nature portfolio

Peer Review File

Molecular basis of collagen galactosylation by GLT25D1

Corresponding Author: Dr Hongjun Yu

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript presents a detailed structural and mechanistic study of human GLT25D1, an essential collagen O-galactosyltransferase. Using cryo-EM, the authors resolve the architecture of GLT25D1, demonstrating a two-lobed structure in which the N-lobe binds UDP-Gal non-catalytically, while the C-lobe contains the catalytic CGT domain. Importantly, the study shows that GLT25D1 predominantly forms oligomers, a finding that may have important implications for its structural stability, regulation, or broader cellular function, although dimerization is not essential for enzymatic activity. The authors further integrate biochemical assays and mutational analysis to elucidate substrate recognition and the effects of disease-related mutations.
Overall, this study makes a valuable contribution to our understanding of collagen glycosylation and opens new mechanistic avenues for further research.

Major Points:
The observation that the non-catalytic N-lobe is critical for enzymatic activity is particularly intriguing and suggests a possible mechanism of allosteric regulation. To clarify this aspect:
• Perform steady-state kinetic analyses (Km and kcat) for both UDP-Gal and peptide substrates, comparing wild-type GLT25D1 with N-lobe mutants (e.g., D166A).
• Use molecular dynamics (MD) simulations to investigate whether UDP-Gal binding in the N-lobe induces conformational changes that affect the CGT active site.
• Conduct Elastic Network Model (ENM) analyses to assess mechanical coupling and potential allosteric communication between the two lobes.

Minor Points:
• Line 376:
The sentence "likely weakening the donor phosphoester bond....." is confusing. Typically, binding of Asp437 and Mn^{2+} would stabilize the UDP-Gal and prime it for catalysis, rather than weaken the bond. Please clarify and rephrase accordingly.
• Figure 4:
The figure does not depict key interactions involved in Mn^{2+} coordination. It would strengthen the figure to explicitly show metal coordination with UDP-Gal and relevant catalytic residues.
• Discussion:
While the manuscript mentions the potential relevance of GLT25D1 in disease, it would benefit from a brief discussion of possible therapeutic opportunities, such as targeting GLT25D1 regulation in collagen-related diseases or cancer.

Reviewer #2

(Remarks to the Author)
INTRODUCTION AND OVERALL ASSESSMENT
This is a detailed molecular investigation of GLT25D1. The paper includes protein expression, structural analysis by single-particle cryo-EM, and enzymology. From a technical point of view, I personally enjoyed how the careful biochemistry is fully displayed in figure 1 and the elegant application of C3-symmetry expansion to convert the hexamer to a dimer computationally. Overall, the figures are excellent and the text easy to follow. I have some concerns below that need addressing but overall I think this is a great piece of work.

MAP-MODEL CHECKS
Overall, the maps and models look good, with the quality of the maps matching what one would expect. However, the one area of ambiguity is the unexplained density between Ala-2 of the substrate and Tyr-400 of the protein in the ternary complex map. It looks like the Tyr could be flipped around to fill it. The authors should confirm that there is no way to do this whilst maintaining clash and stereochemistry values. Figure 5C should be expanded to show density for ligating residues, particularly Tyr-400 and the mystery blob. If local model building can be made to account for that density then that closes the issue, otherwise it should be highlighted and explicitly mentioned in the text.

MAJOR COMMENTS
The issues in the ternary complex map as above.
How the peptide was synthesised and purified was not reported. This should go in even if its standard
The recent work of Peng et al (Nat Comms, 2024) on the 'KOGG' complex made of GLT25D1/ColGalT1 and LH3/PLOD3 should be cited and the relationship between the two works made clear (the focus of the two papers is clearly different).
The energy window for the GIF should be reported.
The map resolution range in Table in Suppl Table 1 looks weird. I've never really known what the Nature family of journals mean with this value in their standard table but I typically read it as the range in the local resolution metric. If so, then the smallest value should match (or be slightly better than) the nominal map resolution above. But assuming that is the case, then it's not clear what the lower value should be, as the value will depend a lot on input parameters and nominally the lowest resolution feature will be set by box size, which is completely arbitrary. Rest of the stats look good.

