

Structural basis for regulation of CELSR1 by a compact module in its extracellular region

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The manuscript by Bandekar et al., probes the structural features of extracellular region of CELSR using cryo-EM and complements the study with cellular experiments to link these features with functional outcomes. Overall, the study is well-designed and executed, and it provides a significant advance in our understanding of adhesion receptor activation and signaling. While I find the authors' analogy of the overall architecture with ourorobos amusing, I am not sure if this analogy is indeed essential, but I leave it up to their discretion. I wholeheartedly support the publication of this manuscript, and I have only a couple of minor comments that the authors may consider addressing during revision.

1. If the side-chains can be confidently modelled at the presented resolution, it may be a good idea to show the density maps for the interacting residues in main figure (Figure 2).

2. The scale bar is missing in some of the microscopy panels in Figure 5 and Supplementary Figure S5, and negative staining and cryo-Em micrographs (Supplementary Figure S1 and S2). Also, some of the microscopy panels in Figure 5 appear to be merged perhaps during PDF conversion, and it should be fixed. Statistical analysis can also be included in supplementary Figure S5 presenting aggregation index.

Arun K. Shukla, PhD

Reviewer #2

(Remarks to the Author)

In this paper, Bandekar and colleagues present a partial cryo-EM structure of the ectodomain of the adhesion GPCR, CELSR1. CELSR family proteins mediate adhesion via their N-terminal cadherin repeats but the functions of their 14 other extracellular domains are less understood. Moreover, despite decades of work in animal models demonstrating roles for CELSR proteins in tissue morphogenesis, development of the nervous system, ciliogenesis and planar cell polarity (PCP), very little structural information is available for this class of very large adhesion GPCRs. The authors present a 4.3 angstrom structure consisting of fourteen domains of CELSR1s extracellular region (CADH9-GAIN) and show that it forms a compact module that folds in on itself through interactions between the CADH9 and GAIN domains. They further show a functional requirement for CADH1-8 in cell adhesion and for CADH9-GAIN in G-protein signaling in HEK cells. Biophysical assays and modeling of the full ectodomain in the presence of calcium suggest an antiparallel dimer and a flexible hinge region between CAD repeats 5 and 6. Although the cryoEM structure did not resolve the 8 CADH repeats that engage in adhesion, and the purified protein lacks the 7-pass transmembrane and cytosolic regions of the protein, given how little was known prior to this study, the structural data presented here significantly advances our understanding of CELSR1 architecture. It also makes important predictions for how adhesion may regulate its GPCR activity to be tested in future studies.

In general, the data are presented in a compelling manner and the conclusions are well supported by the data. However, some of the reported findings (necessity and sufficiency of CADH1-8 in adhesion, and functions for EGF and LAM repeats) were recently demonstrated in the fly ortholog of CELSR1, Flamingo, which slightly diminishes the novelty of some of the findings. That paper (Strutt et al, 2023 PMID: 37995695) should be cited and discussed as it is probably the most extensive structure-function study on a CELSR family member to date. Moreover, there is some important context missing about the

unknown relevance of G-protein signaling for CELSR1 function that should be acknowledged. Finally, given the detailed characterization of the CADH9-GAIN binding interface, which is the most unexpected and exciting finding of the paper, some functional testing of this binding interface would greatly strengthen the impact of the paper.

Major points

1. A recent study by Strutt et al in *Drosophila* demonstrates that CAD1-8 of Flamingo is both necessary and sufficient for adhesion. That paper should be cited and discussed.
2. The Strutt et al, 2023 paper also shows that Fmi only engages in adhesion/trans-interactions when one of the two cells also expresses Frizzled, and this asymmetry requires the presence of CAD9-GAIN. Other data in that study suggest the EGF and Lam repeats may interact with Fz. They also show deletion of LamG1 or LamG2 impairs transport to the cell surface. Although it is not clear that CELSR1 shares this need for Frizzled in one cell to engage in adhesion (most evidence suggest it does not), this is all to say that the statement on line 101-102 "No function has been demonstrated for this C-terminal EGF/LamG/HormR/GAIN region", is not correct.
3. The interaction of CADH9 with the GAIN domain is both unexpected and exciting as it places the adhesion region of CELSR1 in direct contact with a region known to regulate GPCR activity. However, the large deletions performed in this study do not shed light on the significance of this binding interface. Point mutations that disrupt this interaction should be tested for their effects on adhesion and G-protein signaling.
4. It should be acknowledged that although CELSR1 shows an ability to signal via GalphaS using BRET assays in cultured cells, a functional role for G-protein signaling has never been demonstrated for CELSR1 in vivo or for planar cell polarity, where CELSR1 function is best described.
5. In the BRET2 assays in figure 6, CELSR1 lacking CADH1-8 shows a greater change in BRET than the wild type protein. Can the authors comment on this? Is it possible the deltaCADH1-8 is constitutively active? Perhaps adhesion relieves the interaction between CADH9 and GAIN? Do cells expressing WT CELSR1 treated with EDTA show a greater BRET change, similar to the deltaCADH1-8 version?

Minor points

According to the model of the full length protein in the membrane, the HormR domain is positioned more membrane proximal than the GAIN domain and is in contact with the 7-pass TM domain. Can the authors comment on how this configuration could lead to GPCR activity?

Reviewer #3

(Remarks to the Author)

Sumit J. Bandekar and co-workers presented 4.3 Å cryo-EM reconstruction of the mCELSR1 ECR, and revealed a compact interdomain bundle including 14 domains. The author discovered that the ECR (extracellular region) of mCELSR1 exhibits dimerization, likely facilitated by the cadherin repeats, arranged in an antiparallel manner. Furthermore, the authors utilized immunofluorescence to validate the effects of Δ CADH1-8 and Δ CADH-Gain on the aggregation of mCELSR1. In addition, the authors employed BRET assays to assess the modulatory role of Δ CADH1-8 and Δ CADH-Gain on the activity of the CELSR1-Gs signaling. These studies revealed that CADH1-8 repeat is required for cell-cell adhesion, whereas the C-terminal CAHD9-GAIN compact module regulates transmembrane-domain mediated G protein signaling. Overall, these conclusions may have important impacts in adhesion GPCR field, providing an important example for the study of adhesion GPCR huge extracellular domain structures and functions. However, there are several questions need to be addressed to improve the overall manuscript quality before publications. I would like to recommend for minor revision before publication in Nature communications.

1. The overall cryo-EM density map of mCELSR1 ECR is suboptimal in this study, it is recommended to incorporate the sidechain density when discussing the residue interactions in Figure 2.
2. (1) In Figure 5, the relative expression level of mCELSR1 mutants should be presented. Because receptor expression level affected their biological functional assays. (2) Moreover, it remains to be determined whether the interactions observed in the wild-type (WT) are consistent with those in various mutations. (3) Please specify the experimental conditions involve the presence of calcium ions.
3. In Figure S5, cell-surface expressions were tested using immunofluorescence. However, the expression levels should be further validated through western blot because some mutations may cause truncated expression.
4. In Figure 1, the colors in Figure 1b-f are inconsistent.
5. In Figure 2, the representation of the three key interfaces was not very friendly. It would be better to modify the cartoon transparency and display the sticks of different domains in different colors. Similarly, in Figure S4, it was not a good choice to show the potential map for interdomain interfaces of mCELSR1 ECR by Coot.
6. In this manuscript, it indicated that the CADH9-GAIN module regulated Gs activity through the 7TM. Considering the density of mCELSR1 is suboptimal, I will suggest to perform computational simulation or design alanine screening methods to reveal which residues of CADH9-GAIN domain contribute to modulation of G protein signaling.
7. In line 283, the sentence "Evidence collected and others is consistent with a model of the CADH1-8 mediated antiparallel dimer driving the adhesive activity of CELSRs" should be cited with corresponding reference.
8. It is recommended to list the distances between residues that are considered to form interactions in this study, for

example, the hydrophobic interactions mentioned in line 170 to 179. Also in this paragraph, there are several residues (E2195, E1188, R2252, Y1592) that are mentioned to from interactions are not indicated in the corresponding figure. Is this a simple negligence or is there a reasonable explanation? In addition, when describing interaction interfaces, it would be better to present the sidechains of two sides in two different colors.

