 500), whereas the main text cites the same workflow as Supplementary Fig. 5 (line 188). Please verify the correct figure reference. In addition, please use a consistent format for figure citations throughout the manuscript (e.g., “Supplementary Fig.” vs. “Fig. S”).

A: Revised.

Reviewer2:

Thank you for the opportunity to review the revised manuscript. The authors have addressed the majority of the comments and concerns raised in the previous review, and the revisions have substantially improved the manuscript. I have a few remaining minor comments:

- The authors demonstrated by Western blot that OST α protein levels are reduced in the mutants when analyzed in whole cell lysates. However, this observation is not discussed in the Results section and is only mentioned in the Supporting Information. The authors conclude that the reduced activity is due to the amino acid substitutions. Should the transport activity be normalized to the protein expression levels measured by Western blot? How do the authors distinguish between reduced transport activity caused by altered substrate binding vs from lower protein abundance? If space allows, could the author add a sentence about the protein expression measured in the results.

A: We thank the reviewer for this important suggestion. We acknowledge that reduced OST α abundance in mutant constructs may contribute to the decrease in measured transport activity. Unfortunately, reliable quantitative normalization between transport-activity assays and Western-blot signals is technically challenging in our system. We have now added a sentence in the Results section to explicitly discuss this caveat: the observed reduction in transport activity could arise either from impaired substrate-binding/transport function or from decreased protein expression of OST α .

- In their response to the reviewers, the authors state that they analyzed protein expression at the membrane. However, according to the Methods section, the Western

blots were performed using whole cell lysates rather than enriched membrane fractions. Whole-cell lysates contain proteins from all cellular compartments.

A: We thank the reviewer for pointing out this inconsistency. This was an inaccurate statement in our previous response. Indeed, our Western blot analyses were performed on whole-cell lysates, not purified membrane fractions. Therefore, we cannot specifically report the membrane-localized protein abundance from these blots. We have corrected this mistake in our revised response letter. In the manuscript, we have clarified that the protein level measured represents total cellular OST α , rather than membrane-only protein.

Reviewer3:

I thank the authors for their detailed response. Some points are resolved; regarding the lipid scramblase analogy, I would only suggest citing the specific GPCR scramblase studies rather than a general reference.

However, there are two points that, in my opinion, are not yet adequately addressed.

"GPCR-like" architecture

I am not satisfied with the continued description of the fold as "GPCR-like," and I think the authors' own data argue against it. The closest structural homolog they report is TMEM184C (a non-GPCR); among retinal proteins, the best match is a microbial (type-1) rhodopsin (KR2), which superposes better than bovine rhodopsin, the actual GPCR. Microbial and animal (GPCR) rhodopsins are a textbook case of convergent 7-TM evolution: they share no sequence homology and differ in helix – loop topology and retinal geometry. A shared 7-TM bundle is therefore not a GPCR signature, and calling OST α "GPCR-like" on the basis of a hit to a microbial opsin is misleading.

I ask the authors to describe the fold as a 7-TM architecture throughout the manuscript, and to remove the "GPCR-like" framing. If a comparison to GPCRs is retained, it should be presented explicitly as a convergent structural resemblance of the 7-TM bundle.

A: Thank you for your valuable suggestion. We have deleted the description "GPCR-like" from the manuscript.

Molecular dynamics

I thank the authors for the substantially expanded SMD reporting. My concern about whether the simulations support the central mechanistic claim, however, is not resolved. First, it is not clear which method underlies the energetics. The Methods and main text describe steered MD only, yet Supplementary Fig. 7f presents a "One-dimensional free-energy profile ... obtained from the full WT-MetaD campaign ... reconstructed from the accumulated HILLS file." Well-tempered metadynamics is not mentioned anywhere else (not in the main text, not in the Methods) and none of its parameters are reported (e.g., collective variable and its rationale, bias factor, Gaussian height and width, total sampling time, number of walkers/replicas, convergence assessment, or error estimate). This is the only panel in the paper that legitimately reports a free energy, and it is undocumented. The authors must either fully describe this metadynamics protocol and its convergence, or remove the panel.

Second, the energetic conclusion in the main text ("the energy barrier along the central channel is substantially higher than those of the lateral pathways") is drawn from SMD quantities that are not free energies. The SMD restraint potential (Fig. 7a) is the harmonic spring energy, and the pulling force (Fig. 7b) integrates to work. Neither is a free-energy barrier.

Finally, three non-equilibrium pulls per route cannot support a quantitative barrier comparison. The confidence bands in Fig. 7a,b are very large and there is no work-distribution or Jarzynski/Crooks analysis, so no converged ΔF or error is available.

To support the proposed mechanism energetically, the authors should compute a proper potential of mean force for both candidate routes along an appropriate pathway coordinate, e.g., by umbrella sampling with WHAM, or by the metadynamics they apparently already ran, with a stated convergence criterion (e.g. time evolution of the free-energy surface) and error estimate, and to report all method parameters. Absent this, the SMD results should be presented as qualitative evidence for the translocation

pathway and the substrate – protein contacts, and the free-energy and barrier language should be removed from the main text.

A: Thank you very much for your valuable and insightful comments. You have rightly pointed out an important limitation regarding the molecular dynamics analysis in our study, and we welcome the chance to address it.

Following your comments, we carefully revisited this issue and applied several enhanced-sampling methods, such as metadynamics and umbrella sampling, in an attempt to derive rigorous free-energy profiles for TC translocation. We had actually been exploring these calculations before the initial submission. However, we observed that TC translocation is highly heterogeneous along the central channel and the two lateral grooves. Because the molecule can adopt various microscopic trajectories and orientations during diffusion, the free-energy calculations were highly sensitive to sampling conditions. We were ultimately unable to obtain sufficiently converged and reproducible free-energy landscapes, which remains a major technical hurdle.

Despite this, one qualitative feature consistently emerged from our SMD simulations: TC encountered significantly greater mechanical resistance in the central channel than in the lateral grooves. To further assess the robustness of this observation, and in line with your advice, we expanded our independent SMD simulations from three to ten per pathway. The new data further supports this qualitative distinction.

Importantly, we completely agree with your assessment that SMD-derived restraint energies and pulling forces cannot be directly treated as equilibrium free-energy barriers. In the revised manuscript, we have thus removed the statements concerning free-energy and quantitative energy barriers, softening our interpretation of the SMD results. These simulations are now presented solely as qualitative evidence of the relative translocation difficulty and to highlight substrate – protein interactions.

We are deeply grateful for your methodological guidance, which has helped us improve the manuscript substantially.