nature portfolio

Peer Review File

TM184C is a GPCR-like regulator of intercellular exchange and autophagy

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Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
Review of Lee et al., “Ancient Superdark GPCR-Like Transmembrane Protein 184C Regulates mTOR Signaling and Integrates Endolysosomal and Autophagic Networks”.

In this work, the authors explore the largely uncharted “dark proteome,” focusing on identifying and characterizing proteins that exhibit G protein-coupled receptor (GPCR)-like folds. The GPCR superfamily, recognized as the largest and most physiologically relevant group of membrane proteins, plays a pivotal role in cellular signaling and is implicated in many biological processes. The researchers employ a combination of sequence and structural approaches to identify similarities between the dark proteome and the GPCR protein family (i.e., the sequence clustering algorithm goFo and the structural comparison tool TM-align). It serves as an exemplary approach for identifying uncharacterized proteins and shedding light on their potential roles. The authors extensively characterized one of the most intriguing hits, the “superdark” Transmembrane Protein 184C, through a plethora of experiments, highlighting its expression in autophagosomes and late endosomes and its role in cellular projections.

Furthermore, this study presents an important discovery of interactions between parts of the mTOR complex and GPCRs, as well as GPCR-like proteins. The authors provide compelling evidence of direct physical interactions between mTOR and the newly characterized TM184C, but also between mTOR and some extensively studied GPCRs, such as the β2 adrenergic receptor, adenosine A2A receptor, and vasopressin receptor 2. This direct interaction, specifically localized to the transmembrane domain of GPCRs, suggests a novel mechanism by which mTOR can modulate GPCR activity and downstream signaling pathways. This observation opens avenues for future research to elucidate the precise molecular mechanisms underlying this interaction and to explore its implications for various physiological and pathological processes. The work is well-rounded and effectively highlights interdisciplinary approaches and would benefit from addressing the areas outlined in the major comments to strengthen its impact further.

In short, we support publication in Nature upon major revisions and constructively addressing the points below. We hope the authors find these comments helpful while revising this work for resubmission.

Major comments

A. The rationale behind employing such an elaborate protocol as ChaSiT is unclear, and the conclusions extracted are vague to a certain extent. If the objective of applying this method is to trace the sequence to the ancestor and the relationships between the other sequences, there are more established phylogenetic reconstruction tools. For example, tools like PSI-BLAST are later used to determine the "ancient origin" of TM184C. However, the rationale for combining these approaches is not sufficiently explained, making the section “All 7TMP structural homologs are related” somewhat confusing. Additionally, the conclusions drawn from this analysis are vague and are not fully supported by the data provided. The claim “Together, these findings reveal deep sequence similarities among 7TMPs across all domains of life” is confusing and too generic. Or “While ChaSiT paths capture meaningful sequence patterns (which sequence patterns?), they do not necessarily represent evolutionary trajectories (then again, what is the purpose of using such an elaborate protocol?)". The clarity of the manuscript would be much improved if the authors could please rewrite this section, providing a more explicit rationale for using the ChaSiT method and more robust support for their conclusions. Alternatively, the authors should consider using a more traditional phylogenetic analysis that would return results for similar GPCRs in a more easy-to-

understand manner.

B. After exploring the Pharos database, the authors found several 7TMPs classified as “superdark.” To help clarify their functional hits, the authors could use a classical phylogenetic reconstruction or the ChaSiT method to look for similar GPCRs that are better characterized (known Tclin, Tchem, Tbio). This would help better characterize their structural hits, and inform on function and structural homology to other GPCRs or potential functional ligands.

C. Are the results of the structure search with the comparison algorithm used, TM-align, robust to another seed than rhodopsin as a starting point? Other than being prototypical, is rhodopsin located in the center of the structural ensemble of 7TMPs (does it represent the most common substructure, or could authors use another prototypical GPCR like 2 adrenergic receptor)? If not, the authors should better explain why they used rhodopsin. It would also strengthen the author’s argument if they could perform an explicit robustness analysis using several GPCR seeds from different sub-families.

D. The expression of PRRT3 and PRRT4 is very low in all of the included assays. In the case of SLC51A/B, a known heterodimer, the expression was rescued when the counterpart was co-expressed. Could the authors explore whether PRRT3 and PRRT4 require hetero-oligomerization or a chaperone for successful membrane localization? To investigate this, PRRT3 and PRRT4 could be expressed in a different cell line (potentially one where these proteins are inherently expressed) to evaluate their combined expression and gain further insights into their function. Alternatively, the low expression could be due to HEK cells endogenously expressing these proteins, impairing the expression of the transfected genes. The evidence would be more convincing if they could investigate whether the six receptors are endogenously expressed in HEK cells using RT-PCR or a similar approach.

E. The search for relevant motifs and "codes" is appreciated. Could the authors use tools like the Short Linear Motif database [http://elm.eu.org/] to validate further functional significance of the proteins being studied? Additionally, this could strengthen the evidence for some of the potential interactions revealed in the experimental portion of the manuscript. Similarly, the authors could broaden their analysis of activation motifs to encompass all GPCR family motifs, not just those similar to Class C or Class F GPCRs.

F. The claim “TM184C does not appear to recruit G proteins” warrants further clarification. Figure 3b suggests slight G protein recruitment, while Figures 3c and d indicate G protein dissociation upon expression of TM184C. Could the authors refine the conclusion to allow for the possibility of G protein coupling, as suggested by the results presented in these figures? To further investigate this, the authors could consider imaging experiments to determine whether G proteins and GPCR-like 7TMs colocalize or employ more proximal G protein readouts (e.g., GTPase assays, BODIPY) or downstream transcriptional reporters to clarify coupling at various expression levels of the investigated receptors. These experiments are not needed if the authors suitably modify their interpretations to allow for this possibility.

G. The characterization of the interaction between GPCRs and mTOR suggests that it is only partially modulated by the addition of increasing concentrations of ligand. Does this imply that mTOR complex and GPCRs interact in the basal state? Additionally, if mTORC2 is present on the membrane and truly interacting with the TM184C transmembrane domain, the authors could investigate this further using colocalization experiments. It would also be beneficial to determine if pharmacological antagonists of any of the tested GPCRs or the mTOR complex disrupt the interaction. Furthermore, while the starving experiments are informative, the results show different trends for TM184C and other GPCRs. This would benefit from the authors elaborating on the cellular scenarios in which this interaction occurs and the processes that might modulate the association/dissociation.

H. Expanding the discussion section would be highly beneficial. Could the authors include a discussion of the limitations of the methodologies used, opportunities that may arise from the findings, and potential applications of the interdisciplinary approach used or newly discovered functions of TM184C?

Other comments:

A. The authors claim that Foldseek has lower accuracy than TM-align. While this might be true, this conclusion requires a more exhaustive comparison and is not fully supported by the presented data in the current form. For TM-align, the authors had to develop additional structure-based algorithms for “ranking structure similarity and discerning the correct 3-dimensional fold topology”. Instead, for Foldseek, only two searches were performed, and none of their geometric heuristics to remove false positive matches were used. This is an uneven comparison, as Foldseek options are not systematically explored beyond the two mentioned. Please rewrite this section to minimize the impact of the claim or perform a detailed comparison to support this conclusion.

B. For the parts describing methodology, the authors sometimes provide scattered details between text and figure legends, which are difficult to connect comprehensively. An example can be found in the “Calculating all-versus-all BLASTp sequence similarity,” where parts of the explanation are in Figure S1.3a and parts in the text. These should be combined in the methods section for easier comprehension of the methodological details.

C. It would be interesting if the authors could employ additional pocket-searching algorithms, such as Fpocket, to identify more potential binding pockets in TM184C.

D. The authors state that “GPCRs recruit mTORC2,” but their analysis is limited to only four class A GPCRs and TM184C. Could the authors expand their investigation to include additional GPCR classes to provide broader support for this claim?

Alternatively, they might consider revising the section title to something more specific, such as “Some/Several GPCRs recruit mTORC2,” to reflect the scope of their findings better.

E. The sentence “No other GPCR can be traced to archaea or bacteria by sequence alone…” needs to reference some literature or provide a suitable analysis to substantiate that claim.

F. The supplementary figure numbering is off (e.g., 1.2). Could the authors please provide unique, consecutive names for all figures to ensure clarity and ease of reference?

G. Supplementary Figure panel S1.2e is referenced but missing from the supplementary materials.

H. Please explicitly state the number of technical and biological replicates for each experimental figure and include statistical analyses where relevant.

I. It appears that Figure 2a and Supplementary Figure S2.1a are the same; could the authors please check this and remove one if they are redundant?

J. Figure 2b and Supplementary Figure S2.1b show a set of two Western blot experiments with differences between expression levels of PRRT3 and PRRT4. Could the authors explain this divergence?

K. Given the observation that TM184C localizes near kinesin motors, could the authors perform imaging of relevant kinesin motors and colocalization analysis to further substantiate the claim that those two proteins are localized in similar areas of the cell?

L. Please explicitly state which experiments utilized cells stably expressing TM184C and those using transient transfections.

M. In Figure 3, the authors refer to the same protein using both ADRB2 and β2AR. For consistency, please choose one term and use it throughout the figure and manuscript.

N. Given the structural similarity to GPCRs, could these GPCR-like folds respond to conventional GPCR-targeting drugs and potentially contribute to adverse drug reactions? It would be interesting if the authors could consider testing this hypothesis using drugs that target GPCRs that are phylogenetically or structurally related to TM184C or include a brief discussion point about this possibility.

(Remarks on code availability)

Referee #2

(Remarks to the Author)
The manuscript by Lee et al. uses large-scale AlphaFold2 predictions to uncover two human protein families, TM184 and PRRT, that structurally resemble G protein-coupled receptors (GPCRs) despite lacking sequence similarity. These "superdark" proteins exhibit key GPCR functions, such as β-arrestin recruitment and GRK-mediated phosphorylation. Notably, TM184C binds to mTORC2, modulating mTOR signaling, and localizes to autophagosomes, endosomes, and lysosomes, where it moves along microtubules and participates in vesicle and organelle transfer. Evolutionary analysis traces TM184C to archaea, with functional conservation shown in yeast. The findings highlight a previously unrecognized GPCR-like regulator of endolysosomal and autophagic pathways, emphasizing the power of AI-based tools to explore the dark proteome.
While the approach is creative and useful to identify novel GPCR superfamily proteins, this reviewer has serious concerns about the strength of the central claims—particularly the assertions that TM184C is GPCR-like and that it functionally regulates mTORC2 (not mTORC1) despite its endo-lysosome localization. These connections are speculative and not sufficiently substantiated by the presented data. Significant experimental clarification is needed to justify the manuscript’s core conclusions. Additionally, several figures lack controls, quantification or statistical rigor. For example, vesicle transfer, projection formation, and lysosomal localization should be quantified across multiple cells and replicates. Overall, the major conceptual and technical weaknesses significantly dampen the enthusiasm for this work.
Major comments:
1. The proposed endolysosomal localization of TM184C needs to be supported with quantitation of co-localization with various markers for endomembranes, including early, late and recycling endosomes, Golgi and ER, mitochondria. Also, the lysosomal targeting signal should be deleted/mutated to determine its functional importance.
2. Conceptually, it is difficult to reconcile how endolysosomal TMEM184C could interact with mTORC2. By now, the overwhelming consensus from cellular, biochemical and proteomic-based studies is that mTORC1 is on endolysosomes whereas, due to its role as an Akt kinase and membrane tension-sensing kinase, mTORC2 is most likely at the plasma membrane.
3. The claim that TM184C regulates mTORC2 signaling is central to the manuscript but not convincingly demonstrated. It is puzzling that S6K phosphorylation (canonical mTORC1 substrate) was used to measure mTORC2 kinase activity instead of

other well-established substrates like SGK or PKC.
- The authors claim mTOR to be one of the strongest binding partners of TM184C by IP-MS. The data is not shown in the manuscript.
-The co-IP data for TM184C and mTORC2 components Rictor but not mTORC1 component Raptor are intriguing but should be validated in vitro using purified proteins. Also, in Fig 4a IgG is not an appropriate negative control; an unrelated, FLAG-tagged GPCR should be used as negative control to demonstrate specificity.
- co-IP should be done in both direction (IP: Rictor or Raptor, WB: TM184C)
- Is TM184C required for mTORC2 complex assembly, localization, or kinase activity? Does TM184C bind RICTOR directly, or is the interaction mediated through scaffolding proteins like arrestins?
- immunofluorescent experiment validating co-localization of mTORC2 with TM184 must be presented. It is important to show that mTORC1 does not co-localize with TM184C.
- FigS4, the IP quality showing TM184A,B,C binding to mTORC2 must be improved. There is no TM184A pulled-down, but binding to Rictor is the strongest, questioning whether the proposed interaction is non-specific. Negative control must be included.
-What is the functional or causal mechanisms of TM184C-mTORC2 and projection formation and intercellular vesicle transfer?
- The increase in BRET signal under nutrient repletion is used to infer dynamic regulation of TM184C-mTOR association (Fig. 5c), yet no kinetic quantification or specificity control is shown. Also, two important points are overlooked that cast strong doubt on the interpretation of this experiment: 1- mTORC2 is not on lysosomes and 2- FBS does not control mTORC1 localization to endolysosomes, nutrients (e.g. amino acids) do.
The type and time of nutrient starvation done in this manuscript needs to be clarified: from fig5c, I would assume that serum starvation was performed, which is different to nutrient starvation.
-is TM184C phosphorylation mTOR dependent?

4. TM184C vesicles being transferred between cells is a big statement that needs to be validated in multiple cell lines and thoroughly shown by live cell imaging tracking.
What is the mechanism and function of this transfer? Is this dependent on mTORC2, arrestin binding or phosphorylation status? This data seems nonsensical, as presented.

5. Very little is known about TM184 proteins but they have been proposed to interact with Atg8, and loss of Hfl1 (TM184 homolog in yeast) leads to abnormal vacuole formation (PMID: 30451685). Is there any endo-lysosome defects observed by the loss of TM184C in mammalian cells (number, localization, pH, etc.)?
Additionally, the same paper speculates TM184 to be a potential transporter. What is the evidence here that TM184 is not a transporter but close to a GPCR?

6. Fig7b: increase in LC3b lipidation is interpreted differently depending on the results of bafilomycin A, chloroquine or ConcA treatment, which was not done in this study.
The role of TM184C in autophagy is suggested but not clearly linked to mechanistic consequences and possible connection to projection phenotype.

7. Arrestin recruitment is observed, but this can occur in non-GPCRs and does not, by itself, confer GPCR identity.
What is the functional consequence of arrestin recruitment? Does it lead to downstream signaling, trafficking or degradation?
The structural similarity to GPCRs, while interesting, is insufficient to justify functional homology without canonical signaling. TM184C so far seems close to “7TM arrestin-interacting protein” rather than “GPCR-like”—unless a functional GPCR-like mechanism (e.g., signaling through canonical effectors) is clearly demonstrated.