9. In the BRET experiments, the Δ CADH1-8 construct seems to result in stronger G-protein dissociation than WT, but the significance of this curve is absent. This could be an intriguing finding and a little more discussion would be better.

10. In Figure 5d, the fluorescence intensity of HA- Δ CADH1-8 (green) is notably stronger compared to the others. Please provide a more precise explanation.

Reviewer #4

(Remarks to the Author)

This manuscript reports results from EM and SAXS combined with MD of CELSR1. The well-written manuscript presents structural biology methods results well-supported by cellular experiments. The demonstration of the 14-domain structure in different conformations is a significant finding. With minor revisions, I believe the article is suitable for publication in Nature Communications.

The addition of a calibration curve for the column used in Supp Fig 1 would be better. Additionally, why was Superose® 6 chosen over a column like S200 or S300? Can you provide information about oligomerization states using S200 or S300 in protein gel filtration?

Could you please also share with and without Ca; Gel filtration profile using S300 ?

In Supp Fig 2, the 2D images could be moved to the beginning of the figure and made larger and more detailed.

Additionally, the local resolution map in Supp Fig 2 could be placed at the end of the figure and presented as a larger image with the domains labeled. This would allow for a better understanding of which domains are more flexible or have lower resolution. Also adding 180 degrees turned version can be helpful.

I couldn't understand why Figure 3 named as SEC-MALS analysis. In figure 3 most of the information coming from SAXS and main analysis tool also looks SAXS.

No table showing SAXS recording and evaluation parameters according to the BioSAXS consortium agreement is appended - the table in the Supplementary has no errors, no measure of concentration, no description of the instrument etc etc.

Additionally, submitting SAXS data to The Small Angle Scattering Biological Data Bank would be helpful for future investigators.

Could you Please also add

ln I (s) vs s plot

Kratky plot

Pair distance distribution graphs.

R_g, D_{max}, V_p, MW (kDa) V_c (kDa) Q_p (kDa) values from SAXS analysis,

Please also share Fit quality Fineness and Normalized Spatial Discrepancy (NSD) values for model fitting to pdb.

Minor

Most of the figures have some gray background borders around

Reviewer #5

(Remarks to the Author)

Despite its significance, molecular-level insight into CELSR function has been limited. The authors utilize cryo-EM to reconstruct the mouse CELSR1 ECR at 4.3 Å resolution, revealing a compact module of 14 domains mediated by interactions between CADH9 and C-terminal GAIN domains. In addition, cell-based assays demonstrate that the N-terminal CADH1-8 repeat is crucial for cell-cell adhesion, while the C-terminal CAHD9-GAIN compact module regulates G protein signaling via the transmembrane domain. The molecular insights are interesting, however, I have major concerns regarding the computational part.

Major:

1. The authors used a deposited structure of the human CELSR1 CADH4-7 and investigated the dynamics of the proposed hinge region within the cadherin repeat region of CELSR. This was done in three different conditions (WT, dCa2+, canonical). The authors observe that the hinge region adopts three different bending angles based on the condition when starting from the same initial structure. According to the simulation table, systems were run for only 100 ns. I find it very surprising to observe such a large structural rearrangement within 100 ns. In this respect, I propose that the authors plot the evolution of the angle for the WT, the dCa2+ and the canonical system across the simulation time and discuss the observations.
2. In addition, it would be very interesting to better understand the molecular mechanism that drives the structural rearrangement to different bending angles.
3. For data reproducibility and transparency, starting models, simulation protocols, and data should be submitted to a public repository (e.g., GPCRmd (www.gpcrmd.org) or similar).

Minor:

Figure 4a) The authors do not indicate where these snapshots were taken from.

Figure 4e) Instead of a table, I propose to plot this data.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed the comments, and I recommend the publication of the revised manuscript.

Reviewer #2

(Remarks to the Author)

The revised manuscript has several improvements including a higher resolution structure and mutational analysis of CADH9-GAIN interface on signaling and adhesion. I have a few small questions/clarifications that the authors should address prior to publication.

1) In the revised version of the manuscript, the description of the residues involved in the LAMG1-EGF6 interaction have changed considerably. Almost completely different amino acids are now listed as contributing the hydrophobic core as compared to the previous version. Can the authors comment on whether the change is due to the increased resolution provided by the new structure analysis, or if there was a mistake in the previous version?

2) The BRET assays for GalphaS coupling have been removed from the current version and replaced with a different assay for GPCR activity. However, this assay is not described in the main text, methods, or figure legends. Can the authors please describe what this assay measures and how. Besides the mix up of constructs in the BRET assays described in the rebuttal letter, can the authors comment on why the new signaling assay is preferred over the BRET assay, which I understand to be standard in the GPCR field?

3) Line 96-97. ' In *C. elegans* Fmi, the EGF/LamG/HormR/GAIN region plays a role in the interaction with Frizzled, an Fmi binding partner involved in PCP(70).' The study being referred to was performed in *Drosophila*, not *C. elegans*.

Reviewer #3

(Remarks to the Author)

The authors have satisfactorily addressed my requests, and I no longer have any further questions.

Jin-Peng Sun

Reviewer #4

(Remarks to the Author)

I sincerely thank the authors for addressing most of the comments in the revised manuscript. However, there are still a few aspects that require further clarification, particularly regarding the response to the query:

"Please also share Fit quality Fineness and Normalized Spatial Discrepancy (NSD) values for model fitting to PDB."

The authors replied: "We did not fit any models to PDB files in this work."

This response is unclear, as in the previous version of the manuscript, there was a SAXS envelope that incorporated the Cryo-EM map of structure 8VY2. Given that the Cryo-EM map corresponds to part of the 8VY2 structure, it raises several questions:

How was the Cryo-EM map of 8VY2 fitted into the SAXS envelope in the earlier version of the figure? What were the Fit quality, Fineness, and Normalized Spatial Discrepancy (NSD) values for this fitting? These quantitative metrics are essential for evaluating the model quality and fitting precision.

The authors appear to have replaced the DAMMIN/DAMMIF model with the DENSS model in the revised version. Could the authors clarify why this change was made? While replacing the model is acceptable, it is critical to explain how the new approach improves or differs from the previous analysis.

Even if the DAMMIF model has been removed, the following question remains: How was the 8VY2 Cryo-EM map fitted into the SAXS envelope originally, and what were the corresponding fit quality parameters (NSD, etc.)?

If the authors have opted to use the DENSS model, the electron density map reconstructed from DENSS analysis typically provides SAXS electron density values. In such cases, the SAXS envelope should include a color bar indicating electron density values, which is absent in the current figure. The resulting electron density map should then be used to fit either the 8VY2 Cryo-EM map or a cartoon representation of the structure. Could the authors clarify:

Why this color bar and the corresponding SAXS DENSS analysis electron density values are not shown?