MINOR COMMENTS
92- Put expression system in main text so its clear to the reader.
175- in vitro should be in italices
323 & 328. Not clear why these words are bolded and underlined.
534- GIF quantum energy filter. Quantum should be capitalised. This makes it sounds like it relies on a quantum effect.
539- this should be fluence

Reviewer #3

(Remarks to the Author)
This manuscript presents cryo-EM structures of human GLT25D1 and its ternary complex with substrates, elucidating the enzyme's role in collagen galactosylation. While the structural and biochemical data are rigorously obtained and technically sound, major concerns regarding novelty arise due to substantial overlap with two recently published Nature Communications articles (Nat Commun. 2025, 16(1):3624 and Nat Commun. 2025, 16(1):2436).
1. The Introduction/Discussion must explicitly position this work against these 2025 publications to clarify its unique contributions.
2. The quality of Figure 1 requires improvement
-Figure 1A: Abbreviations "LH/PLODS" require definition in the legend or main text.
-Figure 1B: Claims regarding "dimer" and "hexamer" oligomeric states lack validation. Either add size-exclusion chromatography molecular weight standards to calibrate elution peaks, or remove speculative oligomer labels ("dimer," "hexamer") as these states are not biochemically verified.
-Figure 1C vs. 1D: Content duplication is apparent. Recommend moving Figure 1D to Supplementary Information.

3. Mutant purification (Lines 216–217): The assertion that failed purification of R61A/V143A/D168A/H235A mutants "might be the effect of UDP-galactose" is insufficiently supported. Alternative explanations (e.g., protein misfolding, solubility issues) must be considered or experimentally addressed.

4. Thermal stability assays (Lines 223–226): Reduced Tm values for mutants only indicate global destabilization, not specifically impaired ligand binding. Direct binding assays (e.g., ITC, SPR) are needed to substantiate claims about UDP-galactose's role in structural stability.
W135 validation: The rationale for including W135A (not part of initial screening) requires clarification.

5. No significance testing (e.g., p-values, error bars denoting SD/SEM) is reported for any quantitative data in the whole text (activity assays, thermal shifts, etc.).

6. Lines 306–308, the importance of Mn^{2+} for enzymatic activity should be contextualized with prior literature citations.

7. Alternate use of "hydroxylysine" and "hydroxylated lysine" is confusing.

Reviewer #4

(Remarks to the Author)
Relevant Expertise of Reviewer
I am a biochemistry and structural biologist who works on carbohydrate-processing enzymes, while I can comment on these aspects, I cannot pass comment on the mass spectrometry nor collagen-specific biology as I am not an expert in these.

Overview
The paper details the structure and mechanism of GLT25D1, an important enzyme in the modification of hydroxylysine residues in collagen. Overall, the paper is of high quality with excellent written and visual descriptions of the data. It is a very important piece of work that complements and enhances the recent papers by De Marco et al., (https://doi.org/10.1038/s41467-025-59017-5) and Peng et al., (https://doi.org/10.1038/s41467-025-57768-9). The work is generally robust, and evidence provided is of high quality. It also provides essential new insights including a enzyme UDP/peptide complex and proposed catalytic mechanism. However, I have a few concerns that would warrant consideration and adjustment prior to publication.

Essential Revisions

[1] Enzyme Kinetics
To assess enzyme kinetics, the authors have chosen to compare the activities of GLT25D1 domains and their mutants relative to the WT enzyme at fixed substrate concentrations. While this approach provides a basic comparison, a more rigorous and informative analysis would ideally to have been for authors to have undertaken full Michaelis-Menten kinetic experiments for all constructs, including the mutants. At a minimum, Michaelis-Menten kinetic experiments should be conducted for the full-length, CGT, and NGT constructs—although the latter may exhibit little to no activity. Given the resources available to the authors, incorporating these experiments should be feasible and would significantly strengthen the study's conclusions.

Also, in relation to the kinetics, it is also unclear how the data has been normalised relative to the WT protein, e.g. in Figure 3 the WT activity against gelatin is 40% of the activity of WT. This is a contradiction, and it is likely authors have normalised activity to WT against Hyl peptide - however this needs clarification. The methods do indicate 'The final activity was normalized to the GLT25D1 amount quantified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels.' However, this seems like a very unusual way of normalising activity given protein concentrations have been determined. It is also unclear how the SDS-PAGE normalisation has occurred; did authors quantify band intensities? If so, these values should be provided in the source data files. Still this is a very unusual way to normalise kinetics, and is possibly incorrect, given authors should be using the same quantity of protein in each reaction mix (which they do suggest they are doing within the methods). Please could authors better clarify the mechanism of data collection and normalisation.

