nature portfolio

Peer Review File

Remote On-Off Switching of Protein Activity by Intrinsically Disordered Region

Corresponding Author: Dr Chengdong Huang

Version 0:

Decision Letter:

20th Nov 2024

Dear Dr Huang,

Thank you again for submitting your manuscript "Distant On-Off Switching of Protein Activity by Intrinsically Disordered Region". I sincerely apologize for the delay in responding, which resulted from the difficulty in obtaining suitable referee reports. Nevertheless, we now have comments (below) from the 3 reviewers who evaluated your paper. In light of those reports, we remain interested in your study and would like to see your response to the comments of the referees, in the form of a revised manuscript.

You will see that while reviewers appreciate the results, they raise extensive concerns which will need to be addressed in a revision. In line with comments of reviewer #1, we ask that you work towards providing a precise mechanism by which Sgt2C changes the dynamics of Sgt2N and its interaction with Get4/5. Please experimentally address all requests for further experiments to strengthen the conclusions, including further NMR data. Regarding reviewer #3 comments, please provide all requested controls, and more importantly, consider and strengthen the physiological relevance of the findings reported, and their relationship to published literature.

Please be sure to address/respond to all concerns of the referees in full in a point-by-point response and highlight all changes in the revised manuscript text file.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

We appreciate the requested revisions are extensive. We thus expect to see your revised manuscript within 6 months. If you cannot send it within this time, please let us know. We will be happy to consider your revision as long as nothing similar has been accepted for publication at NSMB or published elsewhere. Should your manuscript be substantially delayed without notifying us in advance and your article is eventually published, the received date would be that of the revised, not the original, version.

As you already know, we put great emphasis on ensuring that the methods and statistics reported in our papers are correct and accurate. As such, if there are any changes that should be reported, please submit an updated version of the Reporting Summary along with your revision.

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Please note that the form is a dynamic ‘smart pdf’ and must therefore be downloaded and completed in Adobe Reader.

When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-portfolio/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in

perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

If there are additional or modified structures presented in the final revision, please submit the corresponding PDB validation reports.

Please note that all key data shown in the main figures as cropped gels or blots should be presented in uncropped form, with molecular weight markers. These data can be aggregated into a single supplementary figure. While these data can be displayed in a relatively informal style, they must refer back to the relevant figures. These data should be submitted with the last revision, prior to acceptance, but you may want to start putting it together at this point.

SOURCE DATA: we urge authors to provide, in tabular form, the data underlying the graphical representations used in figures. This is to further increase transparency in data reporting, as detailed in this editorial (http://www.nature.com/nsmb/journal/v22/n10/full/nsmb.3110.html). Spreadsheets can be submitted in excel format. Only one (1) file per figure is permitted; thus, for multi-paneled figures, the source data for each panel should be clearly labeled in the Excel file; alternately the data can be provided as multiple, clearly labeled sheets in an Excel file. When submitting files, the title field should indicate which figure the source data pertains to. We encourage our authors to provide source data at the revision stage, so that they are part of the peer-review process.

While we encourage the use of color in preparing figures, please note that this will incur a charge to partially defray the cost of printing. Information about color charges can be found at http://www.nature.com/nsmb/authors/submit/index.html#costs

We require deposition of coordinates (and, in the case of crystal structures, structure factors) into the Protein Data Bank with the designation of immediate release upon publication (HPUB). Electron microscopy-derived density maps and coordinate data must be deposited in EMDB and released upon publication. Deposition and immediate release of NMR chemical shift assignments are highly encouraged. Deposition of deep sequencing and microarray data is mandatory, and the datasets must be released prior to or upon publication. To avoid delays in publication, dataset accession numbers must be supplied with the final accepted manuscript and appropriate release dates must be indicated at the galley proof stage. Please find the complete NRG policies on data availability at http://www.nature.com/authors/policies/availability.html.

Nature Structural & Molecular Biology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as ‘corresponding author’ on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. This applies to primary research papers only. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on ‘Modify my Springer Nature account’. For more information please visit please visit www.springernature.com/orcid.

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Note: This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Sincerely,
Kat

Katarzyna Ciazynska, PhD
(she/her)
Senior Editor
Nature Structural & Molecular Biology
https://orcid.org/0000-0002-9899-2428

Referee expertise:

Referee #1: biophysics, IDRs

Referee #2: NMR, biophysics, IDRs

Referee #3: membrane protein biogenesis

Reviewers' Comments:

Reviewer #1 (Remarks to the Author):

The manuscript presents a significant advancement in our understanding of allosteric regulation mediated by intrinsically disordered regions (IDRs). It focuses on Sgt2, a crucial protein in the GET pathway responsible for inserting tail-anchored (TA) proteins into the endoplasmic reticulum (ER). The authors provide a thorough examination of how the C-terminal IDR of Sgt2 (Sgt2C) allosterically regulates the conformational entropy of its N-terminal folded domain (Sgt2N) and its interaction with the Get4/5 scaffolding complex. The authors have compiled an impressive body of data using solution NMR, SEC-MALS, MS, and ITC measurements on a library of protein constructs. These data convincingly reveal that the C-terminal IDR segment of Sgt2 is crucial for auto-inhibiting its interaction with the Get4/5 complex. Notably, this inhibition is achieved without direct binding between Sgt2C and Sgt2N. Instead, NMR relaxations experiments suggest that the IDR induces fast internal motions within Sgt2N, thereby creating an entropic barrier that impedes binding to Get4/5. The authors further show that the dynamic interactions between Sgt2C and its substrates subsequently reduce this entropic barrier, allowing for binding. Although the role of conformational entropy in regulating protein-protein interactions have been well documented, the allosteric modulation by IDRs is not as thoroughly explored. This manuscript addresses this gap with remarkable clarity and depth. The work is meticulously executed and the findings are robustly supported by high-quality figures that clearly illustrate the complex data, providing readers with a comprehensive understanding of the entropic modulation mechanism. Moreover, the authors extend their insights beyond the specific context of Sgt2 by demonstrating that this mechanism can be generalized. They successfully create artificial systems incorporating IDRs to show reduced binding affinities, highlighting the versatility and potential ubiquity of this regulatory mechanism.

The work is carefuly executed, and the results are clearly described, with excellent figures summarizing the complex and extensive data. Additionally, the authors demonstrate the general applicability of the entropic modulation mechanism by constructing artificial systems with IDRs that reduce binding affinities. Overall, this manuscript should be of great interest to the readership of NSMB. It offers deep conceptual insights into a potentially widespread biological mechanism, and provides crucial details about this important regulatory protein. The breadth, quality, and innovative nature of this work make it an outstanding contribution to the field.

Other comments:
1. Stoichiometry in ITC Data:
Based on the cartoon in Figure 6 and the SEC-MALS data in Figure 3B, it appears that two Sgt2 dimers bind one Get4/5 complex. The n-values (stoichiometry) should be reported to confirm this. Do the reported n-values make sense in this context?
2. Sequence Information:
The sequences of the various chimeric constructs presented in Figure 2 should be provided for clarity and reproducibility.
3. Introduction Clarification:
The statement in the Introduction, "Intrinsically disordered regions (IDRs) constitute a substantial portion of the proteome, accounting for approximately 44% of the human proteome ..." is somewhat confusing. If referring to the percentage of residues in the human proteome that are disordered, it aligns with the high end of current estimates (~35%). Clarify that this refers to residues in the proteome. Notably, over 60% of proteins in the proteome contain IDRs 30 residues or longer, which is a different metric.
4. References for NMR Experiments:
The authors should provide references for the NMR methyl-TROSY and dispersion experiments to support their methodology.
5. Mutation Nomenclature:
In paragraph 2 of the "Plausible models of distant on-off switching" section, the cysteine mutations are referred to backwards (C254S and C294S). They should be labeled as S254C and S294C, consistent with the associated figure.
6. Figure 4B Clarification:
Figure 4B lacks the lower plot of chemical shift perturbations mentioned in the legend. Ensure this plot is included or adjust the legend accordingly.
7. Typographical Errors:
In Figure S1E, correct "misture" to "mixture".
In Figure S3E, change "limited sequence conservation" to "limited positional conservation" or alignment.
8. Terminology Correction:
The term "fast-exchange (ps-ns) dynamic properties" is confusing as exchange implies between discrete states. Remove the term "-exchange" to avoid confusion.

Reviewer #2 (Remarks to the Author):

This interesting manuscript reports on the structure and dynamics of the yeast protein Sgt2 that acts as a chaperone in the GET pathway. The function of Sgt2 is to bind nascent tailed-anchored (TAs) proteins as they emerge from the ribosome. The GET pathway involves the interaction of the Get4/5 complex with the N-terminal, folded domain (Sgt2N) of Sgt2. The manuscript addresses the following question: How can Get4/5 distinguish between TA-loaded and TA-unloaded states of Sgt2?

The manuscript describes an IDP regulatory mechanism by which the presence of the disordered C-terminal domain (Sgt2C) elevates the fast time scale internal motions of Sgt2N without directly contacting this domain. The increased

dynamics prevent binding of Get4/5 through an entropic barrier. The binding of clients (TAs) to Sgt2C rigidifies Sgt2N thereby allowing binding of Get4/5, providing an IDR-mediated mechanism by which Stg2 communicates its client-loading status.

I find the described regulatory mechanism intriguing and it introduces a novel concept. The manuscript is therefore of interest to the broad readership of NSMB. Having said that, it is frustrating that the manuscript does not reveal the precise molecular mechanisms by which Sgt2C changes the dynamics of Sgt2N and its interaction with Get4/5. I think the conclusions of the study need to be significantly strengthened by providing additional data and analysis before it can become suitable for publication.

1) The authors have not tested whether the dynamic shielding (model 1) could involve steric clashes with either Get4 or Get5. Previous studies have demonstrated a high-affinity binding of the ubiquitin-like (UBL) domain of Get5 to the N-terminal domain of Sgt2 (line 88). The authors work with a more complex system, the Get4/5 complex. Are the same results obtained when using only the UBL domain of Get5 for the interaction (i.e. no interaction is detected with Stg2FL)?

2) To prove the absence of long-range interactions between the C- and N-terminal domains, PRE experiments were carried out. Did the authors verify that the PRE label does not interfere with the autoinhibitory effect by checking that PRE-labeled Stg2FL does not bind Get4/5?

3) To provide further proof for the absence of an interaction between the C- and N-terminal domains of Stg2, the authors should compare the dynamics of the disordered domain in isolation and in the context of the full-length protein. 15N R2 or R1rho relaxation rates should be measured and reported along the sequence. A plot comparing the R2 or R1rho rates of Sgt2C in isolation and within Sgt2FL should be added (this should be possible since the resonances of the C-terminal tail are visible in the Sgt2FL HSQC spectrum). Please also add the superposition of the 1H-15N HSQC spectrum of Sgt2FL and Sgt2C to Extended Data Fig. 3. This superposition is essential to evaluate any differences in conformational behaviour of Sgt2C in the context of the full-length protein. This comparison should also be complemented by an I/I0 plot, where I are the intensities of the isolated Stg2C domain and I0 the intensities of the C-terminal domain within Stg2FL.

4) The authors compare HSQC spectra of Sgt2NT without and with an excess of Sgt2C (Fig. 4c). An I/I0 plot should accompany these data. It would also be useful to see the corresponding NMR spectra of 15N Sgt2C with an excess of Sgt2NT and the corresponding I/I0.

5) The authors should provide additional proof for the strict non-binding of Get4/5 to Sgt2FL (beyond the ITC and MALS experiments). Thus, SPR data should be shown for binding of Sgt2FL to Get4/5, and NMR titration data of 13C methyl-labelled Sgt2FL with increasing molar fractions of Get4/5 (or the UBL domain of Get5).

6) What is the difference between the proposed models 2 and 3? Can they really be considered as separate models? Why can slow conformational exchange dynamics (model 2) not act as a conformational entropy modulator (model 3)?

7) With respect to model 2, CPMG dispersion is observed only in the T domain and not in the N domain. Figure 4f shows only dispersion curves in the T domain. It would make more sense to show some flat dispersion curves for residues in the N-domain, either directly in Figure 4f or in the Supporting Information. The absence of CPMG dispersion in the N-terminal domain does not rule out very slow conformational exchange processes, these would need to be detected with for example CEST experiments.

8) For model 3: I have doubts about the analysis of the methyl relaxation data. If I remember correctly, the overall molecular tumbling time (correlation time) would need to be known in order to extract the order parameters from the relaxation data. The correlation time would change between the constructs used (Stg2FL, Stg2NT, Stg2FL-fusionTMD). Which correlation time was used for the different constructs and how were these values derived? One can even imagine that a global correlation time would not be appropriate and that a domain-specific correlation time would need to be applied for each construct. For a “beads-on-a-string” protein each individual domain may behave independently of the others. In general, details of this analysis (extraction of S2) should be provided in the Methods or Supporting Information. It would also be useful to see plots of Ia/Ib versus relaxation delay superimposed for the three different constructs. Was a sample used with a deuterated background to minimize relaxation effects from nearby protons?