8. Fig5. Is the role of TM184C in cancer dependent on mTORC2 or cellular projection?
Rescue experiments with TM184C wild-type vs mutants are needed.
Similar reconstitution experiments would help understand the role of TM184C in mTORC2 regulation, projection formation and vesicle transfer.

9. Yeast rescue assay in Fig 7e: This result is interesting but not well integrated. The relevance of TM184C’s rescue of vacuolar phenotypes should be explicitly linked to the human cell/cancer functional context.
Minor comments:
- Fig4: the band size for TM184C is different in Fig4e vs other figures. Please explain.

- Fig2D: Neither the material and method section, nor the figure legend, mention what the markers for each organelle are for the bystander BRET experiment

- FigS6. Revise the figure legend as panel a and b are supposedly the same staining (magenta and EGFP of TM184C).

- Manuscript page 6, second paragraph: the figure numbering is incorrect

- FigS7d: the expression of all over-expressed proteins mu

(Remarks on code availability)

Referee #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

(Remarks on code availability)

Referee #4

(Remarks to the Author)
Lee et al. leverage protein structure predictions from AlphaFold2 to identify a GPCR-like superdark protein within the AlphaFold database. The authors subsequently assessed homology among the identified candidates and experimentally characterized several to determine their receptor types. Overall, this work is of broad interest, however, there are shortcomings in the computational analysis that would require justification.

No source code:
"All data beyond what is provided in the supplemental data and computer code is available upon request", it was not possible for me to check the work as whole at this stage. Please provide the source code to reproduce the full analysis. I consider “data available upon request” a dated practice that should be avoided, particularly with services like Zenodo and GitHub being available free of charge.

BLAST-based Similarity Networks:
The current ChaSiT analysis appears to be overly permissive due to transitive alignments; nearly all 882,783 proteins become interconnected, raising the concern that transitive searches alone—without explicit structural evidence—might be sufficient to recover these proteins. The authors interpret this extensive connectivity as evidence of homology, however, the chosen threshold (an e-value of 1) seems excessively relaxed. Additionally, since BLAST relies on local alignments, repeated traversals through the similarity network risk artificially linking proteins that are only weakly or potentially unrelated.

For robust homology inference, the authors might consider maximum likelihood approaches or structural alignments (e.g. 3D-Coffee, FoldMason) to build a more reliable multiple sequence alignment.
Alternatively, combining sequence identity and coverage thresholds (e.g., at least 90% bidirectional coverage with ≥40% sequence identity at an E-value of 0.0001) may reduce questionable connections but also not perfectly resolve the issue.

Lastly, while the ChaSiT videos are great, Figure 1e feels like a maze. I am not sure how to improve it, but in its current form it is hard to get to the point. One approach might be to group subclusters or highlight key paths (as minimal spanning tree) to help readers quickly identify the main findings to avoid getting lost in the visual complexity.

Foldseek Search and Data Discrepancies:
I performed a similar search with Foldseek search using the PDB structure 1F88 (chain A&B) against Foldseek’s AlphaFold Database (AFDB) with a total runtime of 82 CPUh. Following the procedure below:

foldseek databases Alphafold/UniProt50 afdb50 tmp
foldseek easy-search 1F88.cif afdb50 1F88_aln tmp --exhaustive-search 1 --alignment-type 1 --cluster-search 1

A total of 1,567,802 hits passed the ≥0.45 TM-score threshold. Of these, 748,264 overlapped with the 882,000 reported in the authors’ Excel file, but 89,573 hits meeting the same TM-score threshold were not present in the provided data. Additionally, I do find the receptors mentioned in Figure 1 as well (Q6TCH7, Q15546, A0A6G0V012, Q9BWF8, and Q8TDN7). These discrepancies could be due to different search parameters, but it would be helpful to clarify that Foldseek can find these hits.

Justification why only one structure was used:
The manuscript bases its structural comparisons solely on transporter 1F88. Expanding the search to include multiple transporters or functionally related structures would likely yield a more comprehensive result set and provide deeper insights. This seems like a missed opportunity.

Convex Hull Calculation:
The manuscript briefly mentions using convex hulls to compare and visualize structural data, but offers no explanation of how these hulls were computed. Readers need this methodological detail to replicate the analyses accurately.

Minor:

- What TM-score normalization (query or target length) was used to identify the hits?

- Please state how much CPU time was spent on the TMalign step.

(Remarks on code availability)
The code is not provided.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
The revised manuscript by Lee et al., “Ancient Superdark GPCR-Like Transmembrane Protein 184C Regulates mTOR Signaling and Integrates Endolysosomal and Autophagic Networks,” is significantly improved compared to the original submission.

Specifically, the removal of the ChaSit method and simplification of the sequence clustering pipeline now make the content much easier to follow for a reader. Additionally, many of the claims made were appropriately toned down by the authors, which is appreciated. The expanded experimental cell-based validation offers in-depth functional characterization of newly discovered and intriguing functions and interactions of the GPCRs.

Given these revisions, we would support this article for publication in Nature after minor revisions:

A. The authors state that they tested IQ-TREE on candidate subsets and found that branch support was uniformly low and not statistically significant. This attempt is appreciated, but it is not discussed in the paper. It would be interesting to briefly mention it in the text and/or refer to supplementary material for a broader discussion of the point.

B. When testing with four different queries beyond rhodopsin, the authors note a 95% overlap. This is fine and indicates robustness, but it is important to check and discuss whether the rare “superdark” hits that are found are within the 95% overlap or might be in the 5% of the non-overlapping set. As the work focuses on the rare discovery of superdark hits, it is important to check where they fall. The current analysis shows that TM-align is overall robust; however, it does not explicitly demonstrate this for the discovery of rare “superdark” hits.

C. The authors’ arguments regarding the comparison of structure-based discovery methods remain unconvincing. They primarily use a single-hit discovery (TM184C) to conclude on the relative performance of TM-align + filtering versus Foldseek. They do not clearly show whether other novel hits would be discovered by Foldseek but not by their approach. This is suitable for a discovery paper, where the goal is to characterize a newly identified and interesting target; however, removing or significantly toning down the performance assessment of the structure-based discovery methods is strongly recommended. We make this comment in the best interest of the authors.

D. We appreciate the authors’ clarification regarding the expression levels of PRRT3 and PRRT4 and the impact of different exposures, which helps to avoid potential misconceptions.

E. We thank the authors for their additional analyses, which confirmed the presence of the LC3B-binding motif and further supported the existence of a unique fold due to the absence of activation of GPCR microswitches.

F. We appreciate the inclusion of the colocalization images. Figure S14 suggests colocalization in certain regions; however, line scans through these areas would provide stronger evidence for true colocalization, primarily since the overlay of currently used colors can’t offer proper interpretation. Please use those and the best colocalization color combinations (e.g., magenta and green or turquoise as used in some other figures). We kindly request that the authors make this straightforward modification throughout the manuscript, using the images already presented.

G. We thank the authors for their comprehensive work in clarifying the details of mTOR-TM184C interactions through new cell-based assays and imaging, which have greatly enhanced our understanding of the specific functions of these interactions.

H. The current discussion provides an excellent summary of this comprehensive paper, though condensing it further could improve clarity. While the discussion highlights many future opportunities, it does not yet address the study’s limitations, which would help provide a balanced view of this work.

Other comments:

K. Similar to our suggestion in comment F from the major comments, we recommend including line scans to better demonstrate colocalization, as well as exchanging colors to more clearly illustrate this colocalization.

M. “Supplement Figure 4” still references and displays β2AR. For readers who may not be familiar with GPCRs, we recommend adding a note to clarify which data pertain to the gene and which to the protein.

Additional minor comments:

1. The authors use “Supplement Figure” or “Supplemental Figure” throughout the manuscript; please revise this to ensure consistency.

2. Since G-protein coupling has not been demonstrated, the authors may consider referring to TM184C as an Arrestin-coupled receptor (ACR-like) rather than GPCR-like.

(Remarks on code availability)

Referee #2

(Remarks to the Author)
In the revised manuscript, the authors present additional data supporting the role of TM184C as a new GPCR-like protein involved in endo-lysosomal integrity and intercellular vesicle trafficking. While these new results are interesting, the fundamental problems raised in the first review remain unresolved.

1. the claim that TM184C is a GPCR-like protein that functionally regulates mTORC2 (rather than mTORC1) is not adequately supported by the data. Specifically, the unquantified co-localization analysis in Suppl. Fig. 14 using a non-validated Rictor antibody is not acceptable evidence, by itself, of TMEM184C spatial association with mTORC2.

2. Related to this, the reverse immunoprecipitation experiments pulling down Raptor or Rictor followed by immunoblotting for TM184C (Fig. S7) are problematic and inconsistent with the data presented in the main figure (Fig. 4a). Figure S7 suggests that both Raptor and Rictor interact with TM184C, whereas Figure 4a is used to argue that TM184C binds specifically to mTORC2. These results are contradictory and undermine the authors’ conclusion. Moreover, appropriate negative controls —such as pulldowns with an unrelated or random protein—are missing, making the results impossible to interpret with confidence. Without such controls, the apparent interactions are just as likely to represent background noise or artifacts from protein overexpression.

3. Similarly, the data in Figure 4e, showing multiple GPCRs binding to mTOR (again, without negative controls), reinforce this reviewer’s concern that the reported TM184C–mTORC2 interaction is non-specific and may not reflect a genuine biological association.

4. Furthermore, the IP–MS data provided by the authors raise additional questions. Although mTOR itself appears as a potential binding partner of TM184C, no other core components of either mTORC1 or mTORC2 complexes (e.g., Raptor, Rictor, mSIN1, mLST8) are detected. This lack of supporting interactors makes it difficult to interpret the mTOR association as functionally meaningful thus the authors’ decision to pursue mTOR signaling on the basis of such limited evidence is questionable. The authors also did not provide any in vitro evidence demonstrating a direct interaction between TM184C and either of the mTOR complexes, neither do they map domains or loops within TMEM184C that could mediate this binding, which is a critical shortcoming given the magnitude and unconventional nature of their claims.

5. The claim that TMEM184C directly regulates mTORC2 is also still unsupported and very superficial. The partial reduction in pAkt (S473) in Fig. 5 cannot be taken as evidence of mTORC2 regulation. For example, TMEM184C could affect levels or activity of RTKs (EGFR etc) at the plasma membrane, PI3K or PTEN activation, AKT localization, phosphoinositide levels, and countless other possibilities that are not explored here.

6. And, finally, taking their logic to the extreme: if TMEM184C regulates mTORC2 at the endolysosomal membrane, but the most affected mTORC2 substrate, AKT S473 is activated at the plasma membrane (and this is not a debatable point, given the mountain of evidence) how does this ill-defined mTORC2 activation by TMEM184C propagate to plasma membrane-bound AKT? Again, hard to follow the logic here.

7. And, finally, despite imaging-based evidence for TMEM184C promoting cellular ‘nanotubes’ and vesicle transfer, not even a preliminary mechanistic explanation is provided (i.e. is a G-protein involved?), again making these data purely observational and of questionable impact.

Taken together, the mTOR-related findings in this manuscript are not credible. The data do not support the proposed mechanism, additional data provided in the updated manuscript did not solve the initial concern and the interpretation conflicts with well-established cellular and biochemical evidence. The authors are clearly skilled in bioinformatic, but their treatment of mTOR signaling is inaccurate and misleading.
The observed cellular phenotype upon TM184C loss are potentially interesting and suggests that TM184C somehow regulates endo-lysosome functions. However, it is far more plausible that the observed reduction in mTORC1/2 signaling is a secondary consequence of lysosomal and autophagy dysfunction rather than a direct regulatory effect. Considering TM184C’s apparent role in membrane dynamics and cellular projections, the authors may find greater mechanistic insight by focusing on candidate interactors involved in microtubule organization, kinetochore function, or ciliary/flagellar structures, rather than mTOR signaling.

(Remarks on code availability)

Referee #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

(Remarks on code availability)

Referee #4

(Remarks to the Author)
I greatly appreciate the authors' efforts in addressing many of the previously raised points.
However, two major concerns remain. In particular, although the network is now better described and justified, it is still not entirely convincing.

Major:

- Figure 1e provides helpful visual context for the underlying relationships. However, I remain unconvinced that it adequately addresses the stated aim: “Rather, our aim was to address a complementary question prompted by the advent of AlphaFold2: do diverse 7TMPs that adopt a GPCR-like fold share minimal sequence signals sufficient to specify that fold?” Many of the observed edges have bit scores around 30, which fall within the range of random similarity and therefore lack statistical support. Because BLASTP uses local alignments, these low-scoring matches often correspond to short fragments that do not overlap across sequences and are unrelated to fold-defining regions. As such, they are more consistent with chance alignments than with any conserved sequence signal specifying the GPCR-like fold. Related to this, how were the threshold of ≥30% identity and a bitscore ≥30 selected and justified?
I fully agree with the authors on the importance of developing tools that leverage structural information. Recent work by Grag et al. (MBE, 2025) proposed a substitution matrix for protein structures which can be used in IQ-TREE. Such approaches might substantially improve branch support and better clarify structural relationships among the diverse GPCRs especially if paired with an structural MSAs.

- Foldseek combined with TMalign rapidly recovers most (748,264 out of 882,000 with an additional 89,573) relevant hits and identifies additional significant ones and this is reliant on the fast 3Di prefilter followed by a TMalign. While Folseek-TM is being slower compared to Foldseek 3Di/AA, it only takes 82 CPUh for 7TMPs and therefore is about 400x faster compared to the 34,000 CPUh solution that the authors suggest. I would expect this to be clarified correctly in regard to sustainability and green computing principles.

Minor:

- The provided code in the github is in its current form hard to execute since it misses documentation and is hard to install. Also, I would suggest having a standalone repository for the code linked in the manuscript instead of combining it with the group’s website source code. I would also strongly recommend making the project easily installable through at least one of PyPi, bioconda, docker, etc.

(Remarks on code availability)
The provided code in the github is in its current form hard to execute since it misses documentation and is hard to install. Also, I would suggest having a standalone repository for the code linked in the manuscript instead of combining it with the group’s website source code. I would also strongly recommend making the project easily installable through at least one of PyPi, bioconda, docker, etc.

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)
I would like to thank the authors for the substantial effort that has gone into this revision. The manuscript has improved markedly in clarity, rigor, and conceptual focus, and it now presents a highly compelling and coherent study.

The decision to move the mTOR-related observations to the Extended Data and to explicitly frame them as preliminary is entirely appropriate and significantly strengthens the manuscript. This allows the core discovery, namely TM184C as a superdark GPCR-like protein regulating vesicle dynamics, autophagy, and intercellular connectivity, to emerge clearly and convincingly.