It is also unclear what the three independent replicates are, and this should be clarified e.g. is this from three entirely separate experimental setups? If so, how many technical replicates in each?

[2] Bound ion
In the NGT domain, authors have modelled a Mn2+ ion, the recent paper by De Marco et al., has indicated by ICP-MS that this is a Ca2+ ion. Please could authors comment on this, the main text suggests this hasn't been investigated.

Also, an important note, the De Marco paper have modelled the galactose differently in their NGT domain, in my opinion the modelling in this paper under review is much more likely to be correct given the highly unlikely puckering and orientation of the galactose in De Marco et al., however this discrepancy should be discussed within the text given its importance. The Peng et al., modelling more closely aligns to the paper under review. However, the UDP in CGT is modelled differently in the Peng et al., paper.

Given the large discrepancy, there should be a discussion of the modelling of the UDP-Gal and UDP in this paper against the other papers to resolve the discrepancy - a supplementary figure displaying densities and modelling of the UDP and UDP-Gal from all papers would be particularly useful.

[3] Cancer Mutants
Lines 340-351, I'm not sure what this passage of text adds to the narrative. The cancer-associated mutations shown in Supp Fig 11 are located across the protein and I think it is ambitious to suggest that mutations within the circled regions all share a mechanistic basis with the other disease mutants. It would have been more useful to have summarised how each cancer-associated mutation could affect structure/activity in table format. I think a more informative narrative is simply stating that mutations in both domains are associated with cancer, indicating the importance of the NGT domain.

[4] Catalytic mechanism
Lines 284-288. Authors shown convincing evidence for D522 acting as catalytic base in the mechanism, however regarding "Furthermore, the invariant hydroxylysine (Hyl) is stabilized within this cleft by the "YW" motif (Y494 and W495), with its ε-amino group anchored by E523 (Fig. 5B). This configuration positions the 5-hydroxyl group of hydroxylysine in close proximity to the β-phosphate of UDP in CGT active site, appropriate for galactose transfer." can authors comment on the predicted distance between the proposed nucleophile (Hyl-O) and the predicted position of the anomeric carbon of the galactose, and the suitability of this distance for a concerted Sn2 mechanism. Would authors suggest in slight contradiction to Figure 5F that the D523-Hyl bond is perhaps broken prior to attack? Importantly, the E523 sidechain is not evident in the density map so it is unclear if it is even forming the proposed interaction with the Hyl. While perhaps beyond the scope of the paper, given it is unclear how easily resources can be accessed, MD and QM/MM simulations would have considerably enhanced the manuscript in this regard.

[5] Terminology
Cryo-EM does not produce 'electron density' maps, this term needs updating throughout the text (see: DOI:

10.1002/pro.3060 and DOI: 10.1016/j.sbi.2019.04.006). Also, when density maps are shown in figures the thresholds should be indicated.

[6] Multimeric state
Given the Marco et al., paper proposes that the enzyme is dimeric, there needs to be discussion in the main text acknowledging this work, and a discussion of the discrepancies.

Other Revisions
[1] Remove the bold and underlining at the start of sentences e.g. 'Additionally', 'Further Analysis'

[2] It would be good to see a discussion regarding why NGT is not active from a structural viewpoint.

[3] Could authors clarify if MS experiments were run once per sample or were there repeats.

Lines 71-74: This part could better describe the motif e.g. presence of Hyp.

Line 96: It is unclear from the text which of the two elution peaks were used to determine that GLT25D1 was active.

Line 100: 'In presence of Mn2+' needs adding.

Line122-126: Unclear if the protein used was a mix of the dimer/tetramer SE peak, or an isolated peak.

Line 172-174: How was significance determined? Also, by eye, it looks like activity of gelatin doesn't change but does on Hyl containing peptide?

Line 180-190: Please include surface area for dimer- dimer interactions that comprise the hexamer.

Line 242 space needed between 'Fig.' and '1D'

Line 251: Please specify in main text how H560 and R351 stabilise loop.

Line 324 "the SVD-associated point mutations L151R and A154P, located in helix α3, play critical roles in stabilizing the domain core fold"

Here it is inferred the point mutations induce stability, which they don't, I would rephrase and be more specific about the effects of point mutants, e.g. L151R is introducing a larger, charged residue into a stabilising hydrophobic core.