9) I would like to see further support for the change in dynamics of the N-terminal domain in the presence of the C-terminal tail. The minimal system containing auto-inhibition would be a construct “Stg2NCshort”. This construct contains the N-terminal domain directly linked to the C-terminal domain spanning residues 221-265 (construct lacking the T domain). The NMR spectrum of Stg2NCshort should be shown along with a plot comparing heteronuclear NOEs of Stg2NCshort and Stg2N. A titration of this construct with the UBL domain of Get5 would also be useful, to confirm the absence of an interaction.

10) Figure 2a: I am surprised to see that a single residue in the IDR can be responsible for changing from “No interaction” to a “Kd of 400 nM” (comparison of the 1-264 and 1-265 constructs). Would this not rather indicate the disruption of a “binding motif” embedded in the IDR? Comparison of the sequences of yeast and mammalian Stg2 reveals conserved residues exactly at this 264/265 position (Extended data Fig. 6).

11) Line 235: The authors state that there is no change in dynamics of Stg2C upon binding to Ysy6-TMD. Relaxation is shown for five residues in Fig 3g; however, these are all located at the border of the binding site and, as such, do not directly

report on the dynamics at the interaction site (where the NMR peaks have essentially disappeared). I find it difficult to imagine a binding regime in which the NMR resonances disappear but the dynamics remain unchanged. Could the authors explain this?

12) Figure 3h: I failed to find information about which residues in the Ysy6-TMD part of the fusion are observed in the NMR spectra? Which residues specifically are reported in Fig. 3h? The authors report an exchange rate constant of 3000 s-1 from CPMG experiments, however, would the kex value not rather be the sum of the kon and the koff rate (as indicated in line 674, I am not sure what Ø means in the equation in line 673)? If the kex is directly equal to the off-rate, then the bound population of Ysy6-TMD would be very low, which does not seem to be consistent with the disappearing NMR signals across the entire Stg2C. The authors should provide the equations according to which the CPMG data were analysed and an analysis of how confidently the parameters can be extracted. Normally, two-field measurements are required to accurately extract the exchange parameters (population, exchange rate and chemical shift).

13) Figure 5 is reporting the use of the C-terminal domain of Sgt2 for modulating the interaction of diverse proteins. The same experiments need to be carried out for the same proteins, but where the Sgt2C tail is replaced by another IDR of the same length (for example Tau protein). It is otherwise not possible to know whether the observed effects originate from the sequence characteristics of Sgt2C or whether its rather a general “dynamic shielding” mechanism whereby the IDR partially prevents partner protein binding.
14) Generally, for the superposition of NMR spectra, please make the resonances in the spectrum on top with outer contours only (peaks with a hole in the middle) so that it is more visible how well the top spectrum overlaps with the bottom spectrum. Currently, a lot of features could be hidden, and it is not visible whether all peaks on top actually have peaks below them. This applies to all superpositions of spectra throughout the manuscript, please amend this.

Minor points:

1) Extended Data Figure 1a: Please provide error bars over the three replicates for the SPR experiments in the table (SgtNT + Get4/5).

2) Extended Data Figure 1e: Correct the spelling mistake (Misture -> Mixture).

3) Extended Data Figure 1b, c, d, e: I assume that the molecular masses in the MALS figures were derived from the experiments. The legend should contain the theoretical masses derived from sequence for comparison.

4) Extended Data Figure 2a: Please add labels corresponding to the assignment of the different peaks in the 1H-15N HSQC spectrum. Please deposit the assignment of this domain in the BMRB database and state the degree of completeness for the assignments.

5) Fig 1d: please state in the legend which chemical shifts (Ca, Cb, C’ etc) were used as input for the SSP calculation.

6) Extended Data Figure 2d, top: please indicate the color code in the legend.

7) Extended data Table 1: please provide error bars on the Kd and the thermodynamic parameters. Please add the stoichiometry, N, to the table for each measurement.

8) Rename Figure S4 to Extended Data Figure 4.

9) Extended Data Fig 7b: Why does the stoichiometry change between ITC experiments with different constructs (1:1 for Tau and L20 and 1:2 for Stg2 variants)? Update the legend on the x-axis in all ITC experiments to state which molar ratio is plotted (with protein names).

10) There seems to be no reference to Extended data Fig. 14.

11) Please provide the sequences of the fusion proteins.

12) In Fig. 3d, it seems that the I/I0 ratios are cut at 100. Please provide the data showing all ratios even if they exceed 100.

Reviewer #3 (Remarks to the Author):

The manuscript by Ji et al., investigates the effect both the C-terminal intrinsically disordered region (IDR) of Sgt2, a targeting factor within the GET pathway, and its interaction with the substrate tail anchor (TA) protein have on the downstream interaction with Get4/5. Previous biochemical and biophysical studies had already established the disordered conformation of the Sgt2 C-terminus, along with its role in binding the TA protein transmembrane domain (TMD)(Lin et al., 2021). The novelty of this manuscript lies in the identification of a novel autoinhibition between the Sgt2 IDR and its N-terminal dimerization domain, which prevents its interaction with Get4/Get5. The authors attribute this autoinhibition to an increased flexibility of the N-terminal domain in the presence of the IDR, with binding of the substrate TMD to the IDR leading to rigidification of the N-terminal domain and interaction with Get4/Get5. Most interestingly, transplantation of the Sgt2 IDR on to unrelated protein-protein interaction domains impairs their interactions with canonical binding partners. Whilst this concept is of interest to the field, the main weakness of the manuscript is the suboptimal quality with which some

experiments are explained or represented, which makes it difficult to judge some of the findings. There is also a failure to consider these results and their physiological relevance in the context of existing literature on Sgt2.

My major concerns are as follows:

1. There are no figures showing the SDS-PAGE and/or gel filtration of purified proteins used for downstream experiments, particularly for these poorly soluble or degradation prone constructs (e.g. Sgt2-C, full-length Get4/Get5).

2. There is no comment on why previous studies observe an interaction between full-length Sgt2 and Get4/Get5 by e.g. pull-down assays (Chartron et al., 2011) or yeast two hybrid assays (Chang et al., 2010). Whilst the ITC experiments look convincing, there is no explanation why they are performed in a non-physiological buffer (100 mM MES pH 6.0, 150 mM KCl) and it is not clear how many biological replicates were performed for each experiment. Instead of stating the approximate (~) KD, the KD should be given with errors.

3. In the MALS experiments, the molar mass seems to be rather variable across some peaks. Again, the experimental molar mass should be given with errors. It would also help the reader to give the expected molar mass for the assigned complex.

4. Only one representative solution structure for Sgt2 is shown, when it is more standard to show an ensemble of possible structures from such structure calculations. In this case, this would particularly help to illustrate the range of possible movements between the different domains and how close Sgt2-C is able to approach to Sgt2-N. There should also be a table of restraints and structural statistics as for any structure solution.

5. Given the Sgt2 C-terminus is responsible for autoinhibition, it could still be the fusion of the substrate TMD to the C-terminus that disrupts this autoinhibition, rather than the internal interaction between the TMD and C-terminus. As a control, the authors should test the interaction between Get4/Get5 and Sgt2 fused C-terminally with a non-interacting peptide of similar size. These experiments should be performed with a reconstituted Sgt2-TMD complex (several approaches have been successfully taken in the literature e.g. coexpression with MBP-TMD fusions (Lin et al., 2021) or handover from Ssa1 (Cho and Shan, 2018)) but the authors do not comment further on their “extensive efforts” to obtain such a complex.

6. I don’t think “the high solubility observed in aqueous solutions for both fusion constructs” is sufficient evidence to argue against both Sgt2-C domains of the dimer being required to escort the hydrophobic TA TMD. Indeed, evidence from Cho et al., 2024 suggests both Sgt2-C domains are required and that the overall conformation of the dimer changes substantially upon TA binding. This possibility should be discussed in the context of the observed line broadening. Furthermore, the authors may bias against such a concerted binding with their C-terminal TMD fusions to Sgt2 enforcing a high local concentration of the substrate adjacent to its binding site.

7. More detail should be given on how the IDR fusion to the PDZ domains, SPOC domain and GABARAP could structurally impact their interactions i.e. the position of the C-terminus relative to the binding interface in the (predicted) structure. Again, the protein purifications are not shown and it is questionable how the non-sigmoidal ITC curves obtained for MAG12 and GABARAP can be used to obtain trustworthy KD values. This data should also be added to Extended Data Table 1 to show the evidence for the claimed “entropy penalty due to IDR tail tethering”.

8. The discussion is vague and rambling, discussing the IDR in very generic terms. The authors need to discuss their results in the context of the wealth of literature on Sgt2 and consider the physiological impacts of their findings for the GET pathway. For example, the evidence that Get4/Get5 binds the ribosome to position Sgt2 close to the exit tunnel (Zhang et al., 2021) seems at odds with their model. Similarly, Sgt2 dimer dynamics (Cho et al., 2024) or the structural models of Lin et al., 2021 are not discussed.

More minor comments are as follows:

9. There should be primary data in Figure 2 and Figure 5. In general, the high complexity of the main Figures makes them difficult to understand. It is not necessary to show the full GET pathway in Figure 1 and it is currently redundant with Figure 6.

10. The authors stress several times that the autoinhibition is “mediated remotely by the Sgt2-C domain ~150 residues away”. This statement is misleading; the distance in the primary protein sequence is irrelevant compared to the distance within the tertiary structure. To appreciate this, a full repertoire of solution structures should be shown (see comment 4).

11. Line 140: the authors should state that Extended Figure 3a shows that the domains behave independently, rather than just writing it in the figure legend.

12. The authors state that Sgt2 and SGTA have “little sequence homology” but their alignment shows some conserved motifs. The authors should comment on the position of these motifs relative to the truncations that abrogate the autoinhibition.

13. The signal broadening is not clear from the overlay shown in Extended Figure 8b. In general, NMR spectral overlays where no change is observed should be contoured so that the bottom spectrum is visible.

14. The authors should comment that although the addition of TFE did not change spectral quality, there is a clear effect on

helix formation in the CD. This could be rationalized in terms of the model presented by Lin et al., 2021. How does the TA TMD binding site in Figure 3d compare with this model?

15. The authors should be careful to state that HDX-MS shows changes in solvent accessibility. It is their interpretation that this shows the domains change their dynamic properties. It is not clear from the figure legends what construct was the reference for calculating ΔHDX. The HDX would also be a useful experiment to conduct with the TA TMD fusions.

Version 1:

Decision Letter:

20th Mar 2025

Dear Dr. Huang,

Thank you again for submitting your manuscript "Distant On-Off Switching of Protein Activity by Intrinsically Disordered Region". Thank you for engaging with us and sharing your provisional responses to the reviewers, and your patience in waiting for this decision.

We now have comments (below) from the 3 reviewers who evaluated your paper. In light of those reports, we remain interested in your study and would like to see your response to the comments of the referees, in the form of a revised manuscript.

You will see that reviewer #3 still has remaining technical concerns ('additional reviewer comments'). Please address their points regarding the molar mass calculations.

Please be sure to address/respond to all concerns of the referees in full in a point-by-point response and highlight all changes in the revised manuscript text file. If you have comments that are intended for editors only, please include those in a separate cover letter.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

We expect to see your revised manuscript within 6-10 weeks. If you cannot send it within this time, please contact us to discuss an extension; we would still consider your revision, provided that no similar work has been accepted for publication at NSMB or published elsewhere.

As you already know, we put great emphasis on ensuring that the methods and statistics reported in our papers are correct and accurate. As such, if there are any changes that should be reported, please submit an updated version of the Reporting Summary along with your revision.

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When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-portfolio/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

EXTENDED DATA FIGURES

When re-submitting your manuscript, please ensure that any supplementary figures and tables that are crucial to the manuscript’s conclusions are converted into Extended Data figures and tables to increase visibility of these data. Extended Data figures and tables are online-only (present in the online PDF and full-text HTML versions of the paper), peer-reviewed display items that provide essential background to the article but are not included in the main article due to space constraints. A maximum of ten Extended Data display items (figures and tables) is permitted.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

Please note that all key data shown in the main figures as cropped gels or blots should be presented in uncropped form, with molecular weight markers. These data can be aggregated into a single supplementary figure item. While these data can be displayed in a relatively informal style, they must refer back to the relevant figures. These data should be submitted with the final revision, as source data, prior to acceptance, but you may want to start putting it together at this point.

SOURCE DATA: we urge authors to provide, in tabular form, the data underlying the graphical representations used in figures. This is to further increase transparency in data reporting, as detailed in this editorial (http://www.nature.com/nsmb/journal/v22/n10/full/nsmb.3110.html). Spreadsheets can be submitted in excel format. Only one (1) file per figure is permitted; thus, for multi-paneled figures, the source data for each panel should be clearly labeled in the Excel file; alternately the data can be provided as multiple, clearly labeled sheets in an Excel file. When submitting files, the title field should indicate which figure the source data pertains to. We encourage our authors to provide source data at the revision stage, so that they are part of the peer-review process.

Data availability: this journal strongly supports public availability of data. All data used in accepted papers should be available via a public data repository, or alternatively, as Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. Please note that for some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories can be found below:
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We require deposition of coordinates (and, in the case of crystal structures, structure factors) into the Protein Data Bank with the designation of immediate release upon publication (HPUB). Electron microscopy-derived density maps and coordinate data must be deposited in EMDB and released upon publication. Deposition and immediate release of NMR chemical shift assignments are highly encouraged. Deposition of deep sequencing and microarray data is mandatory, and the datasets must be released prior to or upon publication. To avoid delays in publication, dataset accession numbers must be supplied with the final accepted manuscript and appropriate release dates must be indicated at the galley proof stage.

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We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Sincerely,

Katarzyna Ciazynska, PhD
(she/her)
Senior Editor
Nature Structural & Molecular Biology
https://orcid.org/0000-0002-9899-2428

Reviewers' Comments:

Reviewer #1 (Remarks to the Author):

The authors have thoroughly addressed my comments. I now support the acceptance of this manuscript in NSMB.

Reviewer #2 (Remarks to the Author):

I thank the authors for carefully revising the manuscript and adding several new data supporting the conclusions of the manuscript. I still have one point that I insist should be further addressed:

The authors have added details on the extraction of the methyl order parameters. Specifically, dynamic light scattering was used to determine the hydrodynamic radii of the different protein constructs, and the rotational correlation times (tau_c) were calculated using the Stokes-Einstein-Debye equation. The reported tau_c values are 157 ns (Sgt2NT), 344 ns (Sgt2FL), and 328 ns (Sgt2-Ysy6TMD)*. However, given the molecular weight of these proteins, these values appear significantly overestimated. For example, the molecular weight of Sgt2NT (~48 kDa) suggests a rotational correlation time in the range of 25–35 ns, which is far from the reported 157 ns.

This overestimation likely stems from applying the Stokes-Einstein-Debye equation, which assumes a spherical molecule, whereas these constructs exhibit an elongated, “beads-on-a-string” topology with significant flexibility. Furthermore, the correlation time measured for the full-length constructs may not be directly applicable to the isolated N-terminal domain for the calculation of S2, given that it is connected to the rest of the protein via a disordered linker.

It is therefore possible that the results presented in Fig. 4j and 4l are artifacts arising from incorrect rotational correlation time estimations between constructs. To address this, I recommend determining tau_c from 15N R2 and R1 relaxation rates for Sgt2N and Sgt2N-Cshort (focusing on residues within the folded N-terminal domain). The methyl order parameters should then be extracted for Sgt2N and Sgt2N-short using the determined tau_c values to confirm that the observed effects align with panel 4j. Please also add the equations used to extract S2 from Ia/Ib.

Since the manuscript revolves around the discovery of the “conformational entropy modulator” model, it is crucial that the data analysis is conducted with the highest level of rigor.

*Unless I am mistaken, these values should actually be 1566 ns, 3441 ns and 3183 ns (using the reported equation and assuming a water viscosity of 0.00089 Pa.s at 25C).

Reviewer #3 (Remarks to the Author):

The revised manuscript by Ji et al., includes numerous additional experiments and controls in response to the reviewer’s comments. My previous concerns, which were largely related to the accuracy with which the authors represented their data and a lack of discussion of the results in the context of existing literature on Sgt2, have been mostly addressed. As a result, this work furthers both our understanding of protein targeting by the GET pathway and general allosteric regulation by IDRs and is suitable for publication providing a few outstanding comments are addressed:

1. Most primary research papers on the GET pathway do not start with a schematic like that in Figure 1a and, given this schematic is unnecessarily more complicated than its corresponding description in the introduction, does not help the reader. I strongly recommend to simplify Figure 1 by removing panel a.

2. Extended Data Tables 1 and 2 were not included with the manuscript submission so it is not possible to assess whether they incorporate the requested information. The reference in the ITC methods section presumably needs to be updated to Extended Data Table 2.

3. The legend for Extended Data Figure 1a incorrectly states that the standard buffer for ITC experiments was HEPES.

4. The reported error values for some molar mass estimations from MALS are surprisingly small given the visual quality of the data e.g. the steeply sloping molar mass curve in Extended Data Figure 1h makes an error of 0.7 kDa seem highly implausible. This implies a very narrow subset of the peak was used for the calculation. The authors should show the windows that were selected within the SEC-MALS traces (e.g. by scale bars) for molar mass calculation.

5. The ITC curves from Extended Data Figure 14 should be included above or below the schematic in Figure 5 as it is important to directly show the quality of the primary data. The curves for the untethered systems could be shown in black to avoid creating inconsistencies with the established colour scheme. In the new paragraph for the Tau-PDZ/SPOC/GABARAP fusions, the authors refer to Extended Data Figure 15 which does not exist.

ADDITIONAL REVIEWER COMMENTS:
- the line for molar mass in Extended Figure 1h goes from ~110-150 kDa in the newly indicated peak selection window. It isn't logical that the corresponding molar mass calculation has an error of 0.7 kDa. I selected this curve as it is a clear example of where the error estimation seems to be wrong, but the orange peak in Extended Fig. 1e and the green curve in Extended Fig. 1g are also polydisperse with very low errors. The authors should double check how they are performing their analysis in Astra as there is clearly a discrepancy with the curves shown in the paper. Although I requested that the errors are given, it is better to not show any errors at all, rather than show them incorrectly - it is ultimately a relatively minor contribution to the paper.

- the authors say they show the curves for untethered systems in black in the much improved Figure 5, however they are still in green in the Figure provided - I assume this was a mistake.

Version 2:

Decision Letter:

Our ref: NSMB-A49383B

26th Mar 2025

Dear Dr. Huang,

Thank you for submitting your revised manuscript "Distant On-Off Switching of Protein Activity by Intrinsically Disordered Region" (NSMB-A49383B). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Structural & Molecular Biology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about 2 weeks. Please do not upload the final materials and make any revisions until you receive this additional information from us.

To facilitate our work at this stage, it is important that we have a copy of the main text as a word file. If you could please send along a word version of this file as soon as possible, we would greatly appreciate it; please make sure to copy the NSMB account (cc'ed above).

Thank you again for your interest in Nature Structural & Molecular Biology Please do not hesitate to contact me if you have any questions.

Sincerely,

Katarzyna Ciazynska, PhD
(she/her)
Senior Editor
Nature Structural & Molecular Biology
https://orcid.org/0000-0002-9899-2428

Version 3:

Decision Letter:

5th May 2025

Dear Dr. Huang,

We are now happy to accept your revised paper "Remote On-Off Switching of Protein Activity by Intrinsically Disordered Region" for publication as an Article in Nature Structural & Molecular Biology.

Acceptance is conditional on the manuscript's not being published elsewhere and on there being no announcement of this work to the newspapers, magazines, radio or television until the publication date in Nature Structural & Molecular Biology.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Structural & Molecular Biology style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

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Due to the importance of these deadlines, we ask that you please let us know now whether you will be difficult to contact over the next month. If this is the case, we ask you provide us with the contact information (email, phone and fax) of someone who will be able to check the proofs on your behalf, and who will be available to address any last-minute problems.

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Sincerely,
Sara

Sara Osman, Ph.D.
Senior Editor
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Reviewers' Comments:

Reviewer #1 (Remarks to the Author):

The manuscript presents a significant advancement in our understanding of allosteric regulation mediated by intrinsically disordered regions (IDRs). It focuses on Sgt2, a crucial protein in the GET pathway responsible for inserting tail-anchored (TA) proteins into the endoplasmic reticulum (ER). The authors provide a thorough examination of how the C-terminal IDR of Sgt2 (Sgt2C) allosterically regulates the conformational entropy of its N-terminal folded domain (Sgt2N) and its interaction with the Get4/5 scaffolding complex. The authors have compiled an impressive body of data using solution NMR, SEC-MALS, MS, and ITC measurements on a library of protein constructs. These data convincingly reveal that the C-terminal IDR segment of Sgt2 is crucial for auto-inhibiting its interaction with the Get4/5 complex. Notably, this inhibition is achieved without direct binding between Sgt2C and Sgt2N. Instead, NMR relaxations experiments suggest that the IDR induces fast internal motions within Sgt2N, thereby creating an entropic barrier that impedes binding to Get4/5. The authors further show that the dynamic interactions between Sgt2C and its substrates subsequently reduce this entropic barrier, allowing for binding. Although the roles of conformational entropy in regulating protein-protein interactions have been well documented, the allosteric modulation by IDRs is not as thoroughly explored. This manuscript addresses this gap with remarkable clarity and depth. The work is meticulously executed and the findings are robustly supported by high-quality figures that clearly illustrate the complex data, providing readers with a comprehensive understanding of the entropic modulation mechanism. Moreover, the authors extend their insights beyond the specific context of Sgt2 by demonstrating that this mechanism can be generalized. They successfully create artificial systems incorporating IDRs to show reduced binding affinities, highlighting the versatility and potential ubiquity of this regulatory mechanism.

The work is carefully executed, and the results are clearly described, with excellent figures summarizing the complex and extensive data. Additionally, the authors demonstrate the general applicability of the entropic modulation mechanism by constructing artificial systems with IDRs that reduce binding affinities. Overall, this manuscript should be of great interest to the readership of NSMB. It offers deep conceptual insights into a potentially widespread biological mechanism, and provides crucial details about this important regulatory protein. The breadth, quality, and innovative nature of this work make it an outstanding contribution to the field.

We sincerely thank the reviewer for the detailed and highly encouraging comments. We deeply appreciate your recognition of the significance of our findings and the thoroughness of our approach. Your positive remarks regarding the quality and depth of our work, particularly on the

role of intrinsically disordered regions (IDRs) in allosteric regulation, are incredibly motivating for our team. The constructive suggestions provided have been invaluable in refining our manuscript, and we have addressed all your points below.

Other comments:

1. Stoichiometry in ITC Data:

Based on the cartoon in Figure 6 and the SEC-MALS data in Figure 3B, it appears that two Sgt2 dimers bind one Get4/5 complex. The n-values (stoichiometry) should be reported to confirm this. Do the reported n-values make sense in this context?

Thank you for pointing this out. Multiple studies, including our own, have demonstrated that the stoichiometry of Sgt2 binding to Get4/5 involves two Sgt2 dimers interacting with one Get4/5 heterotetramer, and our results are consistent with this model. To address your suggestion, we have now included the n-values (stoichiometry) for all ITC experiments in **Extended Table 2**, along with the molar ratios of the corresponding proteins. This additional information further confirms the binding stoichiometry and aligns well with the SEC-MALS data presented in Figure 3B.

2. Sequence Information:

The sequences of the various chimeric constructs presented in Figure 2 should be provided for clarity and reproducibility.

We thank the reviewer for this valuable suggestion. In response, we have now included all amino acid sequences for the chimeric constructs presented in Figure 2 (Extended Data Fig. 5 and Supporting Information Fig. 2) to ensure clarity and reproducibility.

3. Introduction Clarification:

The statement in the Introduction, "Intrinsically disordered regions (IDRs) constitute a substantial portion of the proteome, accounting for approximately 44% of the human proteome ..." is somewhat confusing. If referring to the percentage of residues in the human proteome that are disordered, it aligns with the high end of current estimates (~35%). Clarify that this refers to residues in the proteome. Notably, over 60% of proteins in the proteome contain IDRs 30 residues or longer, which is a different metric.

We thank the reviewer for this insightful comment. To clarify, we have reviewed the latest literature and updated the percentage of disordered residues in the proteome to 30–50%, based on Molecular Cell, 2024, 84, 7, 1186. This ensures alignment with current estimates and addresses potential confusion regarding the metric used."

4. References for NMR Experiments:

The authors should provide references for the NMR methyl-TROSY and dispersion experiments to support their methodology.

We thank the reviewer for the suggestion and have added references for the methyl-TROSY and ^{13}C-CPMG relaxation dispersion experiments in the revised manuscript.

5. Mutation Nomenclature:

In paragraph 2 of the "Plausible models of distant on-off switching" section, the cysteine mutations are referred to backwards (C254S and C294S). They should be labeled as S254C and S294C, consistent with the associated figure.

We thank the reviewer for pointing this out and have corrected the mutation nomenclature in the manuscript.

6. Figure 4B Clarification:

Figure 4B lacks the lower plot of chemical shift perturbations mentioned in the legend. Ensure this plot is included or adjust the legend accordingly.

We thank the reviewer for pointing this out and have corrected the figure legend accordingly.

7. Typographical Errors:

In Figure S1E, correct "misture" to "mixture".

In Figure S3E, change "limited sequence conservation" to "limited positional conservation" or alignment.

We thank the reviewer for these suggestions and have corrected the typographical errors in the revised manuscript, and we have changed it to “alignment”.

8. Terminology Correction:

The term "fast-exchange (ps-ns) dynamic properties" is confusing as exchange implies between discrete states. Remove the term "-exchange" to avoid confusion.

We thank the reviewer for this clarification and have removed the term “-exchange” to avoid confusion in the revised manuscript.

Reviewer #2 (Remarks to the Author):

This interesting manuscript reports on the structure and dynamics of the yeast protein Sgt2 that acts as a chaperone in the GET pathway. The function of Sgt2 is to bind nascent tailed-anchored (TAs) proteins as they emerge from the ribosome. The GET pathway involves the interaction of the Get4/5 complex with the N-terminal, folded domain (Sgt2N) of Sgt2. The manuscript addresses the following question: How can Get4/5 distinguish between TA-loaded and TA-unloaded states of Sgt2?

The manuscript describes an IDP regulatory mechanism by which the presence of the disordered C-terminal domain (Sgt2C) elevates the fast time scale internal motions of Sgt2N without directly contacting this domain. The increased dynamics prevent binding of Get4/5 through an entropic barrier. The binding of clients (TAs) to Sgt2C rigidifies Sgt2N thereby allowing binding of Get4/5, providing an IDR-mediated mechanism by which Stg2 communicates its client-loading status.

I find the described regulatory mechanism intriguing and it introduces a novel concept. The manuscript is therefore of interest to the broad readership of NSMB. Having said that, it is frustrating that the manuscript does not reveal the precise molecular mechanisms by which Sgt2C changes the dynamics of Sgt2N and its interaction with Get4/5. I think the conclusions of the study need to be significantly strengthened by providing additional data and analysis before it can become suitable for publication.

We sincerely thank the reviewer for their thoughtful and constructive feedback. We appreciate your recognition of the novel mechanism described and your insightful suggestions for strengthening our conclusions. We have carefully addressed these points in our revision.

1) The authors have not tested whether the dynamic shielding (model 1) could involve steric clashes with either Get4 or Get5. Previous studies have demonstrated a high-affinity binding of the ubiquitin-like (UBL) domain of Get5 to the N-terminal domain of Sgt2 (line 88). The authors work with a more complex system, the Get4/5 complex. Are the same results obtained when using only the UBL domain of Get5 for the interaction (i.e. no interaction is detected with $Stg2^{FL}$)?

   We thank the reviewer for this thoughtful comment.

   Yes, we have performed this experiment using ITC, although it was not included in the previous manuscript. We have now added this data as Extended Data Fig. 1b in the revised manuscript, along with a revised sentence in the Results section (highlighted in yellow). These results confirm that $Sgt2^{FL}$ does not interact with the UBL domain of Get5, consistent with the

findings for the full-length Get4/5 complex. **This demonstrates that the inability of $Sgt2^{FL}$ to bind Get4/5 is not due to steric clashes.**

2) To prove the absence of long-range interactions between the C- and N-terminal domains, PRE experiments were carried out. Did the authors verify that the PRE label does not interfere with the autoinhibitory effect by checking that PRE-labeled $Stg2^{FL}$ does not bind Get4/5?

   We thank the reviewer for this thoughtful suggestion.

   We have now performed the ITC experiments, which show that spin-labeled $Sgt2^{FL}$ doesn't interact with the UBL domain of Get4/5 (Extended Data Fig.8d in the updated MS). **These data rule out the possibility that the MTSL labelling interferes with the autoinhibitory effect.** This result has been added in the Results section (highlighted in yellow).

3) To provide further proof for the absence of an interaction between the C- and N-terminal domains of Stg2, the authors should compare the dynamics of the disordered domain in isolation and in the context of the full-length protein. ^{15}N R2 or R1rho relaxation rates should be measured and reported along the sequence. A plot comparing the R2 or R1rho rates of $Sgt2^{C}$ in isolation and within $Sgt2^{FL}$ should be added (this should be possible since the resonances of the C-terminal tail are visible in the $Sgt2^{FL}$ HSQC spectrum). Please also add the superposition of the ^{1}H-^{15}N HSQC spectrum of $Sgt2^{FL}$ and $Sgt2^{C}$ to Extended Data Fig. 3. This superposition is essential to evaluate any differences in conformational behaviour of $Sgt2^{C}$ in the context of the full-length protein. This comparison should also be complemented by an I/I_0 plot, where I are the intensities of the isolated $Stg2^{C}$ domain and I_0 the intensities of the C-terminal domain within $Stg2^{FL}$.

   To address the reviewer's thoughtful suggestion, we conducted ^{15}N R_2 relaxation experiments for $Sgt2^{C}$ both in isolation and within the full-length $Sgt2^{FL}$ context. This analysis allowed us to directly compare the dynamics of $Sgt2^{C}$ in the two environments and assess any potential effects of its incorporation into the larger complex. To ensure reliability, we restricted our analysis to signals that were well-resolved in both spectra.

   We included a side-by-side plot comparing the R_2 values of $Sgt2^{C}$ residues in isolation and within $Sgt2^{FL}$ (Extended Data Fig. 4b). Our results show that the R_2 values for isolated $Sgt2^{C}$ range from approximately 2 to 4 s^{-1}**,** which aligns with its expected flexibility as a 13-kDa disordered protein. Within $Sgt2^{FL}$, the corresponding R_2 values range from ~2 to 10 s^{-1}. While this represents a slight increase compared to the isolated domain, it remains consistent with the flexibility of a highly dynamic region tethered to a much larger protein (the dimeric Sgt2 protein has a molecular weight of 74 kD). Importantly, if significant interactions or restricted

motion were present, we would expect substantially higher R_2 values due to slower tumbling. Instead, our data clearly demonstrate that Sgt2^{C} retains its intrinsic flexibility in both contexts.

We have also included a superposition of the ^{1}H-^{15}N HSQC spectra of isolated Sgt2^{C} and Sgt2^{FL} as requested (Extended Data Fig. 3a). The strong spectral overlap indicates minimal perturbations to the Sgt2^{C} chemical environment in the full-length protein, supporting its dynamic nature. Additionally, an I/I_0 plot comparing resonance intensities of Sgt2^{C} in isolation (I) and within Sgt2^{FL} (I_0), where most residues exhibit less than a 50% increase in intensity despite a 5.7-fold decrease in molecular weight (Extended Data Fig. 3a). These results confirms the absence of significant interaction-induced broadening, reinforcing the conclusion that Sgt2^{C} remains dynamically in the context of full-length protein.

These results collectively demonstrate that Sgt2^{C} retains its flexibility whether isolated or part of the full-length protein, and they have been incorporated in the Results (see highlighted in yellow). This finding supports our hypothesis that the autoinhibitory effect of Sgt2^{C} on Sgt2^{N} does not arise from direct interactions but instead is mediated through dynamic and entropic modulation**.**

4) The authors compare HSQC spectra of Sgt2^{NT} without and with an excess of Sgt2^{C} (Fig. 4c). An I/I0 plot should accompany these data. It would also be useful to see the corresponding NMR spectra of ^{15}N Sgt2^{C} with an excess of Sgt2NT and the corresponding I/I0.

We thank the reviewer for this suggestion. We have included the I/I_o plot for the HSQC spectra of Sgt2^{NT} with and without Sgt2^{C} (Extended Data Fig. 8b in the updated manuscript). Additionally, we performed NMR titrations of ^{15}N-Sgt2^{C} with an excess of Sgt2^{NT} and provided the corresponding I/I_o plot (Extended Data Fig. 8a and 8c). These data further confirm the absence of physical interactions between the disordered tail of Sgt2 and its other domains, in aggreement with our previous findings.

5) The authors should provide additional proof for the strict non-binding of Get4/5 to Sgt2FL (beyond the ITC and MALS experiments). Thus, SPR data should be shown for binding of Sgt2FL to Get4/5, and NMR titration data of ^{13}C methyl-labelled Sgt2^{FL} with increasing molar fractions of Get4/5 (or the UBL domain of Get5).

We thank the reviewer for this valuable suggestion. To further support our conclusion, we have performed both SPR and NMR experiments to investigate the interaction between Sgt2^{FL} and Get4/5. These results are now included as Extended Data Fig. 1b and 1c in the updated manuscript. **Both SPR and NMR experiments confirmed that the full-length Sgt2 does not bind to Get4/5, further corroborating our previous ITC and MALS findings.**

6) What is the difference between the proposed models 2 and 3? Can they really be considered as separate models? Why can slow conformational exchange dynamics (model 2) not act as a conformational entropy modulator (model 3)?

We thank the reviewer for raising this important question. The distinction between models 2 and 3 pertains to the timescales and the nature of the dynamics involved. Fast (ps-ns) internal dynamics are associated with local fluctuations and intrinsic disorder, which are well-established contributors to conformational entropy, as highlighted in several studies (*Curr Opin Struct Biol. 2023 Dec;83:102697; Annu Rev Biophys. 2018 May 20;47:41-61; Curr Opin Struct Biol. 2013 Feb;23(1):75-81; Proc Natl Acad Sci U S A. 2017 Jun 20;114(25):6563-6568*). In contrast, slow (µs-ms) internal dynamics involve transitions between distinct conformational states and are more closely linked to conformational selection rather than direct modulation of conformational entropy.

While both timescales can influence protein function, they operate differently: fast dynamics contribute to the thermodynamic ensemble through conformational entropy, while slow dynamics modulate the distribution and interconversion of distinct states. Thus, models 2 and 3 represent distinct conceptual frameworks addressing different aspects of protein dynamics.

We hope this explanation clarifies the rationale for considering these as separate models.

7) With respect to model 2, CPMG dispersion is observed only in the T domain and not in the N domain. Figure 4f shows only dispersion curves in the T domain. It would make more sense to show some flat dispersion curves for residues in the N-domain, either directly in Figure 4f or in the Supporting Information. The absence of CPMG dispersion in the N-terminal domain does not rule out very slow conformational exchange processes, these would need to be detected with for example CEST experiments.

We thank the reviewer for this insightful suggestion. In response, we have added epresentative flat curves for the CPMG data (Extended Data Fig. 9 a and 9b) to illustrate the absence of dispersion in the Sgt2N domain. Additionally, we prepared protein samples and collected ^{13}C-methyl CEST data. Consistent with our CPMG results, slow conformational dynamics were observed exclusively in the Sgt2^{T} domain and not in the Sgt2^{N} domain (Extended Data Fig. 9c and 9d).

While the attachment of Sgt2^{C} induces changes in conformational exchange behavior, as also evidenced by our CPMG data, such changes are unlikely to contribute to the autoinhibitory effect since Sgt2^{T} is not directly involved in Get4/5 binding. These results have been incorporated into the manuscript (highlighted in yellow).

We hope this additional data and clarification adequately address the reviewer's concern.

8) For model 3: I have doubts about the analysis of the methyl relaxation data. If I remember correctly, the overall molecular tumbling time (correlation time) would need to be known in order to extract the order parameters from the relaxation data. The correlation time would change between the constructs used (Stg2^{FL}, Stg2^{NT}, Stg2^{FL}-fusion^{TMD}). Which correlation time was used for the different constructs and how were these values derived? One can even imagine that a global correlation time would not be appropriate and that a domain-specific correlation time would need to be applied for each construct. For a "beads-on-a-string" protein each individual domain may behave independently of the others. In general, details of this analysis (extraction of S^2) should be provided in the Methods or Supporting Information. It would also be useful to see plots of Ia/Ib versus relaxation delay superimposed for the three different constructs. Was a sample used with a deuterated background to minimize relaxation effects from nearby protons?

We thank the reviewer for these critical comments. We agree that the correlation time is crucial for extracting order parameters (S^2) from relaxation data. In the original draft, we noted in the Methods section under the subsection "Fast internal motion (ps-ns) dynamic experiments" that protein hydrodynamic radii were determined using dynamic light scattering (DLS). The correlation times for the different Sgt2 constructs were subsequently derived from these hydrodynamic radii, which were measured as 12.0 nm, 15.6 nm, and 15.2 nm for Sgt2^{NT}, Sgt2^{FL}, and Sgt2^{FL}-Ysy6^{TMD}, respectively (Extended Data Fig. 13b).

To address the reviewer's concerns, we have now added further details in the Methods section (highlighted in yellow in the updated manuscript) explaining how these correlation times were calculated using the Stokes-Einstein-Debye equation. We acknowledge the reviewer's point that, for a "beads-on-a-string" protein, the correlation time might vary across domains. However, after careful inspection, the derived values appear reasonable: most residues within the rigid regions exhibit order parameter values close to 1, while the flexible linker residues have values around 0.1, which aligns with expectations for such systems.

While the derived values appear reasonable, we share the reviewer's concern regarding the potential impact of inaccurate correlation time measurements on the NMR-derived dynamics for such a protein. To address this, we utilized HDX mass spectrometry in the original draft to independently assess the dynamic behavior. The HDX results showed excellent agreement with our NMR data. Furthermore, in response to another reviewer's suggestion, we have now performed additional HDX experiments for the Sgt2-Ysy6^{TMD} complex (see updated Fig. 4m and Extended Data Fig. 13a). These new results are fully consistent with our NMR analysis and further support the proposed dynamic regulation model.

As requested, we have also included a superimposed plot of I_a/I_b versus relaxation delay for representative residues in the three different constructs in updated Fig. 4i. Additionally, all samples used for the dynamic analysis were methyl-labeled and prepared in a deuterated background to minimize relaxation contributions from nearby protons.

We hope these revisions and additional details adequately address the reviewer's concerns.

9) I would like to see further support for the change in dynamics of the N-terminal domain in the presence of the C-terminal tail. The minimal system containing auto-inhibition would be a construct "Stg2NCshort". This construct contains the N-terminal domain directly linked to the C-terminal domain spanning residues 221-265 (construct lacking the T domain). The NMR spectrum of Stg2NCshort should be shown along with a plot comparing heteronuclear NOEs of Stg2NCshort and Stg2N. A titration of this construct with the UBL domain of Get5 would also be useful, to confirm the absence of an interaction.

We appreciate the reviewer's suggestion and have prepared the $Stg2^{N\text{-}Cshort}$ construct as recommended. ITC confirmed the absence of interaction between this construct and the UBL domain of Get5 (Extended Data Fig. 11c). Additionally, we measured and compared the heteronuclear NOEs of $Stg2^{N\text{-}Cshort}$ and $Stg2^{N}$ (Extended Data Fig. 11d), which revealed an overall decrease in NOE values. Notably, residues L33, N34, S41, and E42—key regions involved in binding with the UBL domain of Get5—exhibited significant reductions. Additionally, in the saturated spectrum, the attached truncated $Sgt2^{C}$ fragment displayed negative values, confirming its highly dynamic nature and the absence of stable interaction with the $Sgt2^{N}$ (Extended Data Fig. 11e).

These findings have been incorporated into the manuscript (highlighted in yellow in the updated draft) and align closely with our proposed entropy-driven autoinhibitory model. We believe this data provides strong support for the dynamic changes in the N-terminal domain in the presence of the C-terminal tail.

10) Figure 2a: I am surprised to see that a single residue in the IDR can be responsible for changing from "No interaction" to a "Kd of 400 nM" (comparison of the 1-264 and 1-265 constructs). Would this not rather indicate the disruption of a "binding motif" embedded in the IDR? Comparison of the sequences of yeast and mammalian Stg2 reveals conserved residues exactly at this 264/265 position (Extended data Fig. 6).

We thank the reviewer for this critical comment. Purification of FL-Sgt2, particularly C-terminal partially truncated constructs, is indeed challenging due to the propensity of disordered segments for degradation. For each experiment, we prepared at least three independent samples, verified their integrity using SDS-PAGE analysis (now included in the

supporting information), and ensured consistent results. Like the reviewer, we were initially surprised that a single residue could cause such a significant change in binding affinity. However, we are confident in the reliability of our results.

We appreciate the reviewer highlighting that residues around 264/265 are conserved between yeast and mammalian Sgt2. This observation was previously overlooked, but we have now revised the text to emphasize its importance (see modified text in Results, highlighted in yellow). While this region may initially appear to represent a binding motif, our extensive experiments strongly argue against this possibility (see Fig. 4 and others).

At the start of this project, we also considered that the observed autoinhibition might be mediated by a "physical shielding" mechanism, involving a binding motif in $Sgt2^{C}$. This scenario, commonly reported in other systems, would require strong interactions between $Sgt2^{N}$ and $Sgt2^{C}$ to disrupt the binding of $Sgt2^{N}$ to Get5-UBL (K_d ~ 100 nM). Given NMR's sensitivity to even weak and transient interactions, we conducted extensive experiments but found no evidence of interaction between $Sgt2^{N}$ and $Sgt2^{C}$, let alone the strong interaction needed for this mechanism.

After thorough investigations, we concluded that the autoinhibition mechanism is not physical shielding but dynamic modulation.

The versatility of this IDR further challenges the "physical shielding" model (Fig. 5), as the segment is unlikely to act as a common binding motif for various proteins. While this study is an initial effort to understand such effects, we are continuing to investigate the molecular "codes" embedded within this IDR. Notably, we recently discovered a protein that modulates kinase activity, which completely loses its binding ability to the kinase when attached to $Sgt2^{C}$. This protein, unrelated to the GET pathway, highlights the broader relevance of dynamic regulation mediated by the Sgt2 IDR. We look forward to presenting more details in the future.

11) Line 235: The authors state that there is no change in dynamics of $Stg2^{C}$ upon binding to Ysy6-TMD. Relaxation is shown for five residues in Fig 3g; however, these are all located at the border of the binding site and, as such, do not directly report on the dynamics at the interaction site (where the NMR peaks have essentially disappeared). I find it difficult to imagine a binding regime in which the NMR resonances disappear but the dynamics remain unchanged. Could the authors explain this?

We thank the reviewer for this insightful question. Our results indicate that the dynamics of $Sgt2^{C}$ on the fast timescale (ps-ns) remain largely unchanged upon binding to Ysy6-TMD, as evidenced by the data in Fig. 3g. However, the signals at the interaction site are significantly attenuated. It is important to note that NMR signal intensity is strongly influenced by the

effective transverse relaxation rate (R_2), which is highly sensitive to exchange processes occurring on slower timescales (µs-ms), such as conformational changes or chemical exchange.

In this case, the binding of the TMD to $Sgt2^C$ appears to be highly dynamic, as demonstrated by the CPMG relaxation dispersion data (Fig. 3h). This dynamic binding leads to significant exchange between the bound and unbound states on the µs-ms timescale, which likely results in the attenuation or disappearance of NMR resonances at the interaction site. Although $Sgt2^C$ remains largely disordered, as supported by its low order parameter values, the dynamic exchange between states contributes to the observed signal loss.

We hope this explanation adequately addresses the reviewer's concern.

12) Figure 3h: I failed to find information about which residues in the Ysy6-TMD part of the fusion are observed in the NMR spectra? Which residues specifically are reported in Fig. 3h? The authors report an exchange rate constant of 3000 s-1 from CPMG experiments, however, would the kex value not rather be the sum of the kon and the koff rate (as indicated in line 674, I am not sure what Ø means in the equation in line 673)? If the kex is directly equal to the off-rate, then the bound population of Ysy6-TMD would be very low, which does not seem to be consistent with the disappearing NMR signals across the entire Stg2C. The authors should provide the equations according to which the CPMG data were analysed and an analysis of how confidently the parameters can be extracted. Normally, two-field measurements are required to accurately extract the exchange parameters (population, exchange rate and chemical shift).

We thank the reviewer for these critical comments. Unfortunately, due to the poor quality of the NMR spectra, we were unable to directly assign the specific residues in the Ysy6-TMD region. Based on their positions, these residues are most likely leucines. The spectral overlay of $Sgt2^C$ and the Sgt2-$Ysy6^{TMD}$ fusion strongly suggests that these signals correspond to residues in the TMD, as they appear as additional peaks upon fusion and do not match $Sgt2^C$ based on their NOE patterns. Therefore, we have ambiguously assigned these signals to the Ysy6-TMD. We believe the exact residue assignments are not critical, as the observed signals (from 4 out of 7 leucine residues in the Ysy6-TMD) sufficiently represent the dynamic behavior of the TMD in the chaperoning complex.

All CPMG experiments were conducted using both 600 MHz and 850 MHz spectrometers (e.g., Extended Data Fig. 9a and 9b). However, the use of 850 MHz spectrometer was inadvertently omitted in the original draft, and we have clarified this in the updated Methods section. Upon re-evaluating our data, we realized that the previously reported k_{off} value of 3000 s^{-1} was a

simple copy-and-paste error from Fig. 3g. The correct value, 2600 s^{-1}, has now been updated in the manuscript.

We have also included detailed information about the CPMG parameters in the revised manuscript (highlighted in yellow). The bound state population was calculated to be 13.7 ± 3.8%.

Lastly, we have removed the first equation from the manuscript, as it was not used in the actual calculations. We hope these revisions and clarifications address the reviewer's concerns.

13) Figure 5 is reporting the use of the C-terminal domain of Sgt2 for modulating the interaction of diverse proteins. The same experiments need to be carried out for the same proteins, but where the $Sgt2^C$ tail is replaced by another IDR of the same length (for example Tau protein). It is otherwise not possible to know whether the observed effects originate from the sequence characteristics of $Sgt2^C$ or whether its rather a general "dynamic shielding" mechanism whereby the IDR partially prevents partner protein binding.

We thank the reviewer for this very helpful suggestion. To address it, we prepared protein samples where the $Sgt2^C$ tail was replaced by a 65-residue disordered Tau fragment, as suggested, and evaluated the effects using ITC (updated Fig.5 and Extended Data 14b). These additional experiments provided insights into the regulatory potential of different IDRs. While the Tau fragment showed no effect in some cases, it exhibited similar or even stronger regulatory effects in others, accompanied by an entropy penalty during binding. Alphafold predictions suggest that it is unlikely the attached IDR binds directly to the effector domain (see Supporting Information). These findings highlight the versatility of IDRs and suggest that different tails can exert distinct effects depending on the protein context. The results of these experiments have been incorporated into the updated manuscript (highlighted in yellow).

Furthermore, we believe the mechanism is unlikely to be a general "dynamic shielding" model, which would require the IDR tail to directly approach the binding sites—contrary to our NMR titrations and PRE data. Future work will focus on decoding how specific IDRs modulate binding activity for certain proteins.

14) Generally, for the superposition of NMR spectra, please make the resonances in the spectrum on top with outer contours only (peaks with a hole in the middle) so that it is more visible how well the top spectrum overlaps with the bottom spectrum. Currently, a lot of features could be hidden, and it is not visible whether all peaks on top actually have peaks below them. This applies to all superpositions of spectra throughout the manuscript, please amend this.

We thank the reviewer for this valuable suggestion. In response, we have revised all the spectral superposition figures to display the top spectrum with outer contours only. This adjustment enhances visualization and ensures that the overlap between the top and bottom spectra is clearly visible.

Minor points:

1) Extended Data Figure 1a: Please provide error bars over the three replicates for the SPR experiments in the table (SgtNT + Get4/5).

We thank the reviewer for this suggestion and have added the error bars for the three replicates as requested.

2) Extended Data Figure 1e: Correct the spelling mistake (Misture -> Mixture).

Thank you for pointing this out. We have corrected the spelling mistake in the revised manuscript.

3) Extended Data Figure 1b, c, d, e: I assume that the molecular masses in the MALS figures were derived from the experiments. The legend should contain the theoretical masses derived from sequence for comparison.

Thank you for this helpful suggestion. We have included both the measured molecular masses (with error bars) and the theoretical values derived from sequence in the updated figures and figure legend.

4) Extended Data Figure 2a: Please add labels corresponding to the assignment of the different peaks in the 1H-15N HSQC spectrum. Please deposit the assignment of this domain in the BMRB database and state the degree of completeness for the assignments.

Thank you for your suggestion. We have added the labels to the spectrum and deposited the assignment in the BMRB database (52887). Additionally, we have stated the degree of assignment completeness in the Methods section of the revised manuscript.

5) Fig 1d: please state in the legend which chemical shifts (Ca, Cb, C' etc) were used as input for the SSP calculation.

Thank you for your comment. We have included the information regarding the chemical shifts used (Cα, Cβ, and NH) in the figure legend.

6) Extended Data Figure 2d, top: please indicate the color code in the legend.

Thank the reviewer for this suggestion. The curves represent predictions from different methods: MetaDisorder3D (purple) uses structural alignments, MetaDisorder (blue) is a consensus-based predictor, MetaDisorderMD (green) combines sequence- and structure-based predictions, and MetaDisorderMD2 (red) optimizes these predictions for higher accuracy. And we have added this information in the Figure legend.

7) Extended data Table 1: please provide error bars on the Kd and the thermodynamic parameters. Please add the stoichiometry, N, to the table for each measurement.

We have added the error bars and included all parameters, including the stoichiometry (N), in the updated Extended Data Table 2. Thank you for bringing this to our attention.

8) Rename Figure S4 to Extended Data Figure 4.

Thank you for pointing this out. We have renamed Figure S4 to Extended Data Figure 4 in the revised manuscript.

9) Extended Data Fig 7b: Why does the stoichiometry change between ITC experiments with different constructs (1:1 for Tau and L20 and 1:2 for Stg2 variants)? Update the legend on the x-axis in all ITC experiments to state which molar ratio is plotted (with protein names).

Thank you for raising this issue. The stoichiometry differences arose due to inconsistent use of Sgt2 monomer and dimer concentrations. We have corrected this and rechecked all ITC data to ensure consistency throughout the manuscript. The ITC figure legends have been updated to include protein names, and the figure legends now state the oligomeric information used for concentrations.

10) There seems to be no reference to Extended data Fig. 14.

Extended Data Fig. 14 has been referenced in the Methods section under the section titled "Dynamic Light Scattering (DLS) measurements." This figure was used to derive the rotational correlation times (τc) for S^2 calculation.

11) Please provide the sequences of the fusion proteins.

We have now provided the amino acid sequences for all fusion proteins in the revised manuscript (Extended Data Fig. 5a-g and 14a and Supporting Information Fig. 2).

12) In Fig. 3d, it seems that the I/I_0 ratios are cut at 100. Please provide the data showing all ratios even if they exceed 100.

Thank you for the suggestion. We have updated the figure to display all I/I_0 ratios, including those exceeding 100.

Reviewer #3 (Remarks to the Author):

The manuscript by Ji et al., investigates the effect both the C-terminal intrinsically disordered region (IDR) of Sgt2, a targeting factor within the GET pathway, and its interaction with the substrate tail anchor (TA) protein have on the downstream interaction with Get4/5. Previous biochemical and biophysical studies had already established the disordered conformation of the Sgt2 C-terminus, along with its role in binding the TA protein transmembrane domain (TMD)(Lin et al., 2021). The novelty of this manuscript lies in the identification of a novel autoinhibition between the Sgt2 IDR and its N-terminal dimerization domain, which prevents its interaction with Get4/Get5. The authors attribute this autoinhibition to an increased flexibility of the N-terminal domain in the presence of the IDR, with binding of the substrate TMD to the IDR leading to rigidification of the N-terminal domain and interaction with Get4/Get5. Most interestingly, transplantation of the Sgt2 IDR on to unrelated protein-protein interaction domains impairs their interactions with canonical binding partners. Whilst this concept is of interest to the field, the main weakness of the manuscript is the suboptimal quality with which some experiments are explained or represented, which makes it difficult to judge some of the findings. There is also a failure to consider these results and their physiological relevance in the context of existing literature on Sgt2.

We sincerely appreciate the reviewer's thorough evaluation of our manuscript and the recognition of our study's novelty in uncovering an autoinhibitory mechanism mediated by the Sgt2 IDR. We acknowledge the reviewer's concerns regarding the clarity of some experimental explanations and data representations. In response, we have carefully revised the manuscript to improve the clarity of our descriptions and figure legends. Additionally, we have incorporated further discussion to better contextualize our findings within existing literature on Sgt2, addressing its physiological relevance. We hope these revisions adequately address the reviewer's concerns and enhance the overall clarity of our study.

Thank you for your insightful feedback.

My major concerns are as follows:

1. There are no figures showing the SDS-PAGE and/or gel filtration of purified proteins used for downstream experiments, particularly for these poorly soluble or degradation prone constructs (e.g. Sgt2-C, full-length Get4/Get5).

We thank the reviewer for this suggestion. Yes, purification of FL-Sgt2, $Sgt2^{C}$, and particularly C-terminal partially truncated constructs, is indeed challenging due to the propensity of disordered segments for degradation. For each experiment, we prepared at least three independent samples, verified their integrity using SDS-PAGE analysis (now included in the supporting information), and ensured consistent results.

2. There is no comment on why previous studies observe an interaction between full-length Sgt2 and Get4/Get5 by e.g. pull-down assays (Chartron et al., 2011) or yeast two hybrid assays (Chang et al., 2010). Whilst the ITC experiments look convincing, there is no explanation why they are performed in a non-physiological buffer (100 mM MES pH 6.0, 150 mM KCl) and it is not clear how many biological replicates were performed for each experiment. Instead of stating the approximate (~) KD, the KD should be given with errors.

We thank the reviewer for this comment. After carefully reviewing the studies by Chartron et al. (JBC 2011), Chang et al. (2010), and Xu et al. (Cell Reports 2012), we found that Chartron et al. explicitly noted that truncated Sgt2 forms stable complexes with Get4/5, while full-length Sgt2 does not, stating: '**the full-length proteins do not form a complex stable over SEC.**' This observation was directly mentioned in both the results and discussion sections in this paper, aligning perfectly with our findings.

Regarding other studies such as yeast two-hybrid assays (Chang et al., 2010) or co-precipitation experiments (Xu et al., 2012), these methods detect protein-protein interactions within a cellular environment but do not distinguish between the TA-loaded and unloaded forms of Sgt2. Our findings show that the functional state of Sgt2 is critical for its interaction with Get4/5, with the unloaded form not interacting with Get4/5, while the TA-loaded form may facilitate such interactions.

Thus, the yeast two-hybrid and co-precipitation results might reflect interactions in the TA-loaded state, where endogenous TA clients in yeast cells could promote binding. Importantly, none of these studies directly demonstrated interactions between recombinant $Sgt2^{FL}$ and Get4/5. In contrast, our extensive biophysical experiments, including ITC, SPR, NMR, and SEC-MALS, specifically controlled for Sgt2's functional states, providing a clearer understanding of how Sgt2's state influences its interaction with Get4/5.

We have added a brief comment on this in the updated manuscript, highlighted in yellow in the Discussion section.

Regarding the buffer choice, we used MES buffer for consistency, as NMR spectra of $Sgt2^{C}$ exhibit optimal behavior in this buffer. ITC experiments performed in PBS yielded identical results, as

shown in the example (Extended Data Fig. 1a). Lastly, we have included all error values for the K_d measurements as suggested (updated Figures and Extended Table 2).

3. In the MALS experiments, the molar mass seems to be rather variable across some peaks. Again, the experimental molar mass should be given with errors. It would also help the reader to give the expected molar mass for the assigned complex.

We thank the reviewer for this comment. While some MALS data may appear slightly heterogeneous, particularly for the complexes, we believe this reflects their dynamic architecture. We have now included the errors and theoretical values (see updated Extended Figures 1e-h, and Figure 3b). The small errors and close agreement between measured and theoretical molar masses confirm the reliability of the data.

4. Only one representative solution structure for Sgt2 is shown, when it is more standard to show an ensemble of possible structures from such structure calculations. In this case, this would particularly help to illustrate the range of possible movements between the different domains and how close Sgt2-C is able to approach to Sgt2-N. There should also be a table of restraints and structural statistics as for any structure solution.

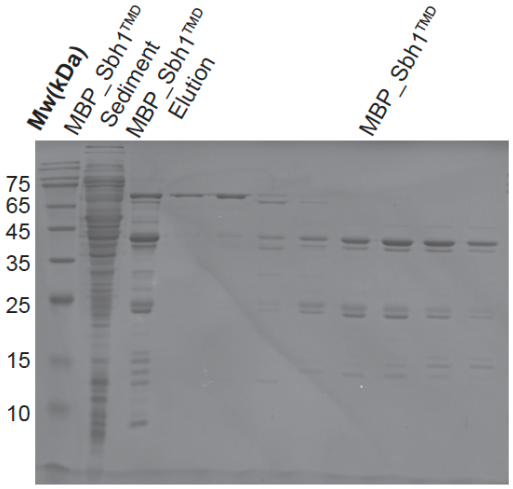
We thank the reviewer for this suggestion. Due to the dynamic "beads-on-a-string" conformation of Sgt2, we initially chose not to show the ensemble structures, as they would depict highly flexible domains with no well-defined arrangement. However, to address the reviewer's comment, we have now included a figure (Extended Data Fig. 2e in the updated manuscript) that shows the full dynamic range of Sgt2's flexibility. This figure demonstrates that the disordered $Sgt2^C$ is randomly distributed with no preferred location, which is consistent with our NMR titration, PRE, and SAXS data.

Additionally, we have included the structural statistics table (Extended Data Table 1), which now also includes the restraints as requested. And the calculated structure has been deposited in PDB (9LLV).

5. Given the Sgt2 C-terminus is responsible for autoinhibition, it could still be the fusion of the substrate TMD to the C-terminus that disrupts this autoinhibition, rather than the internal interaction between the TMD and C-terminus. As a control, the authors should test the interaction between Get4/Get5 and Sgt2 fused C-terminally with a non-interacting peptide of similar size. These experiments should be performed with a reconstituted Sgt2-TMD complex (several approaches have been successfully taken in the literature e.g. coexpression with MBP-TMD fusions (Lin et al., 2021) or handover from Ssa1 (Cho and Shan, 2018)) but the authors do not comment further on their "extensive efforts" to obtain such a complex.

We thank the reviewer for these thoughtful suggestions. In response, we prepared a protein construct by fusing a Tau fragment (a non-interacting peptide) to the C-terminus of Sgt2 and assessed its interaction with Get4/5 by ITC. As expected, the non-interacting peptide did not evoke binding of Sgt2 to Get4/5 (please see Extended Data Fig. 6b in the updated manuscript). This confirms that only specific interactions between $Sgt2^{C}$ and the TMD enable such binding. This result has been incorporated into the manuscript (highlighted in yellow).

Regarding the reconstitution of the Sgt2-TA complex, we acknowledge that previous studies (e.g., Lin et al., 2021; Cho and Shan, 2018) have demonstrated interactions using co-expression or pull-down methods. However, despite extensive efforts, we were unable to obtain sufficient quantities of homogeneous, stable Sgt2-TA complexes suitable for ITC or NMR studies (see the figure for our attempts). Our results are consistent with Cho and Shan (2018), which demonstrated that Sgt2-TA complexes, even when facilitated by Ssa1, are inherently dynamic and kinetically unstable (e.g., Fig. 1C, 1D, and 4F in Cho and Shan (2018)). In such cases, bound TA substrates continuously dissociate from Sgt2 and precipitates, resulting in sample heterogeneity and instability. This dynamic nature of Sgt2-TA interactions was also observed in our NMR analysis (Fig. 3h, i).



Purification of the MBP-Sbh1TMD as shown in Lin, et. al., 2021

To overcome these challenges, we adopted the fusion strategy, which is widely recognized for capturing transiently formed complexes. This approach allowed us to stabilize the interaction and perform the necessary biochemical and structural studies, providing robust and reliable data.

We hope this explanation addresses the reviewer's concerns.

6. I don't think "the high solubility observed in aqueous solutions for both fusion constructs" is sufficient evidence to argue against both Sgt2-C domains of the dimer being required to escort

the hydrophobic TA TMD. Indeed, evidence from Cho et al., 2024 suggests both Sgt2-C domains are required and that the overall conformation of the dimer changes substantially upon TA binding. This possibility should be discussed in the context of the observed line broadening. Furthermore, the authors may bias against such a concerted binding with their C-terminal TMD fusions to Sgt2 enforcing a high local concentration of the substrate adjacent to its binding site.

We thank the reviewer for these insightful comments. Upon further consideration, we agree that the high solubility observed in aqueous solutions for both fusion constructs does not necessarily support the claim that "only one $Sgt2^{C}$ segment is sufficient to effectively escort the hydrophobic TA^{TMD}." This conclusion may have been influenced by the high local concentration of the substrate resulting from the fusion. To clarify, we have revised the sentence as follows: “It is noteworthy that although the dimeric Sgt2 contains two $Sgt2^{C}$ domains, attaching only one $Sgt2^{C}$ segment is sufficient to effectively maintain the hydrophobic TA^{TMD} in solution.”

Regarding the potential open-to-closed conformational changes in Sgt2 upon TA binding (Cho et al., 2024), our NMR analysis of full-length Sgt2-TA^{TMD} fusion constructs did not provide direct evidence for such global conformational transitions. Specifically, we did not observe significant chemical shift perturbations beyond the client binding sites. This absence may be explained by the additional TA^{TMD} introduced by the fusion strategy, which could mask potential cooperative interactions by eliminating competition between the two C-terminal domains.

Interestingly, our NMR analysis of chimeric fusion constructs, in which two $Sgt2^{C}$ domains are linked to a single TA substrate, indirectly supports this dynamic behavior. These constructs exhibit a single set of broadened resonances, indicative of rapid exchange between the two C-terminal domains competing for the same substrate. In the full-length context, such competition likely drives the two $Sgt2^{C}$ into proximity, inducing a closed conformation. **We have incorporated this discussion into the manuscript (highlighted in yellow).**

7. More detail should be given on how the IDR fusion to the PDZ domains, SPOC domain and GABARAP could structurally impact their interactions i.e. the position of the C-terminus relative to the binding interface in the (predicted) structure. Again, the protein purifications are not shown and it is questionable how the non-sigmoidal ITC curves obtained for MAG12 and GABARAP can be used to obtain trustworthy KD values. This data should also be added to Extended Data Table 1 to show the evidence for the claimed “entropy penalty due to IDR tail tethering”.

We sincerely appreciate the reviewer’s insightful comments.

To address the structural impact of IDR fusion, we have now used AlphaFold to predict the fusion construct structures. The models show that the IDR tails do not make direct contacts with the

effector domains, confirming that their influence on binding is not mediated by direct interactions but rather through an entropy-driven mechanism. These structural predictions are now incorporated into the supplementary materials for clarity.

Regarding the ITC data, we acknowledge that the curves for MAGI2 and GABARAP do not exhibit perfectly sigmoidal shapes. This is likely due to the relatively weak interaction affinities and concentration limitations. However, the fitted errors for these two proteins remain within an acceptable range, with the largest error around 20%. We believe this does not impact our overall conclusion. Additionally, we have now included the detailed ITC fitting parameters in Extended Data Table 2, as requested.

Finally, we acknowledge the reviewer's concern about the broader relevance of our findings. While this study serves as an initial effort to investigate such effects, we are continuing to explore the molecular "codes" embedded within the IDR. **Notably, we recently identified a kinase-modulating protein that loses its binding ability when tethered to Sgt2^{C}, further supporting the broader significance of entropy-driven regulation by IDRs**. We plan to present more details in future studies. If the reviewer strongly prefers, we are open to removing the subsection on MAGI2 and GABARAP, as these findings primarily serve as preliminary validation.

We hope this revision sufficiently addresses the reviewer's concerns.

8. The discussion is vague and rambling, discussing the IDR in very generic terms. The authors need to discuss their results in the context of the wealth of literature on Sgt2 and consider the physiological impacts of their findings for the GET pathway. For example, the evidence that Get4/Get5 binds the ribosome to position Sgt2 close to the exit tunnel (Zhang et al., 2021) seems at odds with their model. Similarly, Sgt2 dimer dynamics (Cho et al., 2024) or the structural models of Lin et al., 2021 are not discussed.

We thank the reviewer for this insightful comment.

In our initial manuscript, we focused primarily on the novel regulatory role of the IDR, as we believe the discovery of this entropy-based on-off protein regulation mechanism is the most striking finding of our study. However, we agree with the reviewer that situating our findings within the broader context of the GET pathway, particularly in relation to Sgt2, adds valuable depth to the discussion.

Regarding Zhang et al. (2021): Their model proposes that ribosome-bound Get4/5 recruits Sgt2 and facilitates its capture of nascent TAs. However, our findings indicate that TA-unloaded Sgt2 does not interact with Get4/5, challenging this model. Instead, we propose an alternative explanation: ribosome-bound Get4/5 may enhance interactions by creating a high local

concentration near the ribosome exit tunnel, thereby allowing TA-loaded Sgt2 to efficiently engage with Get4/5. This perspective reconciles our observations with the proposed role of Get4/5 in ribosome-proximal TA targeting.

Regarding Sgt2 dimer dynamics (Cho et al., 2024) and structural models (Lin et al., 2021): We have incorporated these studies into the revised manuscript. Our "beads-on-string" structural model of Sgt2 is consistent with conformational flexibility, facilitating transitions upon TA binding. Furthermore, our NMR analysis of fusion constructs provides indirect evidence for dynamic interactions between Sgt2C domains, likely driving the conformational transitions described in these studies.

These discussions have been added to the revised manuscript (highlighted in yellow in the discussion section) to address the reviewer's suggestions.

More minor comments are as follows:

9. There should be primary data in Figure 2 and Figure 5. In general, the high complexity of the main Figures makes them difficult to understand. It is not necessary to show the full GET pathway in Figure 1 and it is currently redundant with Figure 6.

We sincerely appreciate the reviewer's thoughtful suggestions. As requested, we have added the primary ITC data in Fig. 2 (see Fig. 2a-d in the updated MS).

Regarding Fig. 5, we carefully considered incorporating ITC data but ultimately decided against it for two reasons:

(1) Readability—Fig. 4 already presents a large volume of data (panels a-m), and to maintain clarity, we designed Fig. 5 as a conceptual summary rather than a data-heavy figure.

(2) Color consistency—Integrating ITC curves into Fig. 5 would create inconsistencies with the established color scheme, potentially making the figure harder to interpret.

That said, if the reviewer feels strongly that including ITC data in Fig. 5 would be beneficial, we would be happy to incorporate the current Extended Data Fig. 14b-e into the main figure.

Similarly, we opted to retain the GET pathway depiction in Fig. 1 to provide a comprehensive overview of the broader chaperone system and how downstream components respond to client loading. While Fig. 6 focuses specifically on Sgt2-Get4/5 interactions, Fig. 1 sets the stage for understanding the larger biological context.

We hope the reviewer finds this reasoning acceptable, but we remain open to modifications if needed.

10. The authors stress several times that the autoinhibition is "mediated remotely by the Sgt2-C domain ~150 residues away". This statement is misleading; the distance in the primary protein sequence is irrelevant compared to the distance within the tertiary structure. To appreciate this, a full repertoire of solution structures should be shown (see comment 4).

We appreciate the reviewer's valuable feedback.

In the updated Extended Data Fig. 2e, we now present the solution structure ensemble of Sgt2, which clearly shows that $Sgt2^{C}$ does not make direct contact with $Sgt2^{N}$. This is further supported by our NMR titrations, PRE experiments, and other structural analyses.

To fully address the reviewer's concern, we have removed the phrase "~150 residues away" from the revised manuscript to avoid any potential misinterpretation regarding sequence-based distance versus structural distance.

We hope this revision resolves the reviewer's concern.

11. Line 140: the authors should state that Extended Figure 3a shows that the domains behave independently, rather than just writing it in the figure legend.

Thank you for the suggestion. We have updated the main text to explicitly state the independent behavior of the domains (highlighted in yellow).

12. The authors state that Sgt2 and SGTA have "little sequence homology" but their alignment shows some conserved motifs. The authors should comment on the position of these motifs relative to the truncations that abrogate the autoinhibition.

We have briefly discussed the conserved motifs near residues 264/265 in the updated MS (highlighted in yellow), including their relevance to the truncations that abrogate autoinhibition. Thank you.

13. The signal broadening is not clear from the overlay shown in Extended Figure 8b. In general, NMR spectral overlays where no change is observed should be contoured so that the bottom spectrum is visible.

Thank you for this helpful suggestion. In response, we have revised all the spectral superposition figures to display the top spectrum with outer contours only. This adjustment enhances visualization and ensures that the overlap between the top and bottom spectra is clearly visible.

14. The authors should comment that although the addition of TFE did not change spectral quality, there is a clear effect on helix formation in the CD. This could be rationalized in terms of the model presented by Lin et al., 2021. How does the TA TMD binding site in Figure 3d compare with this model?

We appreciate the reviewer's suggestion. We have updated our manuscript to include a discussion on the effect of TFE on helix formation, despite the lack of spectral improvement (highlighted in yellow). Additionally, our identified TA TMD binding region aligns with the model presented by Lin et al., 2021, as now indicated in the revised manuscript with the phrase "consistent with previous studies."

15. The authors should be careful to state that HDX-MS shows changes in solvent accessibility. It is their interpretation that this shows the domains change their dynamic properties. It is not clear from the figure legends what construct was the reference for calculating ΔHDX. The HDX would also be a useful experiment to conduct with the TA TMD fusions.

We thank the reviewer for this insightful suggestion. To ensure accuracy, we have clarified that HDX-MS primarily measures solvent accessibility, and we now explicitly state that changes in exchange rates suggest altered dynamics rather than proving them directly (highlighted in yellow). Additionally, we have updated the figure legend to clearly define ΔHDX and specify the reference construct used for comparison. Furthermore, we have conducted HDX experiments for Sgt2-Ysy6^{TMD} and incorporated the data (Fig. 5m and Extended Data Fig. 13a), which aligns fully with our Entropy-Modulation model.

We sincerely thank the reviewers and the editor for their careful assessment and constructive feedback on our manuscript. As part of the review process, we previously provided a provisional response addressing the reviewers' key concerns. In this revised submission, we have now comprehensively refined our responses and the manuscript itself, fully addressing all reviewer comments. Specifically, we have clarified methodological issues, improved figure clarity, and enhanced the transparency of our data presentation, including repeating certain experiments to ensure robustness. Below, we provide a detailed point-by-point response to each comment. We are grateful for the opportunity to further improve our work and hope that these revisions meet the reviewers' expectations.

Reviewer #2 (Remarks to the Author):

I thank the authors for carefully revising the manuscript and adding several new data supporting the conclusions of the manuscript. I still have one point that I insist should be further addressed: The authors have added details on the extraction of the methyl order parameters. Specifically, dynamic light scattering was used to determine the hydrodynamic radii of the different protein constructs, and the rotational correlation times (tau_c) were calculated using the Stokes-Einstein-Debye equation. The reported tau_c values are 157 ns (Sgt2NT), 344 ns (Sgt2FL), and 328 ns (Sgt2-Ysy6TMD)*. However, given the molecular weight of these proteins, these values appear significantly overestimated. For example, the molecular weight of Sgt2NT (~48 kDa) suggests a rotational correlation time in the range of 25–35 ns, which is far from the reported 157 ns.

This overestimation likely stems from applying the Stokes-Einstein-Debye equation, which assumes a spherical molecule, whereas these constructs exhibit an elongated, "beads-on-a-string" topology with significant flexibility. Furthermore, the correlation time measured for the full-length constructs may not be directly applicable to the isolated N-terminal domain for the calculation of S2, given that it is connected to the rest of the protein via a disordered linker. It is therefore possible that the results presented in Fig. 4j and 4l are artifacts arising from incorrect rotational correlation time estimations between constructs. To address this, I recommend determining tau_c from 15N R2 and R1 relaxation rates for Sgt2N and Sgt2N-Cshort (focusing on residues within the folded N-terminal domain). The methyl order parameters should then be extracted for Sgt2N and Sgt2N-short using the determined tau_c values to confirm that the observed effects align with panel 4j. Please also add the equations used to extract S2 from Ia/Ib.
Since the manuscript revolves around the discovery of the "conformational entropy modulator" model, it is crucial that the data analysis is conducted with the highest level of rigor. *Unless I am mistaken, these values should actually be 1566 ns, 3441 ns and 3183 ns (using the reported equation and assuming a water viscosity of 0.00089 Pa.s at 25C).

We thank the reviewer for their careful evaluation and for highlighting a critical point regarding our estimation of τc and S^2.

1. Clarification of our mistake

We acknowledge the reviewer's concern regarding the overestimation of τc values based on DLS-derived hydrodynamic radii. As the reviewer correctly pointed out, applying the Stokes-Einstein-Debye equation to our elongated and flexible protein constructs resulted in inflated τc estimates and, consequently, unphysically low S^2 values. This approach is indeed unsuitable for our system.

While our initial draft relied on DLS measurements, we subsequently adopted an alternative strategy based on the approach of Kay et al. (*Nature 2007; 445(7128):618-22*), where τc values were adjusted empirically to ensure that S^2 values remained within the physically meaningful 0–1 range. The final S^2 values reported in the manuscript were derived using this corrected methodology.

We sincerely apologize for inadvertently retaining outdated references to the DLS-derived τc values in earlier manuscript drafts, which led to this confusion. We are grateful to the reviewer for helping us clarify this key issue.

We sincerely apologize for inadvertently retaining outdated references to the initial DLS-derived τc values in earlier manuscript drafts, which caused confusion. We are grateful to the reviewer for helping us clarify this key issue.

2. Why we could not use R_1, R_2 relaxation data to determine τc for $Sgt2^N$ in the context of full-length Sgt2

We appreciate the reviewer's suggestion regarding the use of R1, R2 relaxation data from isolated $Sgt2^N$ (as applied in Schutz and Kay, *eLife 2016*). We initially considered this approach, but after carefully evaluating it using our own experimental data, we found it was not suitable for our system.

Specifically, the isolated dimeric $Sgt2^N$ domain has an expected τc of ~10 ns at 298K. Applying this τc value to the full-length protein would have led to the majority of the calculated S^2 values significantly exceeding 1, which is physically impossible. This suggests that although $Sgt2^N$ is highly flexible within the full-length protein, it does not behave as a fully isolated domain. We also consulted with the authors of Schutz and Kay (*eLife 2016*), who confirmed that this approach may have limitations in such a context.

Ultimately, we followed the methodology outlined by Kay et al. (Nature 2007), adjusting τc empirically to obtain S^2 values within the expected physical range, reflecting the true dynamics of our system.

3. Clarification of Our Analysis

Our methyl S^2 were derived from TQSQ NMR experiments, where the η values were calculated from Ia/Ib ratios. According to kay et al. (*J. Phys. Chem. B* 2011; 115(49):14878–84), S^2 is proportional to the product of η and τc. Since Sgt2 and Sgt2-Ysy6^{TMD} have similar τc (based on their comparable structures and sizes), a larger η corresponds to a larger S^2. When η values are similar, the protein with the smaller τc (e.g., Sgt2^{NT} compared to Sgt2^{FL}) will have a larger S^2. For transparency, we now provide examples of η below (Fig. 1), which clearly illustrate that **even without explicitly calculating S^2, the differences in η already demonstrate distinct dynamic properties between these proteins**. **Therefore, our conclusions remain valid regardless of the exact method used to compute S^2**.

These findings are further **corroborated by HDX-MS data**, reinforcing our conclusions.

	A	B	C	D	E	F	G	H
		sgt2-nt	sgt2-fl	sgt2-ysy6tm	NT	FL	FL_YSY6	
	A144	79	61.91	92.4	0.954106	0.358275	0.57037	0.595831
	I40	80.63	73.96	105.87	0.973792	0.428009	0.653519	0.545783
	A60	61.96	54.59	89.2	0.748309	0.315914	0.550617	0.432395
	L176-2	49.15	28.21	67.84	0.593599	0.163252	0.418765	0.430347
	V51-1	66.29	67.65	83.69	0.800604	0.391493	0.516605	0.409111
	V35-2	74.27	88.24	106.98	0.896981	0.510648	0.66037	0.386333
	I54	74.62	89.50	115.9	0.901208	0.51794	0.715432	0.383268
	A9	66.02	72.71	92.89	0.797343	0.420775	0.573395	0.376568
	A43	54.32	48.98	68.23	0.656039	0.283449	0.421173	0.37259
	A36	67.18	83.15	99.25	0.811353	0.481192	0.612654	0.330161
	A3	59.23	67.41	76.2	0.715338	0.390104	0.47037	0.325234
	M37	51.68	52.45	63.66	0.624155	0.30353	0.392963	0.320624
	A140	52.39	54.2	69.65	0.632729	0.313657	0.429938	0.319072
	I66	57.83	71.03	95.78	0.69843	0.411053	0.591235	0.287377
	L33-2	50.77	59.51	54.81	0.613164	0.344387	0.338333	0.268778
	A121	55.2	70.25	112.1	0.666667	0.406539	0.691975	0.260127
	I8	59.38	79.19	94.18	0.71715	0.458275	0.581358	0.258874
	A72	48.38	56.64	68.62	0.5843	0.327778	0.42358	0.256522
	A215	67.65	98.59	93.72	0.817029	0.570544	0.578519	0.246485
	A30	58.2	79.53	83.2	0.702899	0.460243	0.51358	0.242655
	L149-1	54.15	73.79	102.7	0.653986	0.427025	0.633951	0.22696
	L11-2	38.83	41.83	49.62	0.468961	0.242072	0.306296	0.22689
	V13-2	66.16	98.88	79.75	0.799034	0.572222	0.492284	0.226812
	L69-2	41.53	48.23	68.52	0.50157	0.279109	0.410617	0.222461
	L65-1	51.53	69.51	71.25	0.622343	0.402257	0.439815	0.220086
	A128	70.85	111.4	142.9	0.855676	0.644676	0.882099	0.211
	L11-1	44.94	57.9	54.75	0.542754	0.335069	0.337963	0.207684
	V219-2	17.19			0.207609	0	0	0.207609
	L55-1	48.9	66.73	66.36	0.59058	0.386169	0.40963	0.204411
	A112	56.32	82.74	125.5	0.680193	0.478819	0.774691	0.201374
	V51-2	53.7	77.85	75.39	0.648551	0.450521	0.46537	0.19803
	A204	40.36	50.78	51.75	0.48744	0.293866	0.319444	0.193574
	L149-2	47.06	68.59	96.76	0.568357	0.396933	0.597284	0.171425
	L69-1	53.9	84.57	79.68	0.650966	0.48941	0.491852	0.161556
	A159	52.18	81.65	109.7	0.630193	0.472512	0.67716	0.157682
	A192	46.87	71.93	96.63	0.566063	0.416262	0.596481	0.149801
	A143	54.02	88.32	66.66	0.652415	0.511111	0.411481	0.141304

Fig. 1 TQSQ-derived η values

4. Manuscript revisions

We have now removed all references to DLS-based τc calculations and clearly detailed the empirical τc adjustment approach in the Methods section, citing Kay et al. (*Nature 2007*). We greatly appreciate the reviewer's attention to this issue and believe these clarifications resolve the concern.

Reviewer 3:

The revised manuscript by Ji et al., includes numerous additional experiments and controls in response to the reviewer's comments. My previous concerns, which were largely related to the accuracy with which the authors represented their data and a lack of discussion of the results in the context of existing literature on Sgt2, have been mostly addressed. As a result, this work furthers both our understanding of protein targeting by the GET pathway and general allosteric regulation by IDRs and is suitable for publication providing a few outstanding comments are addressed:

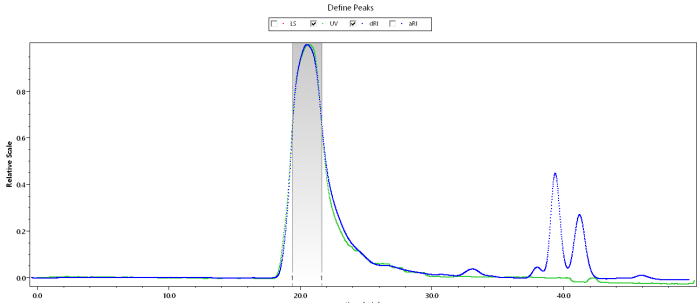
We sincerely appreciate the reviewer's careful assessment of our revised manuscript. We are pleased that the additional experiments and discussion have largely addressed the reviewer's previous concerns. In response to the remaining comments, we have made the following revisions to further improve the clarity and accuracy of the manuscript.

1. Most primary research papers on the GET pathway do not start with a schematic like that in Figure 1a and, given this schematic is unnecessarily more complicated than its corresponding description in the introduction, does not help the reader. I strongly recommend to simplify Figure 1 by removing panel a.
   We thank the reviewer for this suggestion. Figure 1a has been removed, as recommended, to simplify the figure.
2. Extended Data Tables 1 and 2 were not included with the manuscript submission so it is not possible to assess whether they incorporate the requested information. The reference in the ITC methods section presumably needs to be updated to Extended Data Table 2.
   We re-uploaded Extended Data Tables 1 and 2 for the reviewer's convenience. They were inadvertently not visible in the initial submission, and we apologize for the confusion.

3. The legend for Extended Data Figure 1a incorrectly states that the standard buffer for ITC experiments was HEPES.
   We thank the reviewer for pointing out this typo. The incorrect buffer description in Extended Data Figure 1a has been corrected to MES.

4. The reported error values for some molar mass estimations from MALS are surprisingly small given the visual quality of the data e.g. the steeply sloping molar mass curve in Extended Data Figure 1h makes an error of 0.7 kDa seem highly implausible. This implies a very narrow subset of the peak was used for the calculation. The authors should show the windows that were selected within the SEC-MALS traces (e.g. by scale bars) for molar mass calculation.
   Regarding the SEC-MALS error values, we appreciate the reviewer's observation. To clarify, the reported errors were directly obtained from the SEC-MALS instrument software (Wyatt ASTRA), which reflects the uncertainty in the light scattering signal fitting (this is now explicitly mentioned in the Methods section).
   To enhance transparency, we have included figures showing the MALS analysis results for the raw data in Extended Data Figure 1h, including the peak selection process (elution time: 19.40 to 21.64 min). Additionally, we have now marked the selected data windows within the SEC-MALS traces in Extended Data Figure 1h to further illustrate how the

molecular weight was determined. This should provide a clearer representation of the calculation process.



Peak Results

	Peak 1
Masses	
Calculated Mass (µg)	266.24
Mass Fraction (%)	100.0
Molar mass moments (g/mol)	
Mn	1.307×10^5 (±0.539%)
Mp	1.291×10^5 (±0.521%)
Mw	1.327×10^5 (±0.533%)
Mz	1.348×10^5 (±1.188%)
Polydispersity	
Mw/Mn	1.015 (±0.758%)
rms radius moments (nm)	

5. The ITC curves from Extended Data Figure 14 should be included above or below the schematic in Figure 5 as it is important to directly show the quality of the primary data. The curves for the untethered systems could be shown in black to avoid creating inconsistencies with the established colour scheme. In the new paragraph for the Tau-PDZ/SPOC/GABARAP fusions, the authors refer to Extended Data Figure 15 which does not exist.

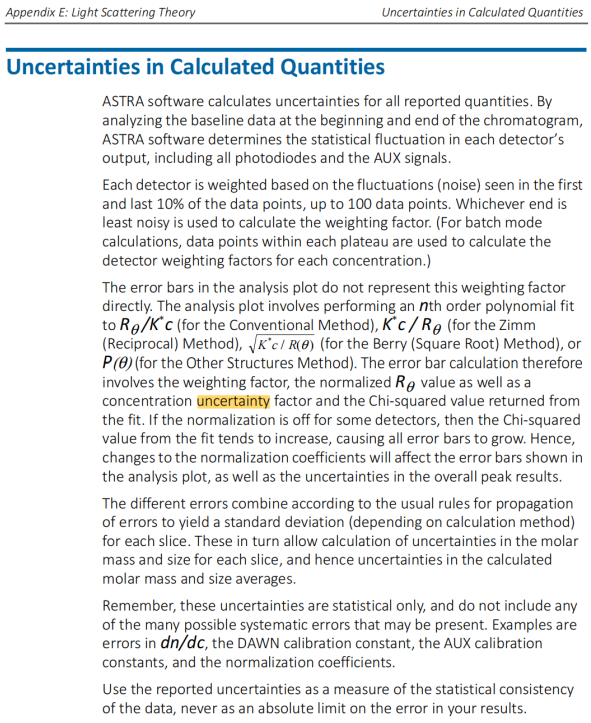
   We thank the reviewer for these suggestions. Figure 5 has been updated to include the ITC curves from Extended Data Figure 14, as suggested. To maintain consistency with the existing color scheme, the curves for untethered systems are shown in black. The incorrect reference to Extended Data Figure 15 has been corrected in the text.

ADDITIONAL REVIEWER COMMENTS:

- the line for molar mass in Extended Figure 1h goes from ~110-150 kDa in the newly indicated peak selection window. It isn't logical that the corresponding molar mass calculation has an error of 0.7 kDa. I selected this curve as it is a clear example of where the error estimation seems to be wrong, but the orange peak in Extended Fig. 1e and the green curve in Extended Fig. 1g are also polydisperse with very low errors. The authors should double check how they are performing their analysis in Astra as there is clearly a discrepancy with the curves shown in the paper.

Although I requested that the errors are given, it is better to not show any errors at all, rather than show them incorrectly - it is ultimately a relatively minor contribution to the paper.

We fully agree with the reviewer's assessment regarding the molar mass error estimations. After carefully revisiting our data analysis and consulting with the instrument manufacturer (Wyatt Technology), we confirmed that the errors reported by the ASTRA software reflect the statistical uncertainty from light scattering signal fitting, rather than being a direct indication of peak polydispersity. As outlined in Wyatt's documentation, these uncertainties are based on detector fluctuations and chi-squared fitting and do not directly correlate with variations seen across sloped lines (please see the figure below for reference).



Appendix E: Light Scattering Theory *Uncertainties in Calculated Quantities*

Uncertainties in Calculated Quantities

ASTRA software calculates uncertainties for all reported quantities. By analyzing the baseline data at the beginning and end of the chromatogram, ASTRA software determines the statistical fluctuation in each detector's output, including all photodiodes and the AUX signals.

Each detector is weighted based on the fluctuations (noise) seen in the first and last 10% of the data points, up to 100 data points. Whichever end is least noisy is used to calculate the weighting factor. (For batch mode calculations, data points within each plateau are used to calculate the detector weighting factors for each concentration.)

The error bars in the analysis plot do not represent this weighting factor directly. The analysis plot involves performing an nth order polynomial fit to R_θ/K^*c (for the Conventional Method), K^*c/R_θ (for the Zimm (Reciprocal) Method), $\sqrt{K^*c/R(\theta)}$ (for the Berry (Square Root) Method), or $P(\theta)$ (for the Other Structures Method). The error bar calculation therefore involves the weighting factor, the normalized R_θ value as well as a concentration uncertainty factor and the Chi-squared value returned from the fit. If the normalization is off for some detectors, then the Chi-squared value from the fit tends to increase, causing all error bars to grow. Hence, changes to the normalization coefficients will affect the error bars shown in the analysis plot, as well as the uncertainties in the overall peak results.

The different errors combine according to the usual rules for propagation of errors to yield a standard deviation (depending on calculation method) for each slice. These in turn allow calculation of uncertainties in the molar mass and size for each slice, and hence uncertainties in the calculated molar mass and size averages.

Remember, these uncertainties are statistical only, and do not include any of the many possible systematic errors that may be present. Examples are errors in dn/dc, the DAWN calibration constant, the AUX calibration constants, and the normalization coefficients.

Use the reported uncertainties as a measure of the statistical consistency of the data, never as an absolute limit on the error in your results.

To further address the reviewer's concern and to enhance data transparency, we have now repeated the SEC-MALS measurements for all samples (biological triplicates) and calculated experimental standard deviations from independent runs. In the revised figures, we now present these replicate-derived errors.

We fully agree with the reviewer that this is a minor aspect that does not impact the main conclusions of our study. We hope this updated analysis resolves the concern. We also respect the reviewer's suggestion that these error bars could be omitted, and we are happy to do so should the reviewer prefer a cleaner presentation without them.

- the authors say they show the curves for untethered systems in black in the much improved Figure 5, however they are still in green in the Figure provided - I assume this was a mistake.

We thank the reviewer for pointing this out and apologize for the oversight. We have corrected the figure, and the curves for the untethered systems are now shown in black as recommended. Thank you for your helpful suggestion.