The most important advance in the revised work is the substantial increase in experimental rigor around the intercellular communication phenotypes. The addition of rescue experiments is particularly impactful, as it establishes a clear causal link between TM184C structure, phosphorylation-dependent regulation, and the observed cellular phenotypes.

The demonstration that the C-terminus and arrestin/GRK-related motifs are required for projection formation and vesicle transfer provides a strong mechanistic anchor for the study. Equally important is the introduction of quantitative analysis frameworks. The development of image-based scoring for bridges and projections, as well as automated detection of vesicle exchange, transforms what would otherwise be qualitative observations into reproducible and quantifiable results. This greatly enhances confidence in the biological conclusions and aligns well with the expectations for rigor in the field.

The manuscript also benefits from improved balance and restraint in its interpretation. The revisions addressing the comparison of structural discovery methods are appropriate, and the reframing as a context-dependent comparison rather than a general benchmark is convincing. Similarly, the clarification of sequence similarity thresholds and their empirical calibration improve the transparency of the network analysis.

The responses to Referee 4's concerns are thoughtful and satisfactory. While the questions raised about sequence-level signal and computational efficiency are non-trivial, the authors revisions clarify the intent and limitations of the approach and place the results in an appropriate context. The manuscript now reads as careful rather than overstated. From a presentation standpoint, the improved organization of the discussion, including the addition of limitations, is very helpful.

Finally, I would like to emphasize that this work stands out for its integration of structural informatics, evolutionary reasoning, and cell biology. The idea that structure-first approaches can reveal completely unanticipated biological functions, and that these can be followed through to mechanistic understanding, is both powerful and timely.

In summary, this is a strong and now well-balanced manuscript that makes a significant contribution to the field. We congratulate the authors on the substantial improvements in this revision and look forward to seeing this work published in Nature.

(Remarks on code availability)

Referee #2

(Remarks to the Author)
Based on the new experiments provided, we support acceptance of the manuscript. However, we feel that the proposed connection to mTORC2 remains insufficiently developed and is not essential to the central conclusions of the study. In its current form, this aspect of the work appears premature and, rather than strengthening the manuscript, risks distracting from its primary message. We therefore strongly recommend that the mTORC2-related data be removed from the present study and instead developed as the foundation of a future, dedicated investigation.

(Remarks on code availability)

Referee #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

(Remarks on code availability)

We thank the reviewers for their thoughtful comments, which helped us substantially improve the manuscript. In Figs. 6–8 and the accompanying supplementary figures, we have presented the highest-quality static images possible to illustrate what are inherently dynamic phenomena—vesicle and organelle movement, interaction, transfer, and exchange. The full magnitude of these findings is best appreciated in the extensive live-cell imaging data provided in the supplementary videos, which capture the continuous and coordinated dynamics of TM184C-mediated communication.

Referee #1 (Remarks to the Author):

Review of Lee et al., "Ancient Superdark GPCR-Like Transmembrane Protein 184C Regulates mTOR Signaling and Integrates Endolysosomal and Autophagic Networks".

In this work, the authors explore the largely uncharted "dark proteome," focusing on identifying and characterizing proteins that exhibit G protein-coupled receptor (GPCR)-like folds. The GPCR superfamily, recognized as the largest and most physiologically relevant group of membrane proteins, plays a pivotal role in cellular signaling and is implicated in many biological processes. The researchers employ a combination of sequence and structural approaches to identify similarities between the dark proteome and the GPCR protein family (i.e., the sequence clustering algorithm goFo and the structural comparison tool TM-align). It serves as an exemplary approach for identifying uncharacterized proteins and shedding light on their potential roles. The authors extensively characterized one of the most intriguing hits, the "superdark" Transmembrane Protein 184C, through a plethora of experiments, highlighting its expression in autophagosomes and late endosomes and its role in cellular projections.

Furthermore, this study presents an important discovery of interactions between parts of the mTOR complex and GPCRs, as well as GPCR-like proteins. The authors provide compelling evidence of direct physical interactions between mTOR and the newly characterized TM184C, but also between mTOR and some extensively studied GPCRs, such as the β2 adrenergic receptor, adenosine A2A receptor, and vasopressin receptor 2. This direct interaction, specifically localized to the transmembrane domain of GPCRs, suggests a novel mechanism by which mTOR can modulate GPCR activity and downstream signaling pathways. This observation opens avenues for future research to elucidate the precise molecular mechanisms underlying this interaction and to explore its implications for various physiological and pathological processes. The work is well-rounded and effectively highlights interdisciplinary approaches and would benefit from addressing the areas outlined in the major comments to strengthen its impact further.

In short, we support publication in Nature upon major revisions and constructively addressing the points below. We hope the authors find these comments helpful while revising this work for resubmission.

We thank you for supporting publication in *Nature* and have worked diligently to address all of your concerns in full, several of which aligned closely with those raised by Referee 4.

Major comments

A. The rationale behind employing such an elaborate protocol as ChaSiT is unclear, and the conclusions extracted are vague to a certain extent. If the objective of applying this method is to trace the sequence to the ancestor and the relationships between the other sequences, there are more established phylogenetic reconstruction tools. For example, tools like PSI-BLAST are later used to determine the "ancient origin" of TM184C. However, the rationale for combining these approaches is not sufficiently explained, making the section "All 7TMP structural homologs are related" somewhat confusing. Additionally, the conclusions drawn from this analysis are vague and are not fully supported by the data provided. The claim "Together, these findings reveal deep sequence similarities among 7TMPs across all domains of life" is confusing and too generic. Or "While ChaSiT paths capture meaningful sequence patterns (which sequence patterns?), they do not necessarily represent evolutionary trajectories (then again, what is the purpose of using such an elaborate protocol?)". The clarity of the manuscript would be much improved if the authors could please rewrite this section, providing a more explicit rationale for using the ChaSiT method and more robust support for their conclusions. Alternatively, the authors should consider using a more traditional phylogenetic analysis that would return results for similar GPCRs in a more easy-to-understand manner.

- This is our most important response to Referees 1 and 4, who raised valid concerns about the ChaSiT approach. We recognize that our motivation was not made sufficiently clear. Our purpose in developing and applying ChaSiT was not to trace individual GPCR or 7TMP sequences to an ancestor, nor to introduce a new method for inferring remote homology among sequence-diverse folds. Rather, our aim was to address a complementary question prompted by the advent of AlphaFold2: do diverse 7TMPs that adopt a GPCR-like fold share minimal sequence signals sufficient to specify that fold? Because structure is more conserved than sequence, our intent was not to reconstruct phylogeny but to quantify sequence similarity associated with a common 7TM architecture. The analysis demonstrates a sequence-to-structure correspondence across the entire 7TM scaffold, consistent with the idea that AF2 leverages recurring sequence features to recover the fold, without implying evolutionary relatedness among these remote proteins. In a sense, this represents an inverse AF2 analysis, identifying the recurring sequence signals that encode the fold. Importantly, these features are broadly distributed across the scaffold (Fig. 1g) rather than clustered in local motifs, addressing Referee 4's concern about potential local bias in the BLASTP component of our pipeline. We have revised the text to make these objectives and conclusions explicit. We have also replaced the ChaSiT algorithm and associated data with a simpler, more transparent pipeline, which we summarize here and describe in Fig. S3 (more details are provided in our response to Referee 4).
    - Step 1: Collect hits by structural homology
    - Step 2: Use MMseqs2 to reduce 1.5M hits to a representative list of 39,808 sequences
    - Step 3: Calculate an all-verus-all BLASTP similarity matrix of the 39,808 sequences
    - Step 4: Convert the similarity matrix to an adjacency network with metadata in edges
        - Percent similarity
        - Bitscore
        - E-value
    - Step 5: Use a breadth-first search (BFS) goForward method to collect connected nodes
        - We can start at any node, but we chose the archaeal node with the most connections in Figs. 1e-f.
        - The rules of goForward are simple. Using the edge thresholds listed below:

        - Once a node is encountered, it can't be encountered again
        - No network rings are allowed
    - Consistent with searches for remote to moderate homology, we used a 30% threshold for percent similarity. Instead of using e-value, which is dependent on database size, we now use a minimum bitscore of 30. The details are further explained and justified in our response to Referee 4 and the revised manuscript.
  - Step 6: Build a maximum spanning forest from the forward results, which we visualize in Figs. 1e-f and Fig. S3d using the open source software Cytoscape. We also provide the Cytoscape files so that referees and readers can inspect them for complete transparency.
- Again addressing Referees 1 and 4, we appreciate the suggestion to apply traditional maximum-likelihood (ML) phylogenetic reconstruction. We agree that such methods are appropriate when analyzing homologous sequences for a single gene or for highly related genes (e.g., metabolic or ribosomal genes) under a well-specified substitution model, which is grounded in sequence-based theory. In contrast, our dataset comprises many non-orthologous GPCR-like proteins connected primarily by remote structural similarity, for which concatenation or joint tree inference lacks a coherent evolutionary framework and risks severe model misspecification. Nevertheless, we tested IQ-TREE on candidate subsets and found that branch support (e.g., SH-aLRT/ultrafast bootstrap) was uniformly low and not statistically significant, consistent with these limitations. For this reason, we approach the problem as one of similarity and network structure rather than classical phylogeny. Because structure is more conserved than sequence, we believe structural biology/informatics is entering a new era in which large numbers of AF2 models will enable the development of next-generation phylogenetic tools. Our work underscores this need.
- As a side note, regarding our use of PSI-BLAST to explore the "ancient origin" of TM184C: this was an ad hoc analysis motivated by curiosity. After demonstrating that human TM184C can rescue its yeast homolog Hfl1—a striking result—we asked how far back the TM184 family could be traced. In our experience, we have never been able to use PSI-BLAST or related tools to connect a human GPCR directly to an archaeal homolog. Remarkably, with TM184C we could, and we considered this significant enough to report.

B. After exploring the Pharos database, the authors found several 7TMPs classified as "superdark." To help clarify their functional hits, the authors could use a classical phylogenetic reconstruction or the ChaSiT method to look for similar GPCRs that are better characterized (known Tclin, Tchem, Tbio). This would help better characterize their structural hits, and inform on function and structural homology to other GPCRs or potential functional ligands.

- Pharos does not contain a category called "superdark." We introduced this term to describe proteins that may belong to, or be related to, known families but are effectively hidden by extreme sequence divergence. For example, Tdark GPCRs in Pharos are still recognized as GPCRs, albeit uncharacterized. We have studied and published on these Tdark GPCRs as part of the Illuminating the Druggable Genome (IDG) Consortium, supported by NCATS and the NIH. By contrast, TM184C is not even established as a GPCR or GPCR-like protein, and thus would never appear on the IDG list. Our reference to Pharos was intended only to note that the candidates we identified fall into the Tdark category, meaning simply that they remain understudied, whereas the concept of "superdark" conveys the deeper challenge of detecting relationships obscured by sequence divergence.
- At the outset of this study, once we had our first list of hits, we shared the referee's instinct to mine resources such as Pharos to gain functional insight into superdarks, particularly TM184C. We were especially interested in whether it might have an endogenous ligand. While we hypothesize that it

does, this remains an open question and a focus for future work that will require additional extramural support. As outlined in our earlier response, phylogenetic approaches are not appropriate for these highly divergent proteins. Moreover, when we constructed networks from known GPCRs, we observed that even closely related receptors often engage very different ligands, limiting the predictive value of sequence or structural homology in this context. Looking forward, we are advancing toward a cryo-EM structure of TM184C, and together with computational approaches such as in silico screening, we anticipate these efforts will provide more direct insight into potential ligands and function.

- Lastly, our work illustrates that the most effective way to investigate the structure-function of superdarks is through a broad and integrative experimental strategy, approaching the problem from multiple complementary angles. We believe this manuscript provides a clear example of the strength of such an approach.

C. Are the results of the structure search with the comparison algorithm used, TM-align, robust to another seed than rhodopsin as a starting point? Other than being prototypical, is rhodopsin located in the center of the structural ensemble of 7TMPs (does it represent the most common substructure, or could authors use another prototypical GPCR like β2 adrenergic receptor)? If not, the authors should better explain why they used rhodopsin. It would also strengthen the author's argument if they could perform an explicit robustness analysis using several GPCR seeds from different sub-families.

- This response addresses Referees 1 and 4. To summarize, Referees 1 and 4 raised the concern that relying on a single query structure could limit the generality of our approach. We acknowledge this important point and agree that it should have been addressed more clearly.
    - In reality, when developing our exhaustive structure-based searches we initially used hundreds of GPCR structures from the PDB, which made the calculations considerably longer and much, much more expensive. We observed, however, that the results were largely the same regardless of the query structure. On this basis, we selected bovine rhodopsin (1F88) as our representative query, both for efficiency and because it is symbolic as the first GPCR structure ever solved.
    - To address the concern that we relied on a single query structure, we performed a formal test using four GPCR query structures from different classes. With our pipeline (restricted to ~6 million targets), the four searches yielded ~95% overlap in hit sets, indicating minimal dependence on query choice. We then evaluated Foldseek against the AFDB50-minimal database. Because CPU time is a finite resource, we used this more compact database to reduce computation time and cost; the outcome is not dependent on the AFDB variant. Using e-value = 1 (required to recover TM184C), Foldseek returned ~1,200 hits with only ~54% overlap across queries. At e-value = 0.01, Foldseek returned ~900 hits with ~83% overlap, but this stricter threshold failed to recover our key superdark targets. These results show that Foldseek query results are quite dependent on query structure.
    - We also investigated the quality of the Foldseek hits at both e=1 and e=0.01 using our bovine rhodopsin query structure and AFDB50-minimal. These searches returned 1,455 and 843 hits, respectively. Applying our geometric 7TM/GPCR-like filters to the results removed ~40% (808 of 1,455 structures) of hits at e=1 (indicating many off-target matches) and only a single hit at e=0.01 (but again, this lower cutoff missed the superdark proteins).
    - In summary, these analyses indicate that our structure-only pipeline is largely insensitive to the specific query structure, whereas Foldseek is more sensitive to query choice and e-value threshold. In particular, Foldseek fails to recover our key superdark targets at e-value ≤ 0.01; relaxing the threshold (> 0.01) retrieves them but admits many non-GPCR-like matches, necessitating extensive geometric filtering that removes a substantial fraction of

hits. This trade-off makes it difficult to distinguish signal from noise and to prioritize high-risk experimental follow-ups based solely on raw Foldseek outputs.

D. The expression of PRRT3 and PRRT4 is very low in all of the included assays. In the case of SLC51A/B, a known heterodimer, the expression was rescued when the counterpart was co-expressed. Could the authors explore whether PRRT3 and PRRT4 require hetero-oligomerization or a chaperone for successful membrane localization? To investigate this, PRRT3 and PRRT4 could be expressed in a different cell line (potentially one where these proteins are inherently expressed) to evaluate their combined expression and gain further insights into their function. Alternatively, the low expression could be due to HEK cells endogenously expressing these proteins, impairing the expression of the transfected genes. The evidence would be more convincing if they could investigate whether the six receptors are endogenously expressed in HEK cells using RT-PCR or a similar approach.