How the Cryo-EM map was fitted into the DENSS SAXS-derived electron density map?

Finally, it is currently unclear how the 8VY2 Cryo-EM map has been placed inside the SAXS envelope in the revised figure.

A more detailed explanation and clarification of this fitting procedure are necessary.

To resolve these ambiguities, I kindly request the authors to provide the following:

A clear explanation of how the Cryo-EM map of 8VY2 was fitted into the SAXS bead model in the earlier version of the figure.

The Fit quality, Fineness, and NSD values for the model fitting values of the earlier version of the figure.

A rationale for replacing the DAMMIF model with the DENSS model.

A visualization of the DENSS analysis results, specifically including a color bar indicating electron density values derived from SAXS.

A step-by-step explanation of how the Cryo-EM map was fitted into the DENSS-derived SAXS envelope.

Providing this information will ensure that the structural fitting procedures are transparent, reproducible, and fully understandable to the readers.

Another concern about Dimerization Interpretation in Figures 3d and 3e:

The models shown in Figures 3d and 3e suggest that the CADH1-8 region dimerizes in a U-shaped or V-shaped manner, rather than in a linear, elongated form. This interpretation raises important questions:

If the CADH1-8 dimerization were truly linear, one would expect a more defined and elongated electron density cloud from the SAXS analysis. However, the current SAXS envelope appears to support a curved dimerization model. Could the authors provide further evidence or clarification to support their structural interpretation?

and Figure 3f and Figure 7 suggests a particular dimerization model. Could the authors provide additional evidence or alternative model-based analyses (as a dimer form of CADH1-8) (e.g., molecular dynamics simulations or comparative structural data) to further support this proposed model (whether it is linear instead of having a curve or a U- or V-shape)?

Best
Çağdaş

Reviewer #5

(Remarks to the Author)

The authors have satisfactorily addressed all my questions and concerns.

Version 2:

Reviewer comments:

Reviewer #4

(Remarks to the Author)

Demet Hocam et al. authors have satisfactorily addressed my requests, and I no longer have any further questions.

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Manuscript NCOMMS-24-10882: Response to Reviewer Comments

We thank the reviewers for their time and effort in reviewing our manuscript. We are pleased to hear their very positive comments and recommendation for publication after revision. In response to the reviewer comments, we present our revised manuscript which has taken all comments into account. We believe this revised manuscript is an improved version.

In summary: We improved the resolution of our cryo-EM reconstruction to 3.8 Å. As suggested by a reviewer, we performed site-directed mutagenesis and identified residues in the CADH9/GAIN interface, which, when disrupted, led to increased cellular adhesion. This important result led us to a refined, more comprehensive model where we suggest that the compact CADH9-GAIN module may regulate CELSR1-mediated cell adhesion through conformational change. Either point mutations or the binding of physiological ligands may induce a conformational change in the CADH9-GAIN module and lead to a partial extension or full opening of the compact structure. We now present this new data and refined model in the new figures (Figures 6 and 7). During the revisions, we realized that there was a mix-up of the constructs used in the previous BRET assay, so we have corrected the figures and our interpretations, accordingly. We have removed the BRET assay results and we now include results of a downstream signaling assay. Finally, we re-analyzed the small-angle X-ray scattering data as recommended and we now provide a better SAXS model.

Please use [this link to a Box folder](#) containing the PDB and Map files for the cryo-EM dataset.

Below we respond in detail to the reviewer's comments. The *reviewer's comments* are italicized, and we provide [responses to the reviewers in blue text](#).

Reviewer #1 (Remarks to the Author):

The manuscript by Bandekar et al., probes the structural features of extracellular region of CELSR using cryo-EM and complements the study with cellular experiments to link these features with functional outcomes. Overall, the study is well-designed and executed, and it provides a significant advance in our understanding of adhesion receptor activation and signaling. While I find the authors' analogy of the overall architecture with ouroboros amusing, I am not sure if this analogy is indeed essential, but I leave it up to their discretion. I wholeheartedly support the publication of this manuscript, and I have only a couple of minor comments that the authors may consider addressing during revision.

[We thank the reviewer for their effort, their kind comments, and their acknowledgment that our study represents a significant advance in the field. We appreciate that they support the publication of our work. We respond to further comments below.](#)

1. If the side-chains can be confidently modelled at the presented resolution, it may be a good idea to show the density maps for the interacting residues in main figure (Figure 2).

[We have re-processed the cryo-EM dataset and improved the resolution to 3.8 Å which allows for better modeling of the side chains seen in Fig. 2. Since it is difficult to present a broad interface in such a way where the interactions are clear and the density is visible as well, we have added Supplementary Fig. 5 \(which shows similar views to Fig. 2\) but with the density shown using ChimeraX.](#)

2. The scale bar is missing in some of the microscopy panels in Figure 5 and Supplementary Figure S5, and negative staining and cryo-Em micrographs (Supplementary Figure S1 and S2). Also, some of the microscopy panels in Figure 5 appear to be merged perhaps during PDF conversion, and it should be fixed. Statistical analysis can also be included in supplementary Figure S5 presenting aggregation index.

Arun K. Shukla, PhD

In the revised manuscript, the microscopy and electron micrographs now include scale bars, including Figs. 5, 6 and Supplementary Figs. 1, 2, and 6. Also, we have fixed the issues with the microscopy panels in Fig. 5 being merged and we added statistical analysis to Supplementary Fig. 6.

Reviewer #2 (Remarks to the Author):

In this paper, Bandekar and colleagues present a partial cryo-EM structure of the ectodomain of the adhesion GPCR, CELSR1. CELSR family proteins mediate adhesion via their N-terminal cadherin repeats but the functions of their 14 other extracellular domains are less understood. Moreover, despite decades of work in animal models demonstrating roles for CELSR proteins in tissue morphogenesis, development of the nervous system, ciliogenesis and planar cell polarity (PCP), very little structural information is available for this class of very large adhesion GPCRs. The authors present a 4.3 angstrom structure consisting of fourteen domains of CELSR1s extracellular region (CADH9-GAIN) and show that it forms a compact module that folds in on itself through interactions between the CADH9 and GAIN domains. They further show a functional requirement for CADH1-8 in cell adhesion and for CADH9-GAIN in G-protein signaling in HEK cells. Biophysical assays and modeling of the full ectodomain in the presence of calcium suggest an antiparallel dimer and a flexible hinge region between CAD repeats 5 and 6. Although the cryoEM structure did not resolve the 8 CADH repeats that engage in adhesion, and the purified protein lacks the 7-pass transmembrane and cytosolic regions of the protein, given how little was known prior to this study, the structural data presented here significantly advances our understanding of CELSR1 architecture. It also makes important predictions for how adhesion may regulate its GPCR activity to be tested in future studies.

We thank the reviewer for their time and consideration of our manuscript, and we appreciate that they acknowledge the significance of our work.

In general, the data are presented in a compelling manner and the conclusions are well supported by the data. However, some of the reported findings (necessity and sufficiency of CADH1-8 in adhesion, and functions for EGF and LAM repeats) were recently demonstrated in the fly ortholog of CELSR1, Flamingo, which slightly diminishes the novelty of some of the findings. That paper (Strutt et al, 2023 PMID: 37995695) should be cited and discussed as it is probably the most extensive structure-function study on a CELSR family member to date. Moreover, there is some important context missing about the unknown relevance of G-protein signaling for CELSR1 function that should be acknowledged. Finally, given the detailed characterization of the CADH9-GAIN binding interface, which is the most unexpected and exciting finding of the paper, some functional testing of this binding interface would greatly strengthen the impact of the paper.

We apologize for missing the (Strutt et al. 2023) paper in our survey of the literature, and we agree with the other points in this critique, importantly that the CADH9/GAIN binding interface is unexpected and quite exciting. Please see detailed responses below.

Major points

1. A recent study by Strutt et al in *Drosophila* demonstrates that CAD1-8 of Flamingo is both necessary and sufficient for adhesion. That paper should be cited and discussed.

We include references and discussion of the (Strutt et al. 2023) paper throughout our revised manuscript, including in the discussion section (line 339).

2. The Strutt et al, 2023 paper also shows that Fmi only engages in adhesion/trans-interactions when one of the two cells also expresses Frizzled, and this asymmetry requires the presence of CAD9-GAIN. Other data in that study suggest the EGF and Lam repeats may interact with Fz. They also show deletion of LamG1 or LamG2 impairs transport to the cell surface. Although it is not clear that CELSR1 shares this need for Frizzled in one cell to engage in adhesion (most evidence suggest it does not), this is all to say that the statement on line 101-102 “No function has been demonstrated for this C-terminal EGF/LamG/HormR/GAIN region”, is not correct.

We have amended our introduction section to acknowledge the work done in Strutt et al 2023 and corrected the mentioned sentence (Line 98).

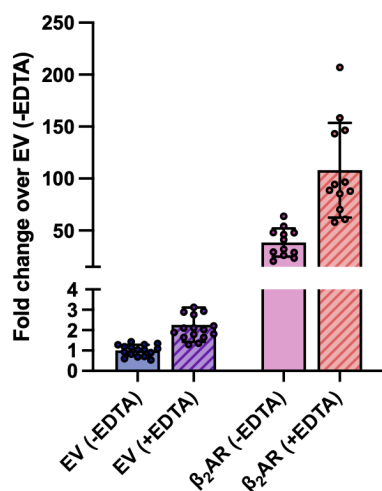
3. The interaction of CADH9 with the GAIN domain is both unexpected and exciting as it places the adhesion region of CELSR1 in direct contact with a region known to regulate GPCR activity. However, the large deletions performed in this study do not shed light on the significance of this binding interface. Point mutations that disrupt this interaction should be tested for their effects on adhesion and G-protein signaling.

We thank the reviewer for the suggestion for this important experiment. We tested three sets of point mutations in the CADH9/GAIN interface that we designed to break the binding interactions and open up the closed compact module (L1163A/F2188A, L1163A/Y2250A, N1184A/R1185A/Y2250A) using cell aggregation and signaling assays, and we found that these mutations resulted in increased CELSR-mediated cell aggregation (see revised Fig. 6) whereas they did not affect signaling (see revised Supplementary Fig. 7). This led us to a refined model where the compact CADH9-GAIN module may regulate CELSR1-mediated cell adhesion through conformational change. This conformational change could be driven by point mutation as we show, or through the binding of a PCP partner, as suggested in the (Strutt et al 2023) paper. Thus these results are indeed very exciting as they agree with the suggested idea in the Strutt paper and provide a mechanistic insight into how CELSR function might be regulated at the molecular level. Under the light of these new results, we have modified the model in Figure 7 and we now provide a better model.

4. It should be acknowledged that although CELSR1 shows an ability to signal via GalphaS using BRET assays in cultured cells, a functional role for G-protein signaling has never been demonstrated for CELSR1 in vivo or for planar cell polarity, where CELSR1 function is best described.

We have amended the discussion of our paper to clarify this (line 387).

5. In the BRET2 assays in figure 6, CELSR1 lacking CADH1-8 shows a greater change in BRET than the wild type protein. Can the authors comment on this? Is it possible the deltaCADH1-8 is constitutively active? Perhaps adhesion relieves the interaction between CADH9 and GAIN? Do cells expressing WT CELSR1 treated with EDTA show a greater BRET change, similar to the deltaCADH1-8 version?



Reviewer Fig. 1: Preliminary signaling assay results showing the effect of EDTA on signaling of empty vector (EV) and β₂AR.

We believe several possibilities could account for ΔCADH1-8 showing higher activity in the BRET assay than the WT protein. One explanation is that ΔCADH1-8 expresses higher than WT CELSR1 on the cell surface (see revised Fig. 5b-d), which we observe. The other possibilities the reviewer suggests, including that ΔCADH1-8 is constitutively active, and that adhesion relieves the interaction between CADH9 and GAIN, are both very exciting hypotheses. We look forward to testing these in our future work involving CELSRs.

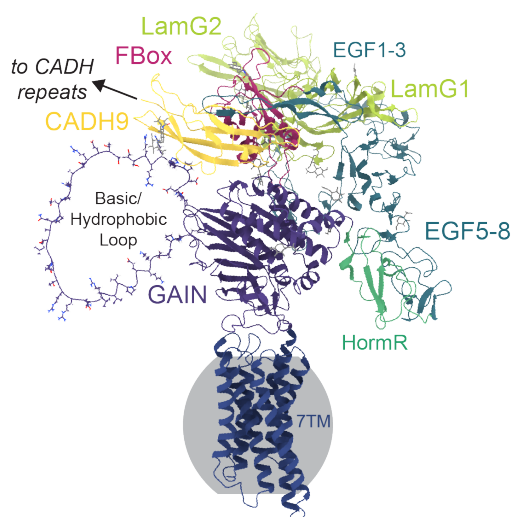
During the revisions, we realized that there was a mix-up of the constructs used in the previous BRET assay, so we have corrected the figures and our interpretations, accordingly. We have removed the BRET assay results and we now include results of a downstream signaling assay with improved assay characteristics.

While the suggested EDTA experiment may be insightful, we believe this experiment will be difficult to optimize and interpret. To address the reviewer's comment, we ran pilot experiments (Reviewer Fig. 1; N=3 independent biological experiments with at least three technical replicates each) using our signaling

assay and found that EDTA affected the signaling levels of cells transfected with both empty vector and a well characterized receptor, β₂AR. This may be due to EDTA affecting overall cell health, or EDTA affecting the assay itself. In future work, we plan to address this question using site-directed mutagenesis to eliminate the Ca²⁺ binding sites in CADH1-8.

Minor points

According to the model of the full length protein in the membrane, the HormR domain is positioned more membrane proximal than the GAIN domain and is in contact with the 7-pass TM domain. Can the authors comment on how this configuration could lead to GPCR activity?



Reviewer Fig. 2: AlphaFold2 model for position of CELSR1 ECR relative to the 7TM.

We believe that the reviewer was referring to the original version of Supplementary Fig. 3e. This conclusion was likely reached due to improper choice of the viewing angle. Here we present a clearer orientation (Reviewer Fig. 2) which shows that HormR is not in contact with the 7TM. Although HormR may be close to the membrane, we cannot make any conclusions about its position with respect to the membrane or the 7TM. In our updated figure we include multiple views to show that the HormR domain is not in contact with the 7TM.

We have now removed our claim that the CELSR ECR may modulate 7TM activity. During the revisions, we realized that there was a mix-up of the constructs used in the previous BRET assay, so we have corrected the figures and our interpretations, accordingly. We have removed the BRET assay

results and we now include results of a downstream signaling assay with improved assay characteristics.

Although ECR/7TM coupling is still a possibility, our data do not support it and so we have removed our claims. We think that relative ECR/7TM orientation could still affect intracellular events; the binding of PCP partners such as Frizzled could interact with the ECR to change ECR conformation and/or ECR/7TM orientation in order to mediate intracellular events. Another possibility is that lipids or other membrane-localized components could interact with the ECR to regulate CELSR function.

Reviewer #3 (Remarks to the Author):

Sumit J. Bandekar and co-workers presented 4.3 Å cryo-EM reconstruction of the mCELSR1 ECR, and revealed a compact interdomain bundle including 14 domains. The author discovered that the ECR (extracellular region) of mCELSR1 exhibits dimerization, likely facilitated by the cadherin repeats, arranged in an antiparallel manner. Furthermore, the authors utilized immunofluorescence to validate the effects of Δ CADH1-8 and Δ CADH-Gain on the aggregation of mCELSR1. In addition, the authors employed BRET assays to assess the modulatory role of Δ CADH1-8 and Δ CADH-Gain on the activity of the CELSR1-Gs signaling. These studies revealed that CADH1-8 repeat is required for cell-cell adhesion, whereas the C-terminal CAHD9-GAIN compact module regulates transmembrane-domain mediated G protein signaling. Overall, these conclusions may have important impacts in adhesion GPCR field, providing an important example for the study of adhesion GPCR huge extracellular domain structures and functions. However, there are several questions need to be addressed to improve the overall manuscript quality before publications. I would like to recommend for minor revision before publication in Nature communications.

We thank the reviewer for their effort in reviewing our manuscript, for their acknowledgement of the impact of our work, and their support for its publication with minor revisions.

1. The overall cryo-EM density map of mCELSR1 ECR is suboptimal in this study, it is recommended to incorporate the sidechain density when discussing the residue interactions in Figure 2.

We have reprocessed the dataset entirely, and we have improved the resolution of the reconstruction to 3.8 Å. This allows us to better model side chains. However, it is difficult to present a broad interface in such a way where the interactions are clearly visible, and the density is visible as well. Therefore, we have added Supplementary Fig. 5 which shows similar views to Fig. 2 but with density shown using ChimeraX.

2. (1) In Figure 5, the relative expression level of mCELSR1 mutants should be presented. Because receptor expression level affected their biological functional assays. (2) Moreover, it remains to be determined whether the interactions observed in the wild-type (WT) are consistent with those in various mutations. (3) Please specify the experimental conditions involve the presence of calcium ions.

(1) We have included an updated version of Fig. 5 including the expression levels of each construct.

(2) The reviewer is correct. This question will be clarified by future structural and molecular studies of CELSR dimerization, which we plan to pursue in following work.

- (3) a. The final buffer for the cell aggregation assay contains 20 mM CaCl₂ (see methods).
b. For the cell junction enrichment assay, the cells were maintained in DMEM (Gibco cat # 11995065 - contains 200 mg/L or 1.8 mM of CaCl₂) until they were fixed with PFA, which would lock all interactions present. [See this resource](#).

3. In Figure S5, cell-surface expressions were tested using immunofluorescence. However, the expression levels should be further validated through western blot because some mutations may cause truncated expression.

We present western blotting of the CELSR1 truncation constructs in revised Supplementary Fig. 6 b, c. The constructs tested do not display any evidence of truncation or degradation.

4. In Figure 1, the colors in Figure 1b-f are inconsistent.

We have changed the color scheme throughout the manuscript.

5. In Figure 2, the representation of the three key interfaces was not very friendly. It would be better to modify the cartoon transparency and display the sticks of different domains in different colors. Similarly, in Figure S4, it was not a good choice to show the potential map for interdomain interfaces of mCELSR1 ECR by Coot.

We have adopted the suggestions for coloring the structure (see revised Fig. 2). We also include revised Supplementary Fig. 5 which includes cryo-EM density rendered using ChimeraX.

6. In this manuscript, it indicated that the CADH9-GAIN module regulated Gs activity through the 7TM. Considering the density of mCELSR1 is suboptimal, I will suggest to perform computational simulation or design alanine screening methods to reveal which residues of CADH9-GAIN domain contribute to modulation of G protein signaling.

During the course of our revisions, we realized there was a mix-up of constructs used in the signaling assay. In the revised manuscript we corrected this, and we no longer claim that the CELSR1 CADH9-GAIN module regulates signaling activity. We agree that the ECR/7TM coupling is a very interesting question and we have recently studied this for the aGPCR ADGRL3/LPHN3 in a different manuscript which has just been published (PMID: 39627215). In this other manuscript, we have studied the ECR/7TM conformational coupling and examined a cancer-associated mutant at the ECR/7TM interface. Although, for CELSRs, we have removed our claim that ECR contributes to 7TM modulation in this current manuscript, we still think that this could be possible. We will continue our studies to understand this question for CELSRs in future work and include the reviewer's suggested experiments.

7. In line 283, the sentence "Evidence collected and others is consistent with a model of the CADH1-8 mediated antiparallel dimer driving the adhesive activity of CELSRs" should be cited with corresponding reference.

We have corrected this in our revised manuscript where we cite Strutt et al. 2023, Nishiguchi et al. 2023, Tamilselvan et al. 2024, and Stahley et al. 2021.

8. It is recommended to list the distances between residues that are considered to form interactions in this study, for example, the hydrophobic interactions mentioned in line 170 to 179. Also in this paragraph, there are several residues (E2195, E1188, R2252, Y1592) that are mentioned to form interactions are not indicated in the corresponding figure. Is this a simple

negligence or is there a reasonable explanation? In addition, when describing interaction interfaces, it would be better to present the sidechains of two sides in two different colors.

We have included key distance measurements in the revised Supplementary Table 1. We apologize for the discrepancy between residues listed in the text and residues included in the figure. It was difficult to prepare a clear figure including all interactions and some were omitted for clarity in the original version. In the revised Fig. 2, we decided to trim further to only the most important interactions, and we only discuss these in the text. In the revised Supplementary Table 1, we include a comprehensive list of interactions involved in the 3 key interfaces from Fig. 2. We incorporated the suggestions for coloring side chains as well.

9. In the BRET experiments, the Δ CADH1-8 construct seems to result in stronger G-protein dissociation than WT, but the significance of this curve is absent. This could be an intriguing finding and a little more discussion would be better.

We believe several possibilities could account for Δ CADH1-8 showing higher activity in the BRET assay than the WT protein. One explanation is that Δ CADH1-8 expresses higher than WT CELSR1 on the cell surface (see revised Fig. 5b-d), which we observe. Some other possibilities are that Δ CADH1-8 is constitutively active, or that adhesion between two CELSR molecules through CADH1-8 relieves the interaction between CADH9 and GAIN. We look forward to testing these hypotheses in following work.

During the revisions, we realized that there was a mix-up of the constructs used in the previous BRET assay, so we have corrected the figures and our interpretations, accordingly. We have removed the BRET assay results and we now include results of a downstream signaling assay with improved assay characteristics, which shows that Δ CADH1-8 does not signal stronger than WT.

10. In Figure 5d, the fluorescence intensity of HA- Δ CADH1-8 (green) is notably stronger compared to the others. Please provide a more precise explanation.

We believe there are two reasons that the fluorescence intensity of HA- Δ CADH1-8 is notably stronger. First, the distribution of HA- Δ CADH1-8 is spread all over the cell surface rather than being restricted to the cell-cell junction (see revised Fig. 5h for a close-up view, and Fig5b for a zoomed out view). Secondly, the Δ CADH1-8 construct has a higher cell-surface expression level (see revised Fig. 5b-d). Both of these factors may explain why it appears that the fluorescence intensity of HA- Δ CADH1-8 is stronger compared to the other constructs.

Reviewer #4 (Remarks to the Author):

This manuscript reports results from EM and SAXS combined with MD of CELSR1. The well-written manuscript presents structural biology methods results well-supported by cellular experiments. The demonstration of the 14-domain structure in different conformations is a significant finding. With minor revisions, I believe the article is suitable for publication in Nature Communications.

We thank the reviewer for their acknowledgement of the significance of our work and their support for the publication of our work with minor revisions. We address individual points below.

The addition of a calibration curve for the column used in Supp Fig 1 would be better. Additionally, why was Superose® 6 chosen over a column like S200 or S300? Can you provide information about oligomerization states using S200 or S300 in protein gel filtration?

Could you please also share with and without Ca; Gel filtration profile using S300?

In the revised Supplementary Fig. 1b, we have included vertical lines to show calibration standards for the Superose 6 increase column. We originally tried Superose 6 increase for CELSR1 because we were worried that dimeric CELSR may be too close to the void volume of S200. To expand, we expected the sample may run larger than expected due to the potentially extended cadherin repeat region. The CELSR sample behaved well on the Superose 6 increase column, so we did not try other columns. We have provided information about oligomerization using SEC-MALS-SAXS with and without Ca^{2+} using a Superose 6 increase column, which is a more rigorous method than SEC alone (Fig. 3a, Table 2, and Supplementary Data Files 1, 2) because accurate molecular weights can be calculated from MALS profiles. Our lab does not have an S300 column, so we cannot provide this filtration profile. We asked neighboring labs and they also did not have one.

In Supp Fig 2, the 2D images could be moved to the beginning of the figure and made larger and more detailed.

Additionally, the local resolution map in Supp Fig 2 could be placed at the end of the figure and presented as a larger image with the domains labeled. This would allow for a better understanding of which domains are more flexible or have lower resolution. Also adding 180 degrees turned version can be helpful.

We reprocessed the cryo-EM dataset from scratch, and we included the updated workflow in the revised Supplementary Fig. 2. We made the images larger as the reviewer suggested. We have also made a new figure to showcase local resolution of our reconstruction (revised Supplementary Fig. 3). This figure includes larger images, 180 degree turned versions, and domain labels.

I couldn't understand why Figure 3 named as SEC-MALS analysis. In figure 3 most of the information coming from SAXS and main analysis tool also looks SAXS.

We have updated the name of revised Fig. 3 to "SEC-MALS-SAXS analysis of CELSR1 CADH1-GAIN" since panel a includes SEC-MALS and panels b-e include SEC-SAXS analysis.

No table showing SAXS recording and evaluation parameters according to the BioSAXS consortium agreement is appended - the table in the Supplementary has no errors, no measure of concentration, no description of the instrument etc etc.

We have included revised Table 2 which includes SAXS data collection and processing parameters.

Additionally, submitting SAXS data to The Small Angle Scattering Biological Data Bank would be helpful for future investigators.

We have submitted our datasets to the SASBDB successfully, and we will release them to the public upon manuscript acceptance. They will be under codes SASDVS7 and SASDVT7.

Could you Please also add

In I (s) vs s plot

Kratky plot

Pair distance distribution graphs.

Please see the revised Supplementary Data Files 1 and 2 which contain all relevant plots as generated by BioXTAS RAW.

Rg, Dmax, Vp, MW (kDa) Vc (kDa) Qp (kDa) values from SAXS analysis,

Please see the revised Table 2 and Supplementary Data Files 1 and 2.

Please also share Fit quality Fineness and Normalized Spatial Discrepancy (NSD) values for model fitting to pdb.

We did not fit any models to PDB files in this work.

Minor

Most of the figures have some gray background borders around

There were some errors when inserting PDFs into the word document and we have fixed this issue in the revised manuscript.

Reviewer #5 (Remarks to the Author):

Despite its significance, molecular-level insight into CELSR function has been limited. The authors utilize cryo-EM to reconstruct the mouse CELSR1 ECR at 4.3 Å resolution, revealing a compact module of 14 domains mediated by interactions between CADH9 and C-terminal GAIN domains. In addition, cell-based assays demonstrate that the N-terminal CADH1-8 repeat is crucial for cell-cell adhesion, while the C-terminal CAHD9-GAIN compact module regulates G protein signaling via the transmembrane domain. The molecular insights are interesting, however, I have major concerns regarding the computational part.

We thank the reviewer for the acknowledgement of the significance of our work. We respond to the other points below.

Major:

1. The authors used a deposited structure of the human CELSR1 CADH4-7 and investigated the dynamics of the proposed hinge region within the cadherin repeat region of CELSR. This was done in three different conditions (WT, dCa²⁺, canonical). The authors observe that the hinge region adopts three different bending angles based on the condition when starting from the same initial structure. According to the simulation table, systems were run for only 100 ns. I find it very surprising to observe such a large structural rearrangement within 100 ns. In this respect, I propose that the authors plot the evolution of the angle for the WT, the dCa²⁺ and the canonical system across the simulation time and discuss the observations.

Please see the plot of the bending angles in revised Fig. 4a and updated results section (line 221).

We agree that the observed conformational change is a drastic change to see over a short time, however (1) the importance of Ca²⁺ for cadherin repeat rigidity is a well-studied topic (PMID: 20066110), and (2) the CELSR inter-cadherin repeat region, like other cadherins, consists of mostly loop character with lots of acidic residues to coordinate Ca²⁺ ions (PMID: 38307021). Thus, it is not surprising that in the absence of Ca²⁺ ions (ΔCa²⁺ simulation), or the residues to coordinate them (WT simulation), that this region can quickly transition to disorder.

2. In addition, it would be very interesting to better understand the molecular mechanism that drives the structural rearrangement to different bending angles.

Nearly every interaction between two CADH repeats is driven by the Ca^{2+} ions that are coordinated between them (PMID: 20066110). Thus, the loss of bound calcium will likely induce flexibility between individual cadherin repeats that is captured as bending in our simulation. This is consistent with what is known about the structure and biochemistry of cadherin repeats (PMID: 20066110).

-Quoting (PMID: 20066110): “Crystal structures of ectodomain regions containing multiple EC domains show that the connections between successive domains are rigidified by Ca^{2+} coordination (Nagar et al. 1996; Boggon et al. 2002). Three Ca^{2+} are coordinated by conserved amino acids contributed from the base of one domain, the top of the next, and the linker region between them (Fig. 2). These Ca^{2+} binding sites are among the most highly conserved sequence features of cadherins across all species (Nollet et al. 2000; Posy et al. 2008).”

-Quoting (PMID: 20066110): “Removal of Ca^{2+} leads to a disordering of interdomain orientations, as can be seen by electron microscopy (Pokutta et al. 1994), increased sensitivity to proteolysis, and increased motion between successive domains (Haussinger et al. 2002).”

3. For data reproducibility and transparency, starting models, simulation protocols, and data should be submitted to a public repository (e.g., GPCRmd (www.gpcrmd.org) or similar).

We are in the process of submitting the requested data and protocols to GPCRmd and will arrange to release them upon acceptance of the manuscript.

Minor:

Figure 4a) The authors do not indicate where these snapshots were taken from.

The snapshots in Figure 4a were taken from the deposited structure of hCELSR1 CADH4-7 (PDB 7SZ8). In the revised manuscript they are omitted as we decided they were not necessary.

Figure 4e) Instead of a table, I propose to plot this data.

We have now plotted the data represented in Figure 4e.

Manuscript NCOMMS-24-10882A: Response to Reviewer Comments

We thank the editors and the reviewers for their time and effort reviewing our manuscript. We appreciate that 3 of the reviewers recommend our work for publication. Below we respond in detail to the remaining comments from reviewers. The *reviewer's comments are italicized*, and we provide [responses to the reviewers in blue text](#).

REVIEWER COMMENTS

Reviewer #2 (Remarks to the Author):

The revised manuscript has several improvements including a higher resolution structure and mutational analysis of CADH9-GAIN interface on signaling and adhesion. I have a few small questions/clarifications that the authors should address prior to publication.

1) In the revised version of the manuscript, the description of the residues involved in the LAMG1-EGF6 interaction have changed considerably. Almost completely different amino acids are now listed as contributing the hydrophobic core as compared to the previous version. Can the authors comment on whether the change is due to the increased resolution provided by the new structure analysis, or if there was a mistake in the previous version?

[We apologize for the confusion generated by the mentioned changes. The authors debated on several ways to best describe the interfaces in the results and figures. Ultimately, we decided on a compromise. Only the most relevant residues are mentioned in the main text and shown in the main figures so as not to overwhelm the reader. This is why some residues were removed in the revised version of the results section. A comprehensive list of interacting residues for each interface is available in Supplementary Table 1.](#)

2) The BRET assays for GalphaS coupling have been removed from the current version and replaced with a different assay for GPCR activity. However, this assay is not described in the main text, methods, or figure legends. Can the authors please describe what this assay measures and how.

[We apologize that this was not made clearer, but we did describe the new assay in the revised methods section \(please search for "CELSR signaling assay"\) in the methods section. This assay measures cyclic adenosine monophosphate \(cAMP\) levels using the \[Promega GloSensor cAMP reporter system\]\(#\).](#)

Besides the mix up of constructs in the BRET assays described in the rebuttal letter, can the authors comment on why the new signaling assay is preferred over the BRET assay, which I understand to be standard in the GPCR field?

[We chose to move forward with the GloSensor cAMP assay because in our situation it provides improved signal/noise, higher throughput, and it is less technically challenging. Furthermore, we have been looking for an assay for testing the effect of force application to CELSR signaling and the cAMP assay is more adaptable for this purpose. Due to these characteristics, we plan to use this assay in our following CELSR manuscripts.](#)

3) Line 96-97. ' In C. elegans Fmi, the EGF/LamG/HormR/GAIN region plays a role in the interaction with Frizzled, an Fmi binding partner involved in PCP(70).' The study being referred to was performed in Drosophila, not C. elegans.

We apologize for this error, and this has been corrected in the newly revised manuscript.

Reviewer #4 (Remarks to the Author):

I sincerely thank the authors for addressing most of the comments in the revised manuscript. However, there are still a few aspects that require further clarification, particularly regarding the response to the query:

"Please also share Fit quality Fineness and Normalized Spatial Discrepancy (NSD) values for model fitting to PDB."

The authors replied: "We did not fit any models to PDB files in this work."

This response is unclear, as in the previous version of the manuscript, there was a SAXS envelope that incorporated the Cryo-EM map of structure 8VY2. Given that the Cryo-EM map corresponds to part of the 8VY2 structure, it raises several questions:

How was the Cryo-EM map of 8VY2 fitted into the SAXS envelope in the earlier version of the figure?

We used ChimeraX to fit the cyro-EM density map to the SAXS bead model, first by manually placing the two close, then by using ChimeraX's fit in map function.

What were the Fit quality, Fineness, and Normalized Spatial Discrepancy (NSD) values for this fitting? These quantitative metrics are essential for evaluating the model quality and fitting precision.

ChimeraX does not provide such values from the fit in map tool ([link to documentation](#)).

The authors appear to have replaced the DAMMIN/DAMMIF model with the DENSS model in the revised version. Could the authors clarify why this change was made? While replacing the model is acceptable, it is critical to explain how the new approach improves or differs from the previous analysis.

In our discussions with Dr. Maxwell Watkins, a SAXS beamline scientist and our contact at Bio-CAT (Advanced Photon Source Sector 18), he suggested using DENSS as it can potentially yield more detailed information than a simple bead model, which can only provide an envelope and no internal density variation. Thus, we used DENSS as it yields a more information-dense reconstruction than DAMMIN/DAMMIF.

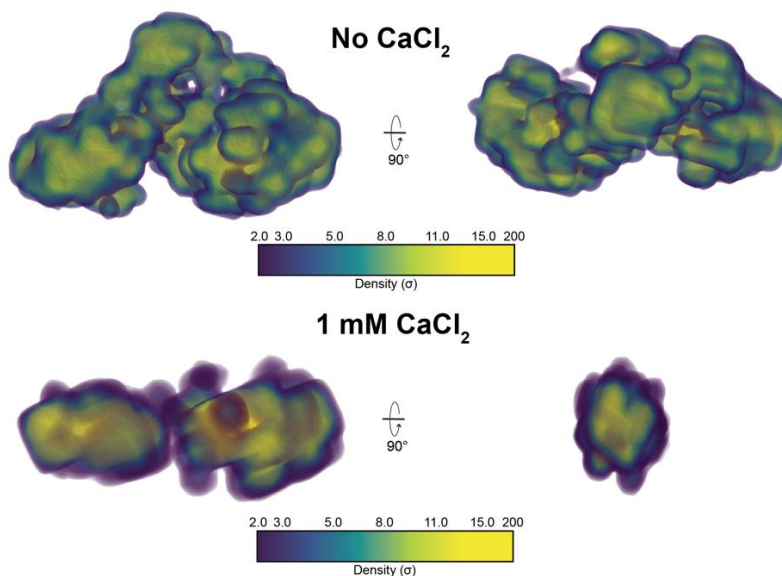
Even if the DAMMIF model has been removed, the following question remains: How was the 8VY2 Cryo-EM map fitted into the SAXS envelope originally, and what were the corresponding fit quality parameters (NSD, etc.)?

We used ChimeraX to fit the cyro-EM density map to the SAXS bead model, first by manually placing the two close, then by using ChimeraX's fit in map function. ChimeraX does not provide such values from the fit in map tool ([link to documentation](#)).

If the authors have opted to use the DENSS model, the electron density map reconstructed from DENSS analysis typically provides SAXS electron density values. In such cases, the SAXS

envelope should include a color bar indicating electron density values, which is absent in the current figure. The resulting electron density map should then be used to fit either the 8VY2 Cryo-EM map or a cartoon representation of the structure. Could the authors clarify: Why this color bar and the corresponding SAXS DENSS analysis electron density values are not shown?

We apologize for this oversight. This is the first time our lab is using DENSS and we were not aware of this standard. Please find the requested figure below.



Reviewer Fig. 1: the two DENSS reconstructions (not to scale) rendered using PyMOL as described in the [DENSS documentation](#).

How the Cryo-EM map was fitted into the DENSS SAXS-derived electron density map?

We used ChimeraX to fit the cyro-EM map to the DENSS SAXS-derived electron density, first by manually placing the two densities close, then by using ChimeraX's fit in map function.

Finally, it is currently unclear how the 8VY2 Cryo-EM map has been placed inside the SAXS envelope in the revised figure. A more detailed explanation and clarification of this fitting procedure are necessary.

We sincerely apologize for any lack of transparency in our SAXS processing and fitting procedures and we are happy to address any issues the reviewer has. What we meant previously is that we did not fit any atomic models (our CELSR1 experimental structure or AlphaFold models) to SAXS-derived bead models or SAXS-derived density maps throughout our manuscript, which is still true. Please find our answers to the reviewer's questions below.

To resolve these ambiguities, I kindly request the authors to provide the following:

A clear explanation of how the Cryo-EM map of 8VY2 was fitted into the SAXS bead model in the earlier version of the figure.

We used ChimeraX to fit the cryo-EM density map to the SAXS bead model, first by manually placing the two close, then by using ChimeraX's fit in map function.

The Fit quality, Fineness, and NSD values for the model fitting values of the earlier version of the figure.

ChimeraX does not provide such values from the fit in map tool ([link to documentation](#)).

A rationale for replacing the DAMMIF model with the DENSS model.

In our discussions with Dr. Maxwell Watkins, a SAXS beamline scientist and our contact at Bio-CAT (Advanced Photon Source Sector 18), he suggested using DENSS as it can potentially yield more detailed information than a simple bead model, which can only provide an envelope and no internal density variation. Thus, we used DENSS as it yields a more information-dense reconstruction than DAMMIN/DAMMIF.

A visualization of the DENSS analysis results, specifically including a color bar indicating electron density values derived from SAXS.

Please see Reviewer Fig. 1 (above).

A step-by-step explanation of how the Cryo-EM map was fitted into the DENSS-derived SAXS envelope.

We used ChimeraX to fit the cryo-EM map to the SAXS density, first by manually placing the two densities close, then by using ChimeraX's fit in map function.

Importantly, we do not make any claims about the fitting of the cryo-EM map to the SAXS density. The only reason we have made this fit is to illustrate that:

- 1) In the absence of Ca^{2+} : The overall shape and size of the cryo-EM and SAXS densities are similar ($\sim 220 \times 140 \times 120 \text{ \AA}$).
- 2) However, comparison of the sizes in the absence and presence of calcium are very different. ($\sim 220 \times 140 \times 120 \text{ \AA}$ (no Ca^{2+}) vs. $\sim 500 \times 220 \times 220 \text{ \AA}$ (1 mM Ca^{2+})) Thus, our conclusion is that there is a change in the size and overall shape of the molecule by addition of Ca^{2+} .

Instead of the fit to the SAXS data, we present an AlphaFold3 model of the putative CELSR1 dimer species. We apologize for the miscommunication. We have clarified this point in the main text.

Providing this information will ensure that the structural fitting procedures are transparent, reproducible, and fully understandable to the readers.

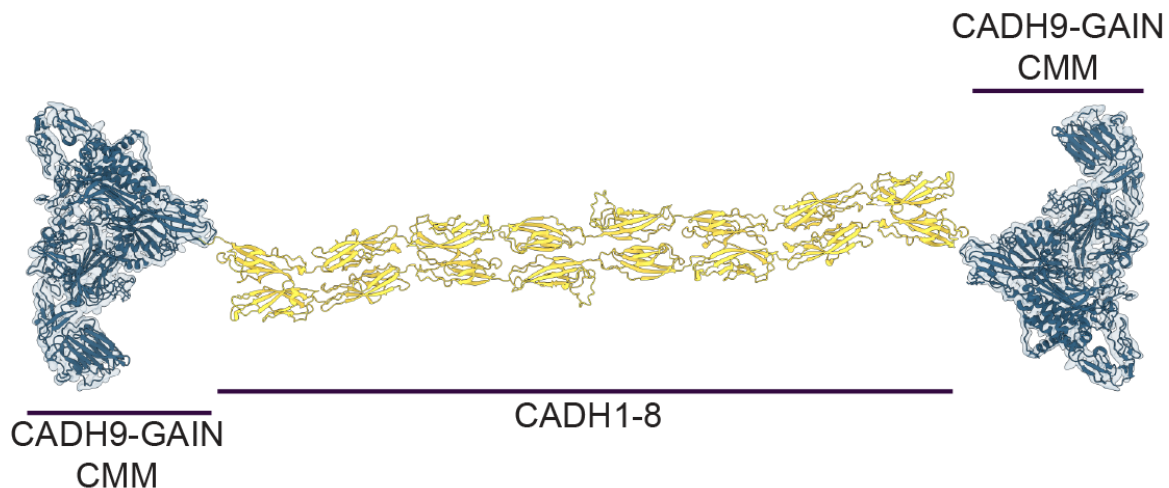
Another concern about Dimerization Interpretation in Figures 3d and 3e:

The models shown in Figures 3d and 3e suggest that the CADH1-8 region dimerizes in a U-shaped or V-shaped manner, rather than in a linear, elongated form. This interpretation raises important questions:

If the CADH1-8 dimerization were truly linear, one would expect a more defined and elongated electron density cloud from the SAXS analysis. However, the current SAXS envelope appears to support a curved dimerization model. Could the authors provide further evidence or clarification to support their structural interpretation?

and Figure 3f and Figure 7 suggests a particular dimerization model. Could the authors provide additional evidence or alternative model-based analyses (as a dimer form of CADH1-8) (e.g., molecular dynamics simulations or comparative structural data) to further support this proposed model (whether it is linear instead of having a curve or a U- or V-shape)?

The reviewer is correct that our SAXS data alone is not sufficient to distinguish between the extended vs curved (U or V shaped) dimerization models. Although we present a linear model for dimerization for simplicity (Fig. 3f), we acknowledge that this species is likely one of several present at a given time. Our molecular dynamics (Fig. 4) as well as other published structural (PMID: 37094146) and simulation-based data (PMID: 38307021) suggest that a hinge exists in the CADH repeat region which could allow this region of the protein to be present in multiple conformations in addition to a linear dimer. The cartoon we presented in Fig. 3f and the model presented in Fig. 7 is based not only on our data, but also on another work (PMID: 37094146, see Figs. 3 and 5 in this work) which used atomic force microscopy to determine low resolution images of the CELSR2 ECR. Furthermore, in the previous version of the manuscript we had not attempted AlphaFold3 to generate a prediction of the CADH1-8 dimer, which we now have successfully done (Reviewer Fig. 2). This prediction agrees with the extended antiparallel mode of dimerization proposed by us and others. Although we present the simplest model, we do not mean to exclude others, and we have clarified this in the text.



Reviewer Fig. 2: AlphaFold3 prediction of the CADH1-8 dimer species agrees with an antiparallel extended species. The sequence for CELSR1 CADH1-8 was input into AlphaFold3 to generate a model for the dimer species and then aligned to an AlphaFold3 model of the full-length extracellular region.

We do appreciate the complexity of the CADH1-8 dimer, but we wanted to keep our analysis and interpretation relatively simple for this manuscript. We plan to explore the CADH1-8 dimer in further detail in the future. Our following work will focus on detailed structural analysis of the CELSR1 dimer species through cryo-EM and detailed modeling based on our SAXS data, and further analysis of the hinge region. We will test structural hypotheses using deletions and point mutations in SEC-MALS-SAXS as well as our cell aggregation experiment.

Best
Çağdaş