Line 336 "Indeed, neither G377R nor R471W significantly", while this is quite possibly the case, as band intensity has not been calculated and statistical tests not performed, whether the result is 'significant' cannot be determined, the sentence needs to be less absolute.

Line 375, β-pyrophosphate should be β-phosphate

Line 456-467: Please provide more details regarding how the SDM was performed including primer sequences.

Line 464: How were cells transferred to BMMY media.

Line 466: 'following the manufacturer's protocol' it is unclear from the text which protocol this is, as the sentence doesn't refer to a very specific technique.

Line 469-470: 'was applied to Ni-NTA affinity chromatography' Ni-NTA is the technique, do you mean Ni-NTA resin? Was this gravity flow purification or FPLC-mediated?

Line 470: 'After washing', what was the wash buffer and how much was applied?

Line 472-472: What ratio of HRV3C to protein did you use? Where was the HRV3C sourced?

Line 478: How was protein concentration quantified? If by A280 please provide ext.co and Mw used.

Line 482: How was the hydroxylated peptide synthesised?

Line 484: I would prefer the concentration of protein to be reported in molarity to enable immediate comparison with the donor and acceptor substrate concentrations.

Line 526: How were grids glow discharged?

Line 525-532: It is unclear whether the dimer or tetramer peaks from the SE were used for the unliganded and ternary state. The latter cryo-EM data suggest tetramer was used for the unliganded CTD structure and dimer for ternary structure, if this is

not the case, can authors comments on why they see dimer in the latter?

Line 563: Can the authors check the 275,468 number, a C3-symmetry expansion of 125,162 would suggest 375,486 particles.

Line 596: ‘Interactive’ not ‘manual’ rebuilding

Line 802: I think Figure 1A is a little confused as it doesn’t specifically show lysine residues as the modified amino acids. I think a chemical diagram showing each step would have been better but is not essential.

Line 838: Use red or pink to describe asterisk, not salmon as this is confusing.

Line 857: Trimer not ‘timer’

Line 870: The UDP in the diagram is all orange, making the phosphates difficult to ascertain and in contrast to the insert. I would change this to yellow to match insert.

Line 891: You are missing a few hydrogen bonds in this diagram suggested in main text e.g. K470 to Ala in peptide.

Line 913: For figure 6 the ‘C’ and ‘D’ are mapped incorrectly in legend, C should be B and D should be C.

Line 929-930 and 1001-1002: For Sup Figure 1D and E and 7D and E, please provide the corrected, unmasked, masked, and randomised FSC curves.

Line 970: Trimer not ‘timer’

Line 980-986: Can you provide uniport identifiers in parentheses after organism’s name in legend.

Line 1066: Most and least conserved not ‘conservative’.

Reviewer #5

(Remarks to the Author)
This manuscript presents a rigorous structural and biochemical dissection of GLT25D1, a collagen galactosyltransferase essential for post-translational modification of hydroxylysine residues. The authors employ high-resolution cryo-EM, structure-guided mutagenesis, and enzymatic assays to map the catalytic site, elucidate substrate specificity, and rationalize the effects of disease-associated mutations. The work is technically sound and clearly presented, and it provides a valuable resource for understanding the molecular mechanism of collagen glycosylation.

However, in considering the broader impact expected for publication in Nature Communications, I am uncertain whether the conceptual advance reaches the necessary threshold. While the structural insights into GLT25D1 are new and welcome, they pertain to a single, well-known enzyme, and the study largely confirms or refines prior expectations (e.g., involvement of the CGT domain in catalysis, recognition of the “X-Hyl-Gly” motif). The physiological or translational implications are discussed but not experimentally explored, and the potential connections to disease, though interesting, remain somewhat speculative.

Minor comments:
Line 73: the authors refer to a Gly-X-Y sequence. Y is the symbol for tyrosine, but it seems it is not used to denote tyrosine here
There are several instances where "timer" has been written in place of "trimer" by mistake.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have done a great job and have thoroughly addressed my previous comments. I have only two minor points for further clarification:

a.-) Could the authors explain why the CGT domain exhibits a better Km towards gelatin than the wild-type enzyme? It seems the NGT domain may negatively affect binding to gelatin.
b.-) How many replicates were performed for the molecular dynamics simulations? I have not found this in Methods.

Reviewer #2

(Remarks to the Author)
Dear authors, fellow reviewers, and editorial team,
The authors have addressed the concerns I had in my original review. I'm particularly glad to see that Tyr flipping into the density in a visually pleasing fashion.
I lack the subject specific knowledge to comment on the other reviewers comments.
Yours sincerely,
Jamie Blaza
University of York

Reviewer #3

(Remarks to the Author)
The authors have answered all my concerns. I am now recommend the publication of this work.

Reviewer #4

(Remarks to the Author)
The authors have made considerable improvements to the manuscript since the initial review improving its overall quality. The results support the conclusions, and the results are of significance to the field. The methodology is sound and meets the expectations of the field and is sufficiently detailed to be able to be reproduced. My points raised within the initial review have been addressed and the work is now of high quality.

Although, I have three very minor edit suggestions, linked to the new edits made:
[1] Line 181 in revised manuscript needs to say "against gelatin" as the peptide with Hyl is significantly less.
[2] Line 1050 p value number is same for *** and ****, they should be different.
[3] Line 1094 the hydrogen bonds differ between "B" and "D" even though they are simply rotated images, this likely results from incorrect H-bonds in "B". Tied to this Line 298-299 "its interaction with acceptor peptide only relies on the B-carbon of this residue" while in fact H-bonding suggests involvement of T562 hydroxyl. Did the authors mean "sidechain"?

These edits are minor and do not detract from my enthusiasm for the work.

Reviewer #5

(Remarks to the Author)
All of my comments have been addressed in the revision.

Point-to-point responses to the reviewers' comments

We greatly thank the insightful and valuable suggestions from reviewers. Our responses below are in blue.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The manuscript presents a detailed structural and mechanistic study of human GLT25D1, an essential collagen O-galactosyltransferase. Using cryo-EM, the authors resolve the architecture of GLT25D1, demonstrating a two-lobed structure in which the N-lobe binds UDP-Gal non-catalytically, while the C-lobe contains the catalytic CGT domain. Importantly, the study shows that GLT25D1 predominantly forms oligomers, a finding that may have important implications for its structural stability, regulation, or broader cellular function, although dimerization is not essential for enzymatic activity. The authors further integrate biochemical assays and mutational analysis to elucidate substrate recognition and the effects of disease-related mutations.

Overall, this study makes a valuable contribution to our understanding of collagen glycosylation and opens new mechanistic avenues for further research.

Response: We thank the reviewer for the support and the valuable comments. They are so helpful to improve our manuscript. We have revised our manuscript accordingly.

Major Points:

The observation that the non-catalytic N-lobe is critical for enzymatic activity is particularly intriguing and suggests a possible mechanism of allosteric regulation. To clarify this aspect:

• Perform steady-state kinetic analyses (Km and kcat) for both UDP-Gal and peptide substrates, comparing wild-type GLT25D1 with N-lobe mutants (e.g., D166A).

• Use molecular dynamics (MD) simulations to investigate whether UDP-Gal binding in the N-lobe induces conformational changes that affect the CGT active site.

• Conduct Elastic Network Model (ENM) analyses to assess mechanical coupling and potential allosteric communication between the two lobes.

Response: We thank the reviewer for these insightful suggestions. We have revised accordingly. The corresponding experimental results have now been added as **Supplementary Fig. 11**, and the related description/discussion has been incorporated into the "Discussion" section of the revised manuscript (Lines 419-445).

As recommended, we performed kinetic analyses to compare wild-type GLT25D1 and the N-lobe mutant D166A. To this end, and given the insufficient yield of the synthesized hydroxylysine-containing acceptor peptide, we used gelatin as the

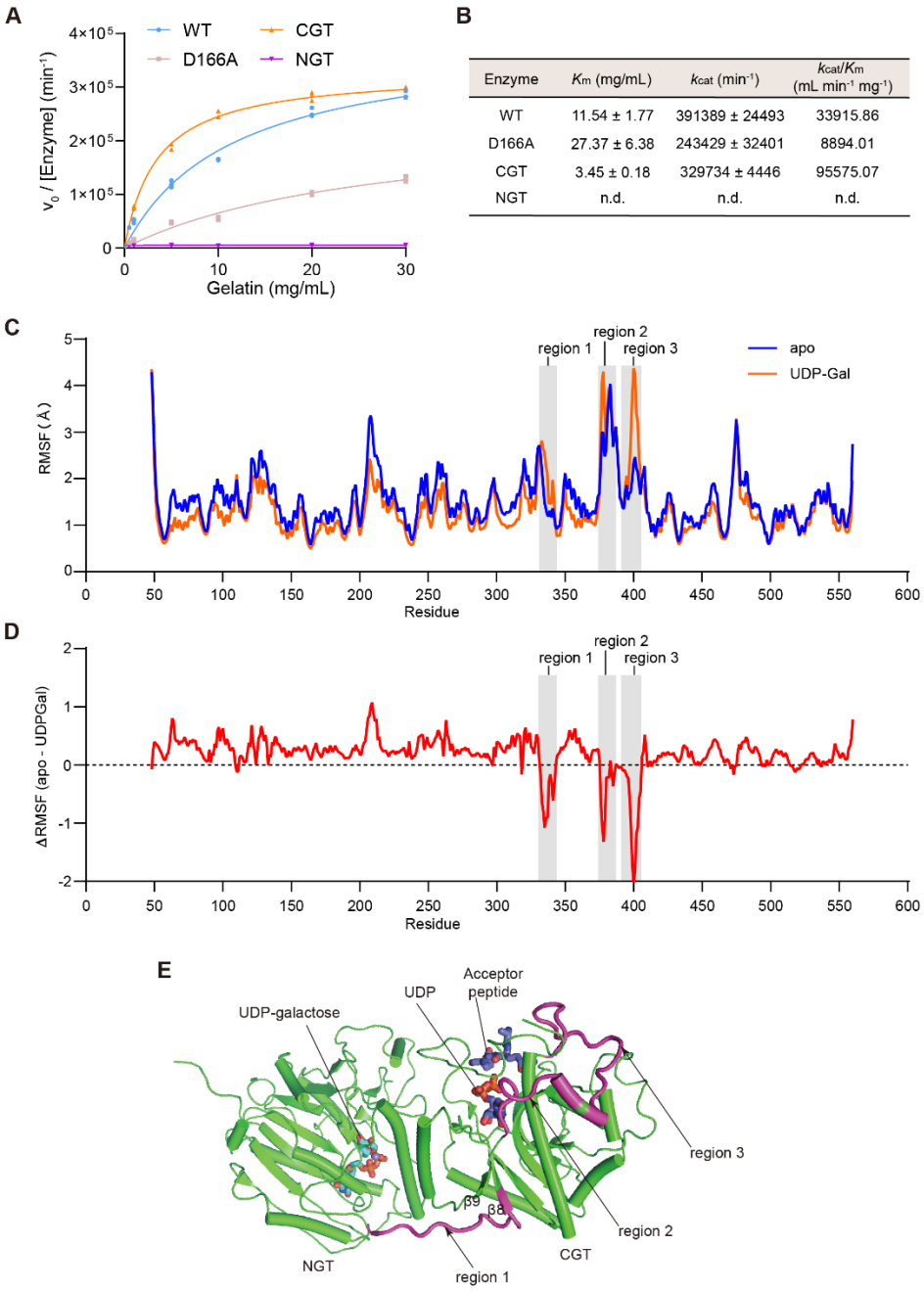
substrate—a well-established alternative for such assays [1-2]. The kinetic parameters obtained for the wild-type (WT) GLT25D1 (**Supplementary Fig. S11A-B**) were consistent with these studies [1-2]. Notably, compared to the wild-type enzyme, the D166A mutant exhibited a ~2.3-fold increase in Km, a ~1.6-fold decrease in kcat, and a ~3.8-fold reduction in catalytic efficiency (kcat/Km) (**Supplementary Fig. S11B**), supporting a potential allosteric regulatory mechanism.

For further investigation, we agree that MD simulations and ENM analyses can provide valuable insights. Based on our team's expertise, we focused our computational efforts on MD simulations. We performed molecular dynamics (MD) simulations of GLT25D1 in the apo state and in complex with UDP-galactose bound to the NGT domain (**Supplementary Fig. S11C-E**). The apo form showed elevated flexibility across the protein, as reflected by broadly increased root-mean-square fluctuation (RMSF) values (**Supplementary Fig. S11C**), indicating structural destabilization in the absence of ligand. Consistently, thermal stability analysis demonstrated that ligand-binding mutations in the NGT domain exhibited a more pronounced destabilizing effect compared to those in the CGT domain (**Supplementary Fig. 12**). In contrast, the MD analysis showed that UDP-galactose binding at the NGT domain restricted global flexibility while selectively increasing RMSF in three localized regions (ΔRMSF <0; **Supplementary Fig. S11D**): region 1 (residues 330–342), which connects the NGT and CGT domains; and regions 2 and 3 (residues 375–385 and 387–405, respectively), which line the substrate-binding site in CGT domain (**Supplementary Fig. S11E**). These regions are spatially connected: β8 in region 1 runs parallel to β9 immediately preceding region 2, and regions 2 and 3 are directly linked. These observations suggest the presence of allosteric communication between the ligand-binding sites in the NGT and CGT domains.

References

[1] Perrin-Tricaud, C., Rutschmann, C. & Hennet, T. Identification of domains and amino acids essential to the collagen galactosyltransferase activity of GLT25D1. ***PLoS ONE*** 6, e29390 (2011).

[2] De Marco, M., Rai, S.R., Scietti, L. et al. Molecular structure and enzymatic mechanism of the human collagen hydroxylysine galactosyltransferase GLT25D1/COLGALT1. ***Nat Commun*** 16, 3624 (2025).



Enzyme	K_m (mg/mL)	k_{cat} (min^{-1})	k_{cat}/K_m (mL min^{-1} mg^{-1})
WT	11.54 ± 1.77	391389 ± 24493	33915.86
D166A	27.37 ± 6.38	243429 ± 32401	8894.01
CGT	3.45 ± 0.18	329734 ± 4446	95575.07
NGT	n.d.	n.d.	n.d.

Supplementary Figure 11. Enzyme kinetics and molecular dynamics analysis of GLT25D1.

A, Enzyme kinetics of wild-type (WT) GLT25D1 and its variants, including the NGT mutant D166A, the isolated CGT domain, and the isolated NGT domain. Data are presented as mean ± SD from three independent replicates. Please refer to Methods section for experimental details.

B, Enzyme kinetical parameters, corresponding to the analysis in (**A**).

C, RMSF (root-mean-square fluctuation) curves from molecular dynamics simulations of apo-GLT25D1 (blue curve) and liganded GLT25D1 (with UDP-Galactose bound to NGT domain) (orange curve).

D, Difference in RMSF (ΔRMSF) curve (red) between apo-GLT25D1 and liganded

GLT25D1 (with UDP-galactose bound to NGT domain). Three localized regions (regions 1-3) exhibiting increased RMSF upon ligand binding to the NGT domain are marked with shaded boxes in (**C**) and (**D**).

E, Three regions (regions 1-3) identified in (**C**) and (**D**) are mapped to the ternary complex of GLT25D1, which contains UDP-galactose in NGT domain, UDP and a hydroxylysine-containing acceptor peptide in CGT domain. Specifically, region 1 (residues 330–342) connects the NGT and CGT domains; regions 2 and 3 (residues 375–385 and 387–405, respectively) line the substrate-binding site in CGT domain. These three regions are spatially connected: β8 in region 1 runs parallel to β9 immediately preceding region 2, and regions 2 and 3 are directly linked.

Minor Points:

• *Line 376:*

The sentence "likely weakening the donor phosphoester bond....." is confusing. Typically, binding of Asp437 and Mn^{2+} would stabilize the UDP-Gal and prime it for catalysis, rather than weaken the bond. Please clarify and rephrase accordingly.

Response: We thank the reviewer for this suggestion. We have now clarified and rephrased the confusing sentence in the manuscript (Lines 383-386 in the "Discussions" section) as follows:

"A manganese (II) ion, coordinated by residue D437 of "EDD" motif (Glu435, Asp436 and Asp437), stabilize the donor diphosphate and primes it for catalysis."

• *Figure 4:*

The figure does not depict key interactions involved in Mn^{2+} coordination. It would strengthen the figure to explicitly show metal coordination with UDP-Gal and relevant catalytic residues.

Response: We thank the reviewer for this suggestion. In response, we have revised **Fig. 4D** to explicitly illustrate metal coordination with donor diphosphate and residue D437. As suggested, we have also shown D522 in Fig. 4D, which is identified as the key catalytic base in our later analysis.

• *Discussion:*

While the manuscript mentions the potential relevance of GLT25D1 in disease, it would benefit from a brief discussion of possible therapeutic opportunities, such as targeting GLT25D1 regulation in collagen-related diseases or cancer.

Response: We thank for this insightful suggestion. In response, we have added a brief discussion of possible therapeutic opportunities in the "Discussion" section (Lines 464-475). The added text now reads as follows:

"The molecular characterization of disease-linked GLT25D1 mutations improves our

understanding of pathogenic mechanism and provides a foundation for developing precise biochemical diagnosis to probe GLT25D1 dysregulation in collagen-related diseases. The distinct structural and functional deficiencies caused by specific variants may correlate with varying clinical severities and subtypes, awaiting further investigation. From a therapeutic perspective, these mechanistic insights suggest that restoring enzymatic activity—either through stabilization of misfolded proteins, or gene-based approaches—could represent viable treatment strategies. Moreover, the association of GLT25D1 dysfunction with both inherited vascular/musculoskeletal disorders and tumors highlights the potential of targeting GLT25D1 in both rare diseases and oncology."

Reviewer #2 (Remarks to the Author):

INTRODUCTION AND OVERALL ASSESSMENT

This is a detailed molecular investigation of GLT25D1. The paper includes protein expression, structural analysis by single-particle cryo-EM, and enzymology. From a technical point of view, I personally enjoyed how the careful biochemistry is fully displayed in figure 1 and the elegant application of C3-symmetry expansion to convert the hexamer to a dimer computationally. Overall, the figures are excellent and the text easy to follow. I have some concerns below that need addressing but overall I think this is a great piece of work.

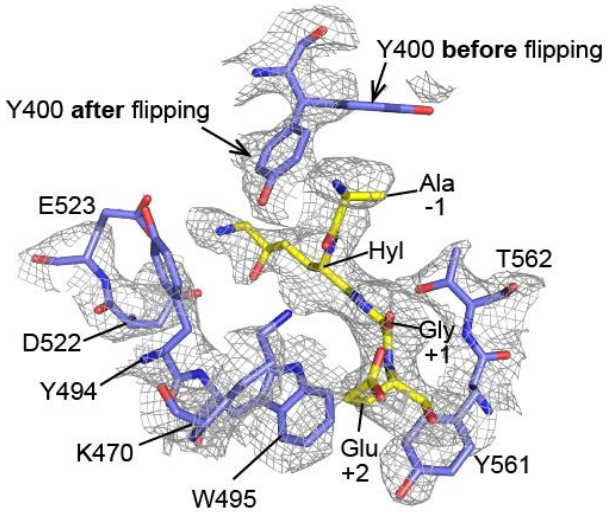
Response: We greatly thank for the professional evaluation and support of our work. We have made the revisions accordingly.

MAP-MODEL CHECKS

Overall, the maps and models look good, with the quality of the maps matching what one would expect. However, the one area of ambiguity is the unexplained density between Ala-2 of the substrate and Tyr-400 of the protein in the ternary complex map. It looks like the Tyr could be flipped around to fill it. The authors should confirm that there is no way to do this whilst maintaining clash and stereochemistry values. Figure 5C should be expanded to show density for ligating residues, particularly Tyr-400 and the mystery blob. If local model building can be made to account for that density then that closes the issue, otherwise it should be highlighted and explicitly mentioned in the text.

Response: We thank the reviewer for this valuable suggestion. Following reviewer's recommendation, we explored flipping Tyr-400 and found that the resulting conformation indeed fits well into the unexplained density (**Response Figure 1**). Accordingly, we have updated the structural model with flipped Tyr-400 in the PDB database and expanded **Figure 5C** to clearly display the density for the acceptor peptide

and its surrounding residues, including Tyr-400.



Response Figure 1. The acceptor peptide (yellow) and its surrounding GLT25D1 residues (blue) are superimposed with their corresponding densities (grey mesh). The arrows indicate Tyr-400 (Y400) before and after side chain flipping.

MAJOR COMMENTS

The issues in the ternary complex map as above.

Response: As described above, we have now addressed this issue as suggested by the reviewer.

How the peptide was synthesized and purified was not reported. This should go in even if its standard.

Response: We thank the reviewer for raising this question. We have now included the source of the hydroxylysine-containing peptide and a corresponding synthesis reference in the "Methods" section (Lines 571-575).

The recent work of Peng et al (Nat Comms