- As shown in Figs. 2a and S4a, human expression data for PRRT4 are not available, and PRRT3 expression is reported only in a limited set of cell lines that we could not obtain (not all primary cell lines can be passaged for in vitro studies). Nevertheless, our data demonstrate that both PRRT3 and PRRT4 express adequately in HEK cells, albeit at low levels, which is common for many GPCRs. For example, the proton sensor GPR4, which we study extensively and included in this work, also expresses at low levels (see IB:FLAG blot in Fig. 4e). Because some GPCRs blot more strongly than others, exposure times must be adjusted to avoid saturating higher-expressing receptors. As a result, lower-expressing receptors can appear faint. The standard solution is to cut the blot and expose the lower-expressing receptors for longer, which we did for the PRRTs. Based on comment J under minor concerns, we believe the figures were misinterpreted because we did not make this clear. In conclusion, the PRRTs do express in HEK cells, though at relatively low levels, which is not unusual for GPCRs. Importantly, the bystander BRET data reveal an interesting subcellular localization pattern for the PRRTs (Fig. 2d), which warrants further study. Unlike SLC51A, which was clearly trapped in the ER and required a chaperone, the PRRTs are not retained in the ER, indicating that they do not require chaperone
- Because we felt the referee's request for RT-PCR stemmed from our lack of clarity—and given that we already have data demonstrating that the PRRTs express adequately—we did not perform this additional experiment. Moreover, when evaluating expression levels, it is not our practice to quantify RNA, as transcript abundance is often poorly correlated with protein levels. We therefore focus on the protein level. To be further responsive to the referee while remaining at the protein level, we performed pulldowns of each superdark, including the PRRTs, confirming that they all express as expected. This data is included in the revised manuscript in Fig. S4b.

E. The search for relevant motifs and "codes" is appreciated. Could the authors use tools like the Short Linear Motif database [http://elm.eu.org/] to validate further functional significance of the proteins being studied? Additionally, this could strengthen the evidence for some of the potential interactions revealed in the experimental portion of the manuscript. Similarly, the authors could broaden their analysis of activation motifs to encompass all GPCR family motifs, not just those similar to Class C or Class F GPCRs.

- To summarize, the reviewer raises two questions: first, whether analysis using a short linear motif database could provide further insight into TM184C function; and second, whether our search for GPCR motifs could be expanded to include the full range of GPCR family motifs.

- For the first question, we searched the TM184C sequence against the Short Linear Motif database. While no GPCR motifs were identified, we detected LC3B-binding motifs, consistent with our observations that TM184C localizes to autophagosomes and participates in autophagy. This finding is now noted in the revised manuscript.
- For the second question, there may have been a miscommunication. As described in the original main text (section *Superdarks TM184C and PRRT3/4 contain arrestin codes*), we searched for Class A and B motifs and found none. We also searched for Class C and F motifs, likewise finding none, but did not explicitly note this in the main text. Our search was guided primarily by Hauser et al. (*Nat. Struct. Mol. Biol. 2021*), specifically Figure 4a, which we now cite in the revised manuscript. We have also revised the text to make clear that there is no compelling evidence for Class C or F motifs in TM184C or the other superdarks.

F. The claim "TM184C does not appear to recruit G proteins" warrants further clarification. Figure 3b suggests slight G protein recruitment, while Figures 3c and d indicate G protein dissociation upon expression of TM184C. Could the authors refine the conclusion to allow for the possibility of G protein coupling, as suggested by the results presented in these figures? To further investigate this, the authors could consider imaging experiments to determine whether G proteins and GPCR-like 7TMs colocalize or employ more proximal G protein readouts (e.g., GTPase assays, BODIPY) or downstream transcriptional reporters to clarify coupling at various expression levels of the investigated receptors. These experiments are not needed if the authors suitably modify their interpretations to allow for this possibility.

- These are great points. Following our computational predictions, we invested 2+ years exploring whether TM184C could recruit or signal through G proteins. One key challenge is that, if TM184C has an agonist, it remains unknown, making standard GPCR assays inherently difficult. Consequently, we focused on constitutive activities, such as β-arrestin and GRK recruitment, as indicators of GPCR-like behavior. While our data provide only circumstantial evidence for G protein binding, they do suggest that TM184C may interact with G proteins at some level. In our original submission, we framed the results conservatively to avoid overinterpretation. In light of the reviewer's insights, we have revised the text to more openly convey the possibility of G protein coupling.

G. The characterization of the interaction between GPCRs and mTOR suggests that it is only partially modulated by the addition of increasing concentrations of ligand. Does this imply that mTOR complex and GPCRs interact in the basal state? Additionally, if mTORC2 is present on the membrane and truly interacting with the TM184C transmembrane domain, the authors could investigate this further using colocalization experiments. It would also be beneficial to determine if pharmacological antagonists of any of the tested GPCRs or the mTOR complex disrupt the interaction. Furthermore, while the starving experiments are informative, the results show different trends for TM184C and other GPCRs. This would benefit from the authors elaborating on the cellular scenarios in which this interaction occurs and the processes that might modulate the association/dissociation.

[redacted]

[redacted]. All three antibodies produced clear and specific staining patterns in HEK293A cells, revealing extensive vesicular co-localization with TM184C–eGFP (Fig. S14).

[redacted] he results are presented in Fig. S15 and Video S25.

- For the third question, there are two parts:
  - [redacted] Live-cell imaging (Videos S27) further revealed that many TM184C-positive vesicles became immobilized near the cell periphery, particularly within extended projections.
  - Part two: For GPCRs, we tested antagonists in our pulldowns and observed no effect. Given the scope of the current study and limitations in time and resources, we plan to pursue more detailed GPCR studies as part of our upcoming renewal of extramural funding. The same applies to efforts aimed at understanding the role of the PRRTs.
- For the fourth question:
  - We appreciate the reviewer's comment regarding the refeeding assay. Although this procedure is somewhat artificial and does not fully replicate physiological

[redacted]

The apparent discrepancy is most likely not a true inconsistency. Across multiple lines of evidence, we have shown that TM184C localizes to the late endocytic pathway (late endosomes, lysosomes, and autophagosomes). [redacted]

H. Expanding the discussion section would be highly beneficial. Could the authors include a discussion of the limitations of the methodologies used, opportunities that may arise from the findings, and potential applications of the interdisciplinary approach used or newly discovered functions of TM184C?

- Thanks for the suggestion. We have done this.

Other comments:

A. The authors claim that Foldseek has lower accuracy than TM-align. While this might be true, this conclusion requires a more exhaustive comparison and is not fully supported by the presented data in the current form. For TM-align, the authors had to develop additional structure-based algorithms for "ranking structure similarity and discerning the correct 3-dimensional fold topology". Instead, for Foldseek, only two searches were performed, and none of their geometric heuristics to remove false positive matches were used. This is an uneven comparison, as Foldseek options are not systematically explored beyond the two mentioned. Please rewrite this section to minimize the impact of the claim or perform a detailed comparison to support this conclusion.

- See response above to Referees 1 and 4. We have an additional response to both referees that addresses the valid criticism of a lack of head-to-head comparison between a full structure-based search and Foldseek. We are puzzled why this standard benchmark wasn't required to get the Foldseek work published. So, the question is, why did we develop an alternative despite the availability of Foldseek? The answer is that Foldseek was released after we had already built our pipeline and begun experimentally validating prioritized hits. Because our study centers on biological discovery rather than benchmarking, we did not initially include a head-to-head comparison that could distract from the main findings. Given Foldseek's emergence prior to submission, we agree that a robust comparison is warranted and have now included it in the

revision, aiming not to dilute the paper's core messages. In our hands, Foldseek's performance depended strongly on the query structure and the e-value threshold: at e ≤ 0.01 it failed to recover our key superdark targets, whereas relaxing the threshold (> 0.01) retrieved them but admitted many non-GPCR-like matches, necessitating extensive geometric filtering and reducing practical precision. By contrast, our structure-based approach was less sensitive to query choice and led directly to TM184C and the connection to archaeal histidine kinases. While Foldseek is undeniably fast, those speed gains come with trade-offs that were misaligned with the goals of this study. We present the comparison in the revised manuscript.

B. For the parts describing methodology, the authors sometimes provide scattered details between text and figure legends, which are difficult to connect comprehensively. An example can be found in the "Calculating all-versus-all BLASTp sequence similarity," where parts of the explanation are in Figure S1.3a and parts in the text. These should be combined in the methods section for easier comprehension of the methodological details.

- We agree and have addressed the issue.

C. It would be interesting if the authors could employ additional pocket-searching algorithms, such as Fpocket, to identify more potential binding pockets in TM184C.

- We appreciate the suggestion and performed the calculation; both methods produced concordant results (Fig. S5f). The code for this analysis is now available on GitHub as part of our pHinder software. We believe that our long-standing virtual screening approach provides a more robust framework than Fpocket.

D. The authors state that "GPCRs recruit mTORC2," but their analysis is limited to only four class A GPCRs and TM184C. Could the authors expand their investigation to include additional GPCR classes to provide broader support for this claim? Alternatively, they might consider revising the section title to something more specific, such as "Some/Several GPCRs recruit mTORC2," to reflect the scope of their findings better.

- [redacted]

E. The sentence "No other GPCR can be traced to archaea or bacteria by sequence alone…" needs to reference some literature or provide a suitable analysis to substantiate that claim.

- We appreciate this point. We previously addressed this issue in our PNAS paper, which demonstrated conserved ionizable circuits from human GPCRs to bacterial opsins. Then as now, we are unable to cite any reference that directly negates a possible sequence connection from GPCRs to archaea. In light of this, and consistent with the referee's concern, we softened this statement in the revision: "To the best of our knowledge, GPCRs cannot be traced to archaea or bacteria by sequence alone, underscoring TM184C's uniqueness and suggesting a potential evolutionary link to primordial GPCR-like folds."

F. The supplementary figure numbering is off (e.g., 1.2). Could the authors please provide unique, consecutive names for all figures to ensure clarity and ease of reference?

- We have done this.

G. Supplementary Figure panel S1.2e is referenced but missing from the supplementary materials.

- Fig S1.2e has been removed in the revision. We will ensure the listing of the supplementary materials is accurate.

H. Please explicitly state the number of technical and biological replicates for each experimental figure and include statistical analyses where relevant.

- We have addressed this issue.

I. It appears that Figure 2a and Supplementary Figure S2.1a are the same; could the authors please check this and remove one if they are redundant?

- We have addressed this issue.

J. Figure 2b and Supplementary Figure S2.1b show a set of two Western blot experiments with differences between expression levels of PRRT3 and PRRT4. Could the authors explain this divergence?

- The figures do not indicate different expression levels of the PRRTs but rather a longer blot exposure to confirm that both proteins are expressed, which is standard practice in such cases. This also helps address the earlier question “D” under major concerns regarding PRRT expression and the possible need for chaperones. Many GPCRs are inherently difficult to detect by Western blotting yet do not require chaperones, as exemplified by the proton sensor GPR4, which we have studied extensively and used this study (Fig. 4e).

K. Given the observation that TM184C localizes near kinesin motors, could the authors perform imaging of relevant kinesin motors and colocalization analysis to further substantiate the claim that those two proteins are localized in similar areas of the cell?

- We conducted the requested experiment, and the results show they are localized (Fig. S11).

L. Please explicitly state which experiments utilized cells stably expressing TM184C and those using transient transfections.

- We have addressed this issue.

M. In Figure 3, the authors refer to the same protein using both ADRB2 and β2AR. For consistency, please choose one term and use it throughout the figure and manuscript.

- We have addressed this issue.

N. Given the structural similarity to GPCRs, could these GPCR-like folds respond to conventional GPCR-targeting drugs and potentially contribute to adverse drug reactions? It would be interesting if the authors could consider testing this hypothesis using drugs that target GPCRs that are

phylogenetically or structurally related to TM184C or include a brief discussion point about this possibility.

- This comment is similar to comment “B” under major concerns, and we refer the referee to that response. In addition, we are actively pursuing our next round of extramural funding to comprehensively explore the possibility of a ligand for TM184C, as well as family members TM184A and TM184B. Our preliminary work indicates that TM184A does not bind heparin as previously reported, underscoring that much remains to be clarified in this area.

Referee #2+3 (Remarks to the Author):

The manuscript by Lee et al. uses large-scale AlphaFold2 predictions to uncover two human protein families, TM184 and PRRT, that structurally resemble G protein-coupled receptors (GPCRs) despite lacking sequence similarity. These "superdark" proteins exhibit key GPCR functions, such as β-arrestin recruitment and GRK-mediated phosphorylation. Notably, TM184C binds to mTORC2, modulating mTOR signaling, and localizes to autophagosomes, endosomes, and lysosomes, where it moves along microtubules and participates in vesicle and organelle transfer. Evolutionary analysis traces TM184C to archaea, with functional conservation shown in yeast. The findings highlight a previously unrecognized GPCR-like regulator of endolysosomal and autophagic pathways, emphasizing the power of AI-based tools to explore the dark proteome.

While the approach is creative and useful to identify novel GPCR superfamily proteins, this reviewer has serious concerns about the strength of the central claims—particularly the assertions that TM184C is GPCR-like and that it functionally regulates mTORC2 (not mTORC1) despite its endo-lysosome localization. These connections are speculative and not sufficiently substantiated by the presented data. Significant experimental clarification is needed to justify the manuscript's core conclusions. Additionally, several figures lack controls, quantification or statistical rigor. For example, vesicle transfer, projection formation, and lysosomal localization should be quantified across multiple cells and replicates. Overall, the major conceptual and technical weaknesses significantly dampen the enthusiasm for this work.

We appreciate the reviewer's emphasis on quantitative rigor and reproducibility. Where quantification was appropriate and meaningful, we have included it in the revised figures and legends. For phenomena such as vesicle transfer, projection formation, and lysosomal localization, these behaviors are consistently and reproducibly observed across all experiments and are best conveyed through representative live-cell imaging rather than numerical reduction. All imaging experiments were performed independently at least three times, with the vast majority repeated more than that, often using multiple batches of stable cell lines and across distinct cell types to ensure biological reproducibility. We have also clarified and, where relevant, added control experiments in the revised figures to further support these conclusions.

Major comments:

1. The proposed endolysosomal localization of TM184C needs to be supported with quantitation of co-localization with various markers for endomembranes, including early, late and recycling endosomes, Golgi and ER, mitochondria. Also, the lysosomal targeting signal should be deleted/mutated to determine its functional importance.

We thank the reviewer for this helpful suggestion. This analysis is now included in the revised manuscript and presented in Figs. S9–S10. To assess vesicular co-localization, we used HEK293A cells stably expressing TM184C–mCherry together with transiently expressed FP-tagged organelle markers. We also developed custom Python-based image analysis code to quantify TM184C co-localization with each organelle marker. The resulting quantitative data are shown in Fig. 10j, and the analysis code is described in the Methods section and available on our GitHub page.

Regarding the lysosomal targeting sequence, this was another excellent suggestion. When we deleted the C-terminal region (TM184CΔ82), which contains this sequence, the protein mislocalized to the plasma membrane and closely resembled TM184A and TM184B, both of which are more prominently membrane-

associated (Fig. 7f and Videos S20-21). TM184CΔ82–eGFP still appeared in vesicles, lysosomes, and autolysosomes, but this likely reflects degradation rather than targeting. Consistent with this interpretation, TM184CΔ82–eGFP could still be transferred intercellularly due to the presence of endogenous TM184C, but none of the vesicular TM184CΔ82–eGFP recycled back to the plasma membrane, indicating that it is transported as transcellular cargo within autolysosomes.

Importantly, [redacted]

2. Conceptually, it is difficult to reconcile how endolysosomal TMEM184C could interact with mTORC2. By now, the overwhelming consensus from cellular, biochemical and proteomic-based studies is that mTORC1 is on endolysosomes whereas, due to its role as an Akt kinase and membrane tension-sensing kinase, mTORC2 is most likely at the plasma membrane.

We thank the referee for raising this important conceptual point. [redacted]

To directly address this in the context of our work, we performed several new experiments:

- Repeating our response to Referee 1:
  - [redacted]. All three antibodies produced clear and specific staining patterns in HEK293A cells, revealing extensive vesicular co-localization with TM184C–eGFP (Fig. S14). [redacted]
  - [redacted]. The results are presented in Fig. S15 and Video S25. [redacted]
  - [redacted] Quoting from the

revised text, “As shown in Fig. 5b, phosphorylated TM184C predominates under fed conditions, whereas in the starved state most TM184C is dephosphorylated. [redacted]

3. The claim that TM184C regulates mTORC2 signaling is central to the manuscript but not convincingly demonstrated. It is puzzling that S6K phosphorylation (canonical mTORC1 substrate) was used to measure mTORC2 kinase activity instead of other well-established substrates like SGK or PKC.

We understand and appreciate this concern. [redacted] However, as you will see below, your comment prompted additional experiments that have significantly expanded our perspective. [redacted]

[redacted] We view these findings as the beginning of an exciting line of investigation, and we believe the current summary provides a balanced and objective framework for understanding TM184C’s role in coordinating these interconnected pathways.

[redacted]. As shown in Fig. 5e, TM184C levels were reduced in all three cell lines relative to controls, confirming efficient knockdown. [redacted]

[redacted]

measured their phosphostatus following TM184C knockdown but observed no significant changes. [redacted]

The authors claim mTOR to be one of the strongest binding partners of TM184C by IP-MS. The data is not shown in the manuscript.

This has been corrected. [redacted]

The co-IP data for TM184C and mTORC2 components Rictor but not mTORC1 component Raptor are intriguing but should be validated in vitro using purified proteins. Also, in Fig 4a IgG is not an appropriate negative control; an unrelated, FLAG-tagged GPCR should be used as negative control to demonstrate specificity.

We appreciate this suggestion. However, as our experiments progressed in response to your feedback, we envisioned a complementary strategy to validate the co-IP data in a cellular context. [redacted] As noted previously, this approach was successful. [redacted]

[redacted] We did perform this control experiment and, in doing so, [redacted]

[redacted]

[redacted]. We did this experiment, and it worked. The results are reported in Fig. S7.

Is TM184C required for mTORC2 complex assembly, localization, or kinase activity? Does TM184C bind RICTOR directly, or is the interaction mediated through scaffolding proteins like arrestins?

These are excellent questions. [redacted] However, TM184C knockout cells exhibit severe growth defects and are difficult to maintain for downstream analyses beyond initial immunoblotting. Moreover, we currently lack small-molecule modulators of TM184C activity, though we are actively pursuing this line of investigation and seeking renewed extramural support for these studies.

[redacted]

[redacted]

Immunofluorescent experiment validating co-localization of mTORC2 with TM184 must be presented. It is important to show that mTORC1 does not co-localize with TM184C.

[redacted]

FigS4, the IP quality showing TM184A,B,C binding to mTORC2 must be improved. There is no TM184A pulled-down, but binding to Rictor is the strongest, questioning whether the proposed interaction is non-specific. Negative control must be included.

We appreciate this feedback. We have redone the immunoblots with LAMP-1 as our negative control. The results are presented in Fig. S6.

What is the functional or causal mechanisms of TM184C-mTORC2 and projection formation and intercellular vesicle transfer?

Our data show that increased TM184C expression correlates with a greater number of cellular projections and intercellular connections (Fig. S12b). However, the causal mechanisms underlying projection formation remain under our active investigation. In contrast—and perhaps more importantly—we have identified a causal mechanism for intercellular vesicle transfer. [redacted] Quoting from the revised text:

“In the motif approach, we mutated the phosphosites in the three arrestin codes to generate a HEK293A cell line stably expressing TM184C-ACM-eGFP. As shown in Fig. 7h and Video S22, TM184C-ACM-eGFP cells appeared rounder than wild-type HEK293A cells or those stably expressing TM184C-mCherry. The defective cells contained larger vesicles that frequently clustered into dense aggregates and often developed multi-membraned “mega-vesicles” up to 10 µm in diameter, typically enclosing a single large acidic compartment detectable with LysoTracker (Video S22). These structures exhibited multiple concentric membrane shells, like nested “vesicles-within-vesicles,” suggestive of progressive engulfment or fusion events.

In addition to their morphological defects, the ACM variant was properly targeted to vesicles yet failed to undergo intercellular transfer. In co-culture with HEK293A cells stably expressing TM184C-mCherry, ACM vesicles were not detected in TM184C-mCherry cells (Fig. 7i, Video S23). This pattern indicates that the C-terminal tail encodes essential information for vesicle transfer and that its disruption exerts a dominant-negative effect, blocking TM184C-ACM-eGFP transfer even in the presence of endogenous TM184C. In line with a causal role for TM184C in maintaining vesicular organization, co-culture of TM184C-ACM-eGFP cells with HEK293A cells stably expressing TM184C-mCherry led to a striking rescue of these defects (Fig. 7i, Video S23). Vesicles from TM184C–mCherry cells transferred into neighboring TM184C-ACM-eGFP-expressing cells, restoring normal morphology and resolving the oversized, multi-membraned aggregates. This rescue resembles the metabolic recovery mediated by tunneling nanotubes, in which healthy cells deliver mitochondria, lysosomes, or other second messengers to stressed counterparts [47,48], and supports the view that TM184C acts as a central regulator of this intercellular vesicle exchange process.

Together, these results reveal that TM184C, unlike its family members TM184A and TM184B, is uniquely dedicated to intercellular vesicle transfer[redacted] Disruption of these motifs abolishes vesicle exchange and induces striking morphological defects, while transfer from neighboring TM184C-expressing cells can restore normal vesicle morphology—mirroring metabolic rescue through tunneling nanotube–like conduits. Thus, TM184C defines a regulated system of intercellular vesicle communication in which GPCR-like and metabolic signaling converge to coordinate the exchange of material between connected cells.”

The increase in BRET signal under nutrient repletion is used to infer dynamic regulation of TM184C-mTOR association (Fig. 5c), yet no kinetic quantification or specificity control is shown. Also, two important points are overlooked that cast strong doubt on the interpretation of this experiment: 1- mTORC2 is not on lysosomes and 2- FBS does not control mTORC1 localization to endolysosomes, nutrients (e.g. amino acids) do.

[redacted]

Regarding the second part of the feedback, we appreciate the opportunity to clarify this point. We recognize that the starvation and re-feeding experiments were not presented as clearly as they could have been in the original submission, and we have revised the text to address this issue.

We approached this question in two steps.

As shown in Fig. 5a, BRET signals for Rluc8-tagged HRAS, TM184C, ADRB2, and AVPR2 were measured under starved, re-fed 0% FBS, and re-fed 10% FBS conditions. HRAS served as a control for localized BRET at the plasma membrane and showed minimal signal across all conditions. In contrast, TM184C displayed a marked increase in BRET upon refeeding, particularly with 10% FBS. The control GPCRs ADRB2 and AVPR2, which primarily localize to the plasma membrane, showed modest, nutrient-independent BRET signals above HRas control levels.

A key aspect of this approach was our observation that the phosphorylation status of TM184C changes in response to nutrient stress and can be detected by immunoblotting using the same method applied in our GRK studies (Figs. 3k-l). As shown in Fig. 5b, phosphorylated TM184C predominates under fed conditions, whereas in the starved state most TM184C is dephosphorylated.

The type and time of nutrient starvation done in this manuscript needs to be clarified: from fig5c, I would assume that serum starvation was performed, which is different to nutrient starvation.

[redacted] The relevant details are provided in the previous response. We thank the reviewer for their thorough and constructive evaluation.

Is TM184C phosphorylation mTOR dependent?

Yes! [redacted]. See Fig. 5b and related text, which is conveniently reproduced above.

4. TM184C vesicles being transferred between cells is a big statement that needs to be validated in multiple cell lines and thoroughly shown by live cell imaging tracking.

We agree that this is an extremely significant claim. We have now performed the requested experiments and confirmed both the occurrence and generality of the transfer phenomenon. Initially, we used live-cell imaging and vesicle tracking, but subsequently developed co-culture assays that enable the study of vesicle transfer across diverse transducible cell types. This approach provided clear evidence of robust intercellular vesicle transfer in multiple cell types, with additional examples currently under investigation. Quoting from the revised text:

“Building on the observation that TM184C vesicles populate microtubule-supported cellular projections, we next asked if these vesicles participate in intercellular communication. Live-cell imaging revealed TM184C vesicles traversing intercellular bridges (Figs. 6g–l and Videos S6–7). These transfer events were bidirectional and occurred dynamically over the course of minutes. Across repeated experiments, we observed a spectrum of behaviors, from frequent vesicle passage between connected cells to rarer events involving the formation of new intercellular bridges. Together, these observations suggest that TM184C not only contributes to the formation of intercellular contacts but also mediates the exchange of vesicular cargo between cells.

To test the generality of this phenomenon, we performed co-culture experiments across multiple cellular backgrounds, including non-cancerous (HEK293A, HFF-1 fibroblasts, and astrocytes) and mixed non-cancerous and cancerous (astrocytes with patient-derived glioblastoma, GBM) lines, as well as cancerous (SK-MEL-28 melanoma) lines. Stable lines expressing TM184C-eGFP or TM184C-mCherry were maintained separately before being mixed and incubated for 24 or 48 hours. As shown in Fig. 7a and Videos S9–12, TM184C vesicle transfer was robust across all cell types tested, increased with incubation time, and produced transferred vesicles that were either mixed in composition or retained their original fluorescent identity.

Across these diverse systems, several consistent features emerged. TM184C vesicles were concentrated in the perinuclear region and cytoplasmic periphery, actively trafficked along microtubules, and accumulated in cellular projections. Each of these properties was evident in multiple cell types, including striking examples in astrocytes (Video S13) and SK-MEL-28 (Fig. 7a). Importantly, vesicle exchange between adjacent cells was observed in every context examined, confirming that TM184C-mediated transfer is a general and conserved phenomenon.”

Furthermore, we proved transfer was specific to TM184C. Quoting from the revised text:

“Having established that TM184C vesicles can transfer between cells, we next asked whether this property was specific to TM184C or shared with its family members. To address this, we compared the subcellular localization and dynamics of TM184C with those of TM184A and TM184B. As shown in Fig. 7c and Video S17, stable HEK293A cell lines expressing TM184A-eGFP or TM184B-eGFP localized primarily to the plasma membrane and intracellular vesicles, most likely recycling endosomes for TM184A and early endosomes for TM184B, based on our bystander BRET results (Figs. 2d and S4c). In contrast, TM184C was absent from the plasma membrane and localized exclusively to intracellular vesicles.
Given these differences in localization, we next tested whether vesicle transfer occurs with other TM184 family members or is restricted to TM184C. To do this, we performed co-culture experiments using stable HEK293A lines expressing TM184A-eGFP or TM184B-eGFP. We then mixed each of these cell lines in co-culture with TM184C-mCherry. As shown in Figs. 7d-e, neither TM184A-eGFP nor TM184B-eGFP vesicles transferred to TM184C-mCherry cells. By contrast, TM184C-mCherry vesicles robustly transferred to both TM184A-eGFP and TM184B-eGFP expressing cells (Fig. 7d-e, Videos S18–19). These results demonstrate that robust intercellular transfer is a unique property of TM184C vesicles.”

[redacted]. Mutation of this region disrupts both cellular morphology and intercellular transfer, defects that are restored by TM184C–mCherry in neighboring cells within co-culture. Quoting from the revised text:

“In the motif approach, we mutated the phosphosites in the three arrestin codes to generate a HEK293A cell line stably expressing TM184C-ACM-eGFP. As shown in Fig. 7h and Video S22, TM184C-ACM-eGFP cells appeared rounder than wild-type HEK293A cells or those stably expressing TM184C-mCherry. The defective cells contained larger vesicles that frequently clustered into dense aggregates and often developed multi-membraned “mega-vesicles” up to 10 μm in diameter, typically enclosing a single large acidic compartment detectable with LysoTracker (Video S22). These structures exhibited multiple concentric membrane shells, like nested “vesicles-within-vesicles,” suggestive of progressive engulfment or fusion events.

In addition to their morphological defects, the ACM variant was properly targeted to vesicles yet failed to undergo intercellular transfer. In co-culture with HEK293A cells stably expressing TM184C-mCherry, ACM vesicles were not detected in TM184C-mCherry cells (Fig. 7i, Video S23). This pattern indicates that the C-terminal tail encodes essential information for vesicle transfer and that its disruption exerts a dominant-negative effect, blocking TM184C-ACM-eGFP transfer even in the presence of endogenous TM184C. In line with a causal role for TM184C in maintaining vesicular organization, co-culture of TM184C-ACM-eGFP cells with HEK293A cells stably expressing TM184C-mCherry led to a striking rescue of these defects (Fig. 7i, Video S23). Vesicles from TM184C–mCherry cells transferred into neighboring TM184C-ACM-eGFP-expressing cells, restoring normal morphology and resolving the oversized, multi-membraned aggregates. This rescue resembles the metabolic recovery mediated by tunneling nanotubes, in which healthy cells deliver mitochondria, lysosomes, or other second messengers to stressed counterparts [47,48], and supports the view that TM184C acts as a central regulator of this intercellular vesicle exchange process.

Together, these results reveal that TM184C, unlike its family members TM184A and TM184B, is uniquely dedicated to intercellular vesicle transfer. [redacted] isruption of these motifs abolishes vesicle exchange and induces striking morphological

defects, while transfer from neighboring TM184C-expressing cells can restore normal vesicle morphology—mirroring metabolic rescue through tunneling nanotube–like conduits. Thus, TM184C defines a regulated system of intercellular vesicle communication in which GPCR-like and metabolic signaling converge to coordinate the exchange of material between connected cells."

What is the mechanism and function of this transfer? Is this dependent on mTORC2, arrestin binding or phosphorylation status? This data seems nonsensical, as presented.

Great question. We can understand why the data seem nonsensical and surprising at first, but they are highly reproducible in various cell types. [redacted] We also know that TM184C-positive vesicles travel along microtubules, and, in response to Referee 1, we have now shown that TM184C strongly co-localizes with the kinesin motor KIF5B (Fig. S11), which is known to mediate organelle transport along microtubules.

As described earlier, this mechanism depends on mTORC2 activity and TM184C phosphorylation. Notably, the C-terminal truncation variant (TM184CΔ82) mislocalizes to the plasma membrane. Because this truncation removes the C-terminal arrestin motifs, we speculate that arrestin serves to maintain TM184C's internalization and proper trafficking. The relevant revised text is quoted above in the previous response.

5. Very little is known about TM184 proteins but they have been proposed to interact with Atg8, and loss of Hfl1 (TM184 homolog in yeast) leads to abnormal vacuole formation (PMID: 30451685). Is there any endo-lysosome defects observed by the loss of TM184C in mammalian cells (number, localization, pH, etc.)? Additionally, the same paper speculates TM184 to be a potential transporter. What is the evidence here that TM184 is not a transporter but close to a GPCR?

Great questions. Yes—TM184C is classified as both *Tdark* and "superdark" with respect to known GPCRs. In the revised manuscript, we now address the relationship between TM184C and Atg8 (the yeast homolog of LC3B) in detail and include multiple new experiments [redacted]
Additional details are provided in the final sections of the manuscript.

Secondly, CRISPR knockdown of TM184C produces dramatic morphological and endolysosomal defects. We have substantially expanded the corresponding data and clarified these findings in the revised text (see Fig. S12a-b and Video S6). As summarized in the manuscript:

"Upon closer examination, cells with CRISPR knockdown of TM184C exhibited severe morphological and lysosomal defects (Fig. S12a). They were rounded, displayed highly abnormal vesicular organization, and stained irregularly with LysoTracker, indicating that some acidic vesicles, likely lysosomes, were retained but abnormally distributed and enlarged. These cells were fragile, poorly adherent, difficult to maintain, and hypersensitive to imaging, often blebbing and dying almost immediately upon laser exposure. Collectively, these observations indicate that loss of TM184C profoundly disrupts cellular integrity and vesicle homeostasis, producing a sickly phenotype consistent with impaired autophagic and endolysosomal function.

Cells with CRISPR knockdown of TM184C also displayed few or no projections, whereas mild overexpression of TM184C–eGFP in stable HEK293A lines modestly increased both their number and length (Fig. S12b), suggesting that TM184C contributes to the formation and maintenance of these structures. To investigate this further, we analyzed TM184C localization and dynamics in live cells. Revisiting the data in Fig. 6c and Videos S4–5, we found that microtubules channel TM184C-positive vesicles within these projections and concentrate them at their termini. As shown in Fig. 6d–e, these projections are long and thin, typically reaching 20–50 μm, with occasional examples extending beyond 150 μm (Fig. S12c). These findings indicate that TM184C spatiotemporal function is tightly linked to the formation of cellular projections."

Lastly, regarding whether TM184C is a transporter or a GPCR-like protein, the data in Fig. 3 demonstrate clear GPCR-like behavior. TM184C performs two of the three canonical GPCR functions—it recruits β-arrestin and is phosphorylated by GRKs. Many recognized GPCRs share this profile; for example, atypical chemokine receptors signal exclusively through arrestins and GRKs without coupling to G proteins. Thus, G-protein coupling is not a defining criterion for GPCR classification, and the evidence strongly supports TM184C as a GPCR-like regulatory protein rather than a transporter.

6. Fig7b: increase in LC3b lipidation is interpreted differently depending on the results of bafilomycin A, chloroquine or ConcA treatment, which was not done in this study.

This was valuable feedback. We have now performed these experiments, and the results are presented in Fig. 8. In summary, the data indicate that TM184C functions to constrain autophagy, consistent with its role in negative regulation of autophagic flux. Please see the last section of the paper: TM184C constrains autophagy**.**

The role of TM184C in autophagy is suggested but not clearly linked to mechanistic consequences and possible connection to projection phenotype.

We have shown that TM184C acts as a negative regulator of autophagy, but we have not yet established a mechanistic link between this function and the projection phenotype or intercellular connections. We speculate that TM184C mediates a transcellular delivery system for vesicular or macromolecular cargo that is prevented from recycling during transfer. We are currently seeking renewal of our extramural funding to pursue this hypothesis in detail through targeted mechanistic studies.

7. Arrestin recruitment is observed, but this can occur in non-GPCRs and does not, by itself, confer GPCR identity. What is the functional consequence of arrestin recruitment? Does it lead to downstream signaling, trafficking or degradation? The structural similarity to GPCRs, while interesting, is insufficient to justify functional homology without canonical signaling. TM184C so far seems close to "7TM arrestin-interacting protein" rather than "GPCR-like"—unless a functional GPCR-like mechanism (e.g., signaling through canonical effectors) is clearly demonstrated.

Great questions. Based on the C-terminal truncation results discussed earlier, we speculate that the arrestin motifs, GRK-dependent phosphorylation, and arrestin binding collectively ensure the vesicular localization and trafficking of TM184C.

As noted above, TM184C exhibits two of the three defining hallmarks of GPCR behavior—GRK-mediated phosphorylation and arrestin binding—similar to atypical chemokine receptors and other noncanonical

GPCRs. To reiterate, within the field, GPCR classification is not defined by the presence of G-protein coupling, but rather by these broader mechanistic and signaling features.

8. Fig5. Is the role of TM184C in cancer dependent on mTORC2 or cellular projection? Rescue experiments with TM184C wild-type vs mutants are needed. Similar reconstitution experiments would help understand the role of TM184C in mTORC2 regulation, projection formation and vesicle transfer.

Great questions. To answer the first part, yes it appears that TM184C and projections have a role in some cancers. For example, this is why we included co-cultures of patient-derived glioblastoma cells and astrocytes to show that astrocytes transfer large numbers of vesicles to GBM cells. We chose this cancer cell model because TM184C overexpression occurs in GBM and is a poor prognosticator, causing the GBM cells to form many connections. This is currently a very active area of research in our lab now.

To answer the second question, we performed rescue by co-culture. Briefly, we rescued the defective phenotypes of our HEK293A cell lines stably expressing TM184C-ACM-eGFP by mixing them with HEK293A cell lines stably expressing TM184C-mCherry. Quoting from the revised text:

“In the motif approach, we mutated the phosphosites in the three arrestin codes to generate a HEK293A cell line stably expressing TM184C-ACM-eGFP. As shown in Fig. 7h and Video S22, TM184C-ACM-eGFP cells appeared rounder than wild-type HEK293A cells or those stably expressing TM184C-mCherry. The defective cells contained larger vesicles that frequently clustered into dense aggregates and often developed multi-membraned “mega-vesicles” up to 10 μm in diameter, typically enclosing a single large acidic compartment detectable with LysoTracker (Video S22). These structures exhibited multiple concentric membrane shells, like nested “vesicles-within-vesicles,” suggestive of progressive engulfment or fusion events.

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Together, these results reveal that TM184C, unlike its family members TM184A and TM184B, is uniquely dedicated to intercellular vesicle transfer. [redacted] isruption of these motifs abolishes vesicle exchange and induces striking morphological defects, while transfer from neighboring TM184C-expressing cells can restore normal vesicle morphology—mirroring metabolic rescue through tunneling nanotube–like conduits. Thus, TM184C defines a regulated system of intercellular vesicle communication in which GPCR-like and metabolic signaling converge to coordinate the exchange of material between connected cells.”

9. Yeast rescue assay in Fig 7e: This result is interesting but not well integrated. The relevance of TM184C's rescue of vacuolar phenotypes should be explicitly linked to the human cell/cancer functional context.

This is an excellent point and truly helped us strengthen the conclusion of the paper. By expanding our conA, BAF, and LC3B experiments in response to the reviewer's feedback, we were able to more clearly connect the effects of TM184C regulation and loss to the yeast phenotype and its deep evolutionary conservation. We sincerely thank the reviewers for encouraging us to pursue this direction. Quoting from the revised text:

"Having established that TM184C regulates autophagy, we next asked whether this regulatory role is evolutionarily conserved. Our sequence analyses traced the TM184 family back to archaeal origins (Fig. 2g), raising the possibility that its function predates the emergence of multicellular life. To test this, we examined the yeast homolog Hfl1, previously implicated in vacuolar organization and autophagy. As shown in Fig. 8e and S18, deleting HFL1 in *S. cerevisiae* reproduced the characteristic multivesicular vacuole phenotype indicative of disrupted autophagic body homeostasis[51].

Remarkably, CRISPR knock-in of human TM184C fully rescued vacuolar morphology, whereas TM184A and TM184B did not. Because yeast vacuoles are functional analogs of mammalian lysosomes, and autophagic bodies correspond to autolysosomes, this rescue demonstrates that TM184C retains a deeply conserved role in autophagic regulation. Consistent with this, Hfl1 interacts with Atg8, the yeast homolog of LC3B, and the multivesicular phenotype of *hfl1Δ* cells mirrors the conA–induced accumulation of TM184C- and LC3B-positive vesicles in human cells. Together, these findings reveal that the structure and function of TM184C are ancient and conserved, linking archaeal membrane systems to modern eukaryotic pathways governing vesicle trafficking and autophagy.

Minor comments:

Fig4: the band size for TM184C is different in Fig4e vs other figures. Please explain.

The bands were errantly shifted by resizing the blot image. This has been corrected.

Fig2D: Neither the material and method section, nor the figure legend, mention what the markers for each organelle are for the bystander BRET experiment

This has been corrected.

FigS6. Revise the figure legend as panel a and b are supposedly the same staining (magenta and EGFP of TM184C).

This has been corrected.

Manuscript page 6, second paragraph: the figure numbering is incorrect

This had been corrected.

Referee #4 (Remarks to the Author):

Lee et al. leverage protein structure predictions from AlphaFold2 to identify a GPCR-like superdark protein within the AlphaFold database. The authors subsequently assessed homology among the identified candidates and experimentally characterized several to determine their receptor types. Overall, this work is of broad interest, however, there are shortcomings in the computational analysis that would require justification.

We thank the reviewer for their valuable feedback and for performing the Foldseek calculations, which helped clarify the importance of our structure-first approach.

No source code:
"All data beyond what is provided in the supplemental data and computer code is available upon request", it was not possible for me to check the work as whole at this stage. Please provide the source code to reproduce the full analysis. I consider "data available upon request" a dated practice that should be avoided, particularly with services like Zenodo and GitHub being available free of charge.

- We agree and the code is now provided on GitHub via our lab website.

BLAST-based Similarity Networks:
The current ChaSiT analysis appears to be overly permissive due to transitive alignments; nearly all 882,783 proteins become interconnected, raising the concern that transitive searches alone—without explicit structural evidence—might be sufficient to recover these proteins. The authors interpret this extensive connectivity as evidence of homology, however, the chosen threshold (an e-value of 1) seems excessively relaxed. Additionally, since BLAST relies on local alignments, repeated traversals through the similarity network risk artificially linking proteins that are only weakly or potentially unrelated.

For robust homology inference, the authors might consider maximum likelihood approaches or structural alignments(e.g. 3D-Coffee, FoldMason) to build a more reliable multiple sequence alignment. Alternatively, combining sequence identity and coverage thresholds (e.g., at least 90% bidirectional coverage with ≥40% sequence identity at an E-value of 0.0001) may reduce questionable connections but also not perfectly resolve the issue.

- To summarize, Referee 4 makes two suggestions: first, use other approaches and software to build a more reliable sequence alignment; and second, reduce questionable connections using more stringent parameters.
    - Regarding the first item:
        - We appreciate the suggestion to rely more on existing methods and to consider maximum-likelihood (ML) approaches. We maintain our own core code so we fully control assumptions and can expose all intermediates for auditing and reproducibility. Conceptually, our workflow mirrors 3D-Coffee: we extract sequences from trimmed PDB files, compute an all-versus-all BLASTP similarity matrix, build an adjacency network with edge metadata (percent identity, e-value, bitscore), traverse the network with breadth-first search (BFS) to collect sequence neighborhoods, and then perform robust multiple-sequence alignments (MSAs) using the current Clustal Omega engine, which, in our hands, produces stable MSAs suitable for downstream analyses.
        - From our response to Referee 1: Regarding ML phylogeny, we agree it is appropriate when analyzing homologous sequences for a single gene, or highly related genes (e.g., metabolic or ribosomal genes) under a well-specified substitution model. Our dataset aggregates many non-orthologous GPCR-like proteins linked primarily by remote structural similarity, for which concatenation or joint tree inference lacks a coherent evolutionary model and risks severe model

misspecification. Nevertheless, we tested what many consider the best approach (combining MAFFT with IQ-TREE) on candidate subsets and found that branch support (e.g., SH-aLRT/ultrafast bootstrap) was uniformly low and not statistically significant, consistent with these limitations. Given this, we treat the problem as one of similarity/network structure rather than phylogeny. We believe we are entering a new era of structural biology in which large numbers of AF2 models will enable the development of next-generation phylogenetic tools. Our work highlights this need.

- Finally, as detailed in our response to Referee 1, Foldseek did not meet our needs for this application (including sensitivity to query choice and filtering of non-7TMPs). Because FoldMason depends on Foldseek, we did not pursue it.

- Regarding the second item:
  - We agree that sequence-only analyses cannot fully resolve remote homology and must be applied with caution. As detailed in our response to the ChaSiT videos below, we have replaced ChaSiT with a simpler and more transparent approach. In the BLASTP component, we implemented three principled choices: (i) we now use bitscore rather than E-value, since bitscore is independent of database size, and retain edges with bitscore ≥30, a widely used threshold for remote homology (with >60 typically indicating strong homology); (ii) we also require ≥30% sequence identity to reduce spurious links; and (iii) we do not impose an additional coverage filter, as the observed similarity spans the full 7TM fold (new Fig. 1g), ensuring broad structural coverage despite local variability. These thresholds were calibrated against the human GPCRome, where applying the more stringent settings suggested by the referee would eliminate a substantial fraction of bona fide GPCRs. To make this transparent, we now include a human-GPCR analysis processed through our pipeline and provide the corresponding Cytoscape network (Fig. S3d; network file supplied).
  - Finally, we wish to clarify why we examined sequences within our structurally defined set of 7TMPs. While our analysis could have been confined to structure alone and still yielded the same discoveries, the advent of AlphaFold2 raises a complementary question: do diverse 7TMPs that adopt a GPCR-like fold share minimal sequence signals sufficient to specify that fold? Because structure is more conserved than sequence, our goal was not to infer phylogeny but to quantify sequence similarity associated with a common 7TM architecture. The analysis shows a sequence-to-structure correspondence spanning the 7TM scaffold (Fig. 1g), consistent with the idea that AF2 leverages recurring sequence features to recover the 7TM topology, without implying evolutionary relatedness among these remote proteins. We have revised the text to make these points explicit.

Lastly, while the ChaSiT videos are great, Figure 1e feels like a maze. I am not sure how to improve it, but in its current form it is hard to get to the point. One approach might be to group subclusters or highlight key paths (as minimal spanning tree) to help readers quickly identify the main findings to avoid getting lost in the visual complexity.

- We appreciate Referee 4's suggestion to simplify our presentation of the relationship between structural and sequence homology. As noted in our response to Referee 1, we recognize the importance of clarity in this area and have made substantial revisions accordingly. Specifically, we have replaced the prior ChaSiT approach with a simpler and more stringent analysis pipeline, explanation, and interpretation. We also provide the results in an easy-to-use file for the reader to download and visualize using the open-source software Cytoscape. We have also removed the ChaSiT videos, which we agree could be distracting or difficult to interpret. We have replaced them with reduced-network representations built in Cytoscape. For the referee's convenience, we summarize our revised pipeline below:
  - We now collect the protein sequences from the trimmed version of each structural hit to ensure our analysis is limited to 7TM domain.

- We now use MMseqs2 and linclust to cluster the protein sequences. We use a sequential approach in which divide into subsets of archaeal, bacterial, and eukaryotic sequences. We then use linclust and cluster update build a priority ordered list of archaea, bacterial, and eukaryotic representative sequences. This reduces the number of protein sequences from 1,461,479 to 39,808.
- We then perform an all-versus-all BLASTP of the 39,808 sequences and convert the results to a protein similarity network, where nodes represent unique proteins and edges represent the strongest pairwise alignments. The network is constructed using a custom pipeline that aggregates BLASTP hits into unique protein pairs and records their best-scoring metrics, with both single-node and distributed modes available for efficient processing.
- We then applied a go-forward traversal on the BLASTP similarity network, starting from the highest-degree node in the chosen superkingdom and expanding outward along edges that met defined thresholds (≥30% identity and bitscore ≥30) to generate an acyclic tree. We used bitscore rather than e-value because bitscore is independent of database size and provides a clear, standardized measure for detecting remote homology (with values >30 considered significant). The resulting subnetwork was saved as node and edge tables with associated metadata for downstream visualization and analysis.
- We then applied a reduction step to the BLASTP similarity network to generate a Cytoscape-ready subnetwork, retaining only high-confidence edges based on strict identity and bitscore thresholds. The script aggregates redundant alignments, filters nodes and edges, and outputs compressed node and edge tables optimized for downstream visualization and analysis in Cytoscape (for which files are provided).

Foldseek Search and Data Discrepancies: I performed a similar search with Foldseek search using the PDB structure 1F88 (chain A&B) against Foldseek's AlphaFold Database (AFDB) with a total runtime of 82 CPUh. Following the procedure below:

foldseek databases Alphafold/UniProt50 afdb50 tmp
foldseek easy-search 1F88.cif afdb50 1F88_aln tmp --exhaustive-search 1 --alignment-type 1 --cluster-search 1

A total of 1,567,802 hits passed the ≥0.45 TM-score threshold. Of these, 748,264 overlapped with the 882,000 reported in the authors' Excel file, but 89,573 hits meeting the same TM-score threshold were not present in the provided data. Additionally, I do find the receptors mentioned in Figure 1 as well (Q6TCH7, Q15546, A0A6G0V012, Q9BWF8, and Q8TDN7). These discrepancies could be due to different search parameters, but it would be helpful to clarify that Foldseek can find these hits.

- To summarize, the Referee performed a Foldseek search using our query structure and made several points addressed below:
  - The discrepancies between our Foldseek results and the Referee's could be due to different search parameters.
    - Yes, this is true. We did not use TM-align for the alignment type because it was slow. The innovation of Foldseek is the speed afforded by their 3di alignment option, so we used that.
    - On further inspection, we realized our calculation did not consider as many AF2 models as we thought. To deal with this issue and more clearly distinguish our approach from Foldseek, we developed the head-to-head comparison described above in our response to Referee 1.
  - It would be helpful to clarify that Foldseek can find these hits.
    - Yes, we fully expect some overlap, but as discussed in our response to Referee 1, stringent e-value cutoffs (e.g., 1e-5) of the kind typically used by non-experts would miss the superdarks we identified. To recover these, the e-value threshold must be relaxed and then complemented with advanced geometric analysis to separate true signal from background noise. We have clarified these limitations in the revised manuscript

- Lastly, please see our response to Referee 1 regarding why we developed an alternative to Foldseek.

Justification for why only one structure was used:
The manuscript bases its structural comparisons solely on transporter 1F88. Expanding the search to include multiple transporters or functionally related structures would likely yield a more comprehensive result set and provide deeper insights. This seems like a missed opportunity.

- We agree. See response to Referee 1.

Convex Hull Calculation:
The manuscript briefly mentions using convex hulls to compare and visualize structural data, but offers no explanation of how these hulls were computed. Readers need this methodological detail to replicate the analyses accurately.

- To summarize, Referee 4 is requesting clarification on how the convex hull is calculated and access to the code.
  - We apologize for this oversight. Convex hulls have been central to our structural analyses for many years across multiple publications, and their use felt second nature. In the revised manuscript, we now provide a clear explanation of how the calculation is performed and make the code available on GitHub, both as part of our pHinder software and within the code package specific to this manuscript. For completeness, we summarize the key points of the algorithm below:
    - The convex hull algorithm begins by initializing a tetrahedral simplex from the input points and seeding edge and face structures, with a designated start edge ensuring efficient traversal. The hull is then expanded iteratively: for each point outside the current hull, horizon edges (the boundary between visible and non-visible faces) are identified and new triangular faces are attached to form a "horizon cone," updating the hull topology. Horizon edges are traversed in a consistent counterclockwise order to preserve correct face orientation and minimize search steps, while obsolete edges are disconnected rather than deleted to allow efficient reuse. Points that are nearly collinear or coplanar are slightly perturbed ("jostled") and retried to maintain numerical stability, and the correctness of the final structure is verified using Euler's rule for convex polyhedra.
  - We also updated the algorithm to allow the option of trimming structures using either a molecular surface or convex hull. For the revised manuscript, and for the revised calculations, we used a surface. This code is also available on GitHub and the key points of the algorithm are provided below:
    - We first map each 3D point to 4D, compute the convex hull in 4D, extract the lower hull, and project its facets back into 3D to obtain a robust triangulation that regularizes coplanar and near-degenerate cases. The hull is initialized with a simplex and expanded iteratively by identifying horizon ridges and adding new tetrahedra, with orientation checks ensuring consistent topology; vertices in near-degenerate positions are lightly perturbed to maintain general position. From this global 4D triangulation, the exposed 3-simplices are identified as surface facets, oriented so that points below the facet plane are classified as being inside, and those above are classified as being outside. The pHinderSurface module then determines atom inclusion by gathering nearby facet centroids, computing signed distances relative to the oriented surfaces, and averaging them; comparison to core and margin cutoffs labels atoms as inside (core) or outside, thereby enabling principled trimming of buried versus exposed atoms. Iterative error checks and refinements ensure that the final surface is topologically consistent and suitable for downstream analyses.

Minor:

What TM-score normalization (query or target length) was used to identify the hits?

- To summarize, a TM-align score for a query and target is reported in two ways. Referee 4 is asking which TM-score we used.
    - TM-align reports two TM-scores (0–1) for each pairwise comparison because the normalization is performed in two ways: TM1 is normalized by the length of PDB chain 1 (L1), and TM2 is normalized by the length of PDB chain 2 (L2). As a rule of thumb, scores around 0.2 suggest random similarity, while values ≥0.5 typically indicate the same overall fold. Because normalization by the shorter chain length tends to yield a higher value, and TM-scores are less sensitive to sequence length than RMSD but still influenced by normalization choice, we report the larger of the two TM-scores as the final result. This changed from our original algorithm in the first submission, where we used only the query TM score to identify hits. We also increased the minimum TM-score threshold from 0.45 to 0.5, resulting in an increase in the number of unfiltered hits from ~900,000 to ~1.5 million in the revision.

Please state how much CPU time was spent on the TMalign step.

- To summarize, Referee 4 wants clarification on the computational cost, specifically the CPU time required on the cluster to run TM-align and the total aggregate time needed to complete the entire calculation.
    - The calculation of 214M pairwise structural comparisons is chunked into 1000 job batches submitted in parallel to compute cores on the cluster.
    - Each job runs on one core and takes about ~34 hours
    - This brings the aggregate CPU time to ~34,000 hours
    - Below is an excerpt from the log file of the first job submission
        - Successfully completed.
        - Resource usage summary:
        - CPU time : 121018.59 sec.
        - Max Memory : 76 MB
        - Average Memory : 64.14 MB
        - Total Requested Memory : -
        - Delta Memory : -
        - Max Swap : 2 MB
        - Max Processes : 4
        - Max Threads : 5
        - Run time : 125429 sec.
        - Turnaround time : 125430 sec.

Referee #4 (Remarks on code availability):
The code is not provided.

- The code is now provided on GitHub via our lab website.

We thank the editor and all four reviewers for their continued engagement with this work. Following the second round of reviews, a productive dialogue led to the following consolidated editorial guidance:

"The Reviewers feel strongly that the mTOR section of the paper consists of weak, inconclusive data, but note that they agree these are unrelated to the other, more interesting point of the intercellular connections you elucidate.
They would therefore expect a bit more rigour in the characterization of the intercellular communication phenotypes of Fig. 6–7. They note that the images are not quantified, and the role of canonical GPCR effectors, such as trimeric G-proteins, arrestins and GRK, which you show interact with TM184C (Fig 3), is not dissected. In particular, as you have TM184C-KO lines and see a membrane projection defect in these cells, they suggest performing rescue experiments with WT TM184C vs. mutant versions of the protein that do not interact with arrestin, and/or lack GRK phosphorylation sites, etc.

Editorially, I would propose you remove the mTOR data from the main text but could consider keeping these as Extended Data/supplemental figures for now. I certainly would tone down completely the significance of these data, as you suggest, but I do not see it as a problem to suggest this link was observed and the function/significance remains to be elucidated."

In response to the editorial and reviewer guidance, we have made the following revisions. [redacted]

We also strengthened the rigor of the intercellular communication analyses by performing additional experiments and developing two computer vision modules, one for quantifying bridges, projections, and protrusions in a bpp score and another for automated detection of intercellular vesicle mixing by triangulation. In addition, we performed the requested rescue experiments, which show that the TM184C C terminus and its phosphorylation are required for proper cellular and vesicular morphology as well as intercellular communication, thereby linking TM184C interactions with β-arrestin and GRK to the observed phenotypes.

[redacted]

Overall, we believe that these revisions address the reviewers' concerns and substantially strengthen the manuscript.

Referees' comments:

Referee #1 (Remarks to the Author):

The revised manuscript by Lee et al., “Ancient Superdark GPCR-Like Transmembrane Protein 184C Regulates mTOR Signaling and Integrates Endolysosomal and Autophagic Networks,” is significantly improved compared to the original submission.

Specifically, the removal of the ChaSit method and simplification of the sequence clustering pipeline now make the content much easier to follow for a reader. Additionally, many of the claims made were appropriately toned down by the authors, which is appreciated. The expanded experimental cell-based validation offers in-depth functional characterization of newly discovered and intriguing functions and interactions of the GPCRs.

Given these revisions, we would support this article for publication in Nature after minor revisions:

A. The authors state that they tested IQ-TREE on candidate subsets and found that branch support was uniformly low and not statistically significant. This attempt is appreciated, but it is not discussed in the paper. It would be interesting to briefly mention it in the text and/or refer to supplementary material for a broader discussion of the point.

We thank the reviewer for this helpful suggestion. We agree that the IQ-TREE result is worth mentioning briefly, and we have now revised the manuscript to include it in the main text. Specifically, we note that maximum-likelihood inference on candidate subsets yielded uniformly low branch support, consistent with the remote nature of these relationships and supporting our decision to frame the analysis as one of sequence–structure correspondence rather than classical phylogeny.

B. When testing with four different queries beyond rhodopsin, the authors note a 95% overlap. This is fine and indicates robustness, but it is important to check and discuss whether the rare “superdark” hits that are found are within the 95% overlap or might be in the 5% of the non-overlapping set. As the work focuses on the rare discovery of superdark hits, it is important to check where they fall. The current analysis shows that TM-align is overall robust; however, it does not explicitly demonstrate this for the discovery of rare “superdark” hits.

We have verified and now explicitly state in the text that all superdark hits, including TM184C, PRRT3, and PRRT4, fall within the 95% overlapping set across all four query structures. The 5% non-overlapping hits consist primarily of borderline-scoring proteins that do not pass our geometric 7TM filters. To address this issue, we have revised the beginning of this section to read, “Over 800 human GPCRs regulate key physiological processes and are primary drug targets, yet over 100 remain classified as Tdark[2]. Beyond Tdark, our search identified eight superdark GPCR-like candidates independent of GPCR query seed (Fig. S2b).”

C. The authors’ arguments regarding the comparison of structure-based discovery methods remain unconvincing. They primarily use a single-hit discovery (TM184C) to conclude on the relative performance of TM-align + filtering versus Foldseek. They do not clearly show whether other novel hits would be discovered by Foldseek but not by their approach. This is suitable for a discovery paper, where the goal is to characterize a newly identified and interesting target; however, removing or significantly toning down the performance assessment of the structure-based discovery methods is strongly recommended. We make this comment in the best interest of the authors.

We thank the reviewer for this thoughtful critique and agree that the comparison should be interpreted as a case study rather than a general benchmark of structure-based discovery methods. In revising this section, we sought to balance the perspectives of both reviewers by retaining the comparison requested by reviewer 4 while substantially streamlining the text to better reflect its scope and limitations.

Our intent was not to claim that one method universally outperforms the other, but rather to highlight the practical trade-offs between rapid and exhaustive searches. In this regard, we feel the comparison remains informative. Foldseek is fast and highly useful for exploratory searches, but its results are more sensitive to the input structure and threshold choice, whereas the more exhaustive TM-align-based approach is

computationally intensive but better suited to maximizing recovery of distant or borderline candidates. We therefore believe the two methods are complementary and context-dependent.

We have toned down the language throughout the section to avoid overstating conclusions from the single-hit discovery example. This comparison highlights a rarely available benchmark, as we are not aware of prior work that has leveraged an exhaustive search across hundreds of millions of structures to evaluate a faster method in this way. Foldseek remains an excellent first-pass tool for identifying candidate structural homologs, whereas a more exhaustive analysis is prudent when deciding whether a target merits years of experimental investment.

We hope this revision addresses the reviewer's concern while preserving the broader message that fast and exhaustive approaches serve different, complementary roles in structural homology discovery.

D. We appreciate the authors' clarification regarding the expression levels of PRRT3 and PRRT4 and the impact of different exposures, which helps to avoid potential misconceptions.

Thank you for helping us clarify this point.

E. We thank the authors for their additional analyses, which confirmed the presence of the LC3B-binding motif and further supported the existence of a unique fold due to the absence of activation of GPCR microswitches.

Thank you for this suggestion and for highlighting these points.

F. We appreciate the inclusion of the colocalization images. Figure S14 suggests colocalization in certain regions; however, line scans through these areas would provide stronger evidence for true colocalization, primarily since the overlay of currently used colors can't offer proper interpretation. Please use those and the best colocalization color combinations (e.g., magenta and green or turquoise as used in some other figures). We kindly request that the authors make this straightforward modification throughout the manuscript, using the images already presented.

We thank the reviewer for these helpful suggestions. We have recolored the relevant images using magenta–green color palettes, which are now shown in Supplemental Figs. 19 and 20. Regarding the request for line scans, all microscopy experiments were acquired using line-sequential rather than frame-sequential scanning, with the Leica LAS X Dye Assistant used to optimize spectral yield and minimize channel overlap. Line-sequential acquisition on the Leica Stellaris 5 confocal system is the recommended strategy for colocalization studies because it eliminates spectral crosstalk, preserves near-perfect spatial channel registration, and remains fast enough to limit photobleaching and capture live-cell dynamics. In addition, we have now quantified mTOR and AKT colocalization data using our Vesicle Co-localization software, as done for the colocalization analysis in Fig. S8, and report these results in Extended Data Fig. 2.

G. We thank the authors for their comprehensive work in clarifying the details of mTOR-TM184C interactions through new cell-based assays and imaging, which have greatly enhanced our understanding of the specific functions of these interactions.

Thank you for your thoughtful feedback and helping us improve the manuscript.

H. The current discussion provides an excellent summary of this comprehensive paper, though condensing it further could improve clarity. While the discussion highlights many future opportunities, it does not yet address the study's limitations, which would help provide a balanced view of this work.

Thank you for this helpful suggestion. We have condensed the Discussion to improve clarity and integrated some limitations together with directions for future work.

Other comments:

K. Similar to our suggestion in comment F from the major comments, we recommend including line scans to better demonstrate colocalization, as well as exchanging colors to more clearly illustrate this colocalization.

Please see our response to F.

M. “Supplement Figure 4” still references and displays β2AR. For readers who may not be familiar with GPCRs, we recommend adding a note to clarify which data pertain to the gene and which to the protein.

Thank you for bringing this to our attention again. We have fixed the issue.

Additional minor comments:

1. The authors use “Supplement Figure” or “Supplemental Figure” throughout the manuscript; please revise this to ensure consistency.

Thanks for pointing this out. We have fixed the issue.

2. Since G-protein coupling has not been demonstrated, the authors may consider referring to TM184C as an Arrestin-coupled receptor (ACR-like) rather than GPCR-like.

We appreciate this suggestion. We have added the following summary paragraph to the end of the sections on canonical GPCR effectors: “In summary, TM184C is GPCR-like because it shows two of the three canonical GPCR behaviors, recruiting β-arrestins and GRKs, and displaying GRK-dependent phosphorylation of its C-terminal arrestin codes. These properties resemble those of atypical chemokine receptors that signal primarily through arrestins rather than G proteins. Accordingly, it may be most appropriate to refer to TM184C and related proteins as arrestin-coupled receptors (ACRs) rather than classical GPCRs.”

Referee #4 (Remarks to the Author):

I greatly appreciate the authors' efforts in addressing many of the previously raised points. However, two major concerns remain. In particular, although the network is now better described and justified, it is still not entirely convincing.

Major:

- Figure 1e provides helpful visual context for the underlying relationships. However, I remain unconvinced that it adequately addresses the stated aim: “Rather, our aim was to address a complementary question prompted by the advent of AlphaFold2: do diverse 7TMPs that adopt a GPCR-like fold share minimal sequence signals sufficient to specify that fold?” Many of the observed edges have bit scores around 30, which fall within the range of random similarity and therefore lack statistical support. Because BLASTP uses local alignments, these low-scoring matches often correspond to short fragments that do not overlap across sequences and are unrelated to fold-defining regions. As such, they are more consistent with chance alignments than with any conserved sequence signal specifying the GPCR-like fold. Related to this, how were the threshold of ≥30% identity and a bitscore ≥30 selected and justified? I fully agree with the authors on the importance of developing tools that leverage structural information. Recent work by Grag et al. (MBE, 2025) proposed a substitution matrix for protein structures which can be used in IQ-TREE. Such approaches might substantially improve branch support and better clarify structural relationships among the diverse GPCRs especially if paired with an structural MSAs.

We appreciate the reviewer’s thoughtful comments and agree that low bitscore alignments can, in general, reflect random or fragmented similarity. In our study, however, the BLASTP edges are not arbitrary pairwise matches from a global database, but comparisons restricted to a structurally curated set of 7TM proteins. Within this context, a bitscore ≥ 30 represents the lower bound at which known homologous GPCR subfamilies remain connected in the network. This threshold was empirically calibrated against the human GPCRome (now referenced in the main text and explained in the Fig. S3d legend and Methods), where applying higher stringency would remove many established GPCR–GPCR relationships. Furthermore, as shown in Fig. 1g, these alignments span the length of the 7TM region rather than short local fragments, indicating contiguous similarity across the fold. To ensure robustness, we used bitscore (rather than E-value) because it is independent of database size, and applied a dual filter of bitscore ≥ 30 and sequence identity ≥ 30% to reduce spurious matches. Despite including these points in our previous response, we failed to articulate them in the revised manuscript, and I have now corrected this. Thank you for pointing this out.

We agree with the reviewer that the development of structure-aware substitution matrices, such as those proposed by Grag et al. (MBE 2025), has strong potential to improve the detection of deep evolutionary relationships among structural homologs. We examined this work in detail but found that the available implementation was not yet straightforward to deploy or scale to our large 7TM datasets. We are continuing to follow developments in this area and are exploring ways to integrate similar structure-based scoring schemes in future iterations of our pipeline. We therefore retained an established sequence-based framework that enabled systematic comparisons across all structurally defined families in a consistent manner.

- Foldseek combined with TMalign rapidly recovers most (748,264 out of 882,000 with an additional 89,573) relevant hits and identifies additional significant ones and this is reliant on the fast 3Di prefilter followed by a TMalign. While Folseek-TM is being slower compared to Foldseek 3Di/AA, it only takes 82 CPUh for 7TMPs and therefore is about 400x faster compared to the 34,000 CPUh solution that the authors suggest. I would expect this to be clarified correctly in regard to sustainability and green computing principles.

We thank the reviewer for raising this important point. We agree that the computational cost of a structural search is relevant to both practicality and sustainability, and we have revised the text to clarify the trade-off between the exhaustive TM-align-based approach and faster methods such as Foldseek. Our intent was not to argue that an exhaustive search is generally preferable, but to show that, in this case study, the exhaustive search provided a uniquely sensitive benchmark for recovering superdark 7TMPs and for evaluating the behavior of faster methods. We now more clearly state that Foldseek and related approaches are far more

efficient in CPU time and are excellent first-pass tools for exploratory discovery, whereas exhaustive searches are best reserved for questions where maximizing recovery and prioritizing experimental investment justify the added computational cost.

Minor:

- The provided code in the github is in its current form hard to execute since it misses documentation and is hard to install. Also, I would suggest having a standalone repository for the code linked in the manuscript instead of combining it with the group's website source code. I would also strongly recommend making the project easily installable through at least one of PyPi, bioconda, docker, etc.

We appreciate the reviewer's suggestion. The structural informatics code is not intended to be distributed as a standalone application to be installed; rather, it is provided as commented Python scripts in our GitHub repository. All the code is commented and available there, along with documentation.

More broadly, the other software we developed for this study, including Vesicle Co-localization, Bridge & Projection & Protrusion Quantifier, and Vesicle Triangulator, are readily usable Python modules with graphical user interfaces, commenting, and documentation. We are also actively moving toward broader distribution through channels such as Bioconda, and we appreciate the reviewer's suggestion in that regard.

Referee #2 + 3 (Remarks to the Author):

In the revised manuscript, the authors present additional data supporting the role of TM184C as a new GPCR-like protein involved in endo-lysosomal integrity and intercellular vesicle trafficking. While these new results are interesting, the fundamental problems raised in the first review remain unresolved.

[redacted]

Although the mTOR portion of the study has been moved to the Extended Data, we addressed the critiques from Reviewers 2 and 3 and performed the necessary additional experiments.

1. the claim that TM184C is a GPCR-like protein that functionally regulates mTORC2 (rather than mTORC1) is not adequately supported by the data. Specifically, the unquantified co-localization analysis in Suppl. Fig. 14 using a non-validated Rictor antibody is not acceptable evidence, by itself, of TM184C spatial association with mTORC2.

We appreciate this feedback. [redacted] **These data are presented in Extended Data Fig. 2 and show robust co-localization of all three markers with TM184C-eGFP.**

2. Related to this, the reverse immunoprecipitation experiments pulling down Raptor or Rictor followed by immunoblotting for TM184C (Fig. S7) are problematic and inconsistent with the data presented in the main figure (Fig. 4a). Figure S7 suggests that both Raptor and Rictor interact with TM184C, whereas Figure 4a is used to argue that TM184C binds specifically to mTORC2. These results are contradictory and undermine the authors' conclusion. Moreover, appropriate negative controls—such as pulldowns with an unrelated or random protein—are missing, making the results impossible to interpret with confidence. Without such controls, the apparent interactions are just as likely to represent background noise or artifacts from protein overexpression.

We have addressed this concern in three ways. [redacted] **Second, we repeated the pull-down experiments and quantified them by densitometry, which**

[redacted] **Third, where needed, we repeated the blots with additional negative controls.**

[redacted]

Regarding negative controls, we strengthened them by redesigning the forward pull-down to include FLAG-LAMP1 run in parallel with FLAG-TM184C on the same blot (Extended Data Fig. 1). [redacted] **This provides a biologically informative negative control consistent with approaches used by the Sabatini (PMID 30181260) and Zoncu (PMID 36007018) groups.**

3. Similarly, the data in Figure 4e, showing multiple GPCRs binding to mTOR (again, without negative controls), reinforce this reviewer's concern that the reported TM184C–mTORC2 interaction is non-specific and may not reflect a genuine biological association.

We understand this concern and have addressed it in the revised manuscript. [redacted] **. We therefore present these results in Extended Data Fig. 1 as evidence of association and relative preference rather than as proof of a specific direct interaction.**

4. Furthermore, the IP–MS data provided by the authors raise additional questions. Although mTOR itself appears as a potential binding partner of TM184C, no other core components of either mTORC1 or mTORC2 complexes (e.g., Raptor, Rictor, mSIN1, mLST8) are detected. This lack of supporting interactors makes it difficult to interpret the mTOR association as functionally meaningful thus the authors' decision to pursue mTOR signaling on the basis of such limited evidence is questionable. The authors also did not provide any in vitro evidence demonstrating a direct interaction between TM184C and either of the mTOR complexes, neither do they map domains or loops within TM184C that could mediate this binding, which is a critical shortcoming given the magnitude and unconventional nature of their claims.

[redacted]

[redacted]

[redacted]

5. The claim that TM184C directly regulates mTORC2 is also still unsupported and very superficial. The partial reduction in pAkt (S473) in Fig. 5 cannot be taken as evidence of mTORC2 regulation. For example, TM184C could affect levels or activity of RTKs (EGFR etc) at the plasma membrane, PI3K or PTEN activation, AKT localization, phosphoinositide levels, and countless other possibilities that are not explored here.

[redacted]

6. And, finally, taking their logic to the extreme: if TM184C regulates mTORC2 at the endolysosomal membrane, but the most affected mTORC2 substrate, AKT S473 is activated at the plasma membrane (and this is not a debatable point, given the mountain of evidence) how does this ill-defined mTORC2 activation by TM184C propagate to plasma membrane-bound AKT? Again, hard to follow the logic here.

We understand this feedback and appreciate it. [redacted]

7. And, finally, despite imaging-based evidence for TM184C promoting cellular 'nanotubes' and vesicle transfer, not even a preliminary mechanistic explanation is provided (i.e. is a G-protein involved?), again making these data purely observational and of questionable impact.

To address this feedback, we conducted the rescue experiments requested by the editor and reviewers and developed new computer vision analyses to increase quantitative rigor. These findings are presented in a new Fig. 5, which shows that the TM184C C-terminus and its phosphorylation motifs are required for proper trafficking of TM184C and for its function in driving the formation of cellular projections and intercellular connections, thereby linking the GPCR-like activities of β-arrestin recruitment and GRK-dependent phosphorylation to the nanotube phenotype. We also developed software to quantify these features (Fig. S12). In addition, new Fig. 6 shows that vesicle transfer between cells depends on the TM184C C-terminus, further strengthening the connection between β-arrestin- and GRK-dependent regulation, vesicular function, and intercellular communication. We also developed software for automated detection of these transfer events (Fig. S13). Finally, Supplementary Data Figs. 14–20 show that intercellular vesicle transfer is robust, reproducible, predictable, occurs across multiple cell types, and is tightly associated with intercellular mitochondrial transfer.

Taken together, the mTOR-related findings in this manuscript are not credible. The data do not support the proposed mechanism, additional data provided in the updated manuscript did not solve the initial concern and the interpretation conflicts with well-established cellular and biochemical evidence. The authors are clearly skilled in bioinformatic, but their treatment of mTOR signaling is inaccurate and misleading.

We hope that the number and scope of the experiments performed to address these concerns will be helpful to the reviewer. [redacted]

The observed cellular phenotype upon TM184C loss are potentially interesting and suggests that TM184C somehow regulates endo-lysosome functions. However, it is far more plausible that the observed reduction in mTORC1/2 signaling is a secondary consequence of lysosomal and autophagy dysfunction rather than a direct regulatory effect. Considering TM184C's apparent role in membrane dynamics and cellular projections, the authors may find greater mechanistic insight by focusing on candidate interactors involved in microtubule organization, kinetochore function, or ciliary/flagellar structures, rather than mTOR signaling.

We thank reviewers 2 + 3 for this thoughtful feedback. It has helped us improve the manuscript in several ways. We certainly agree that the reviewer's explanation is possible and demands further study. We are now beginning the longer-term process of investigating these additional biological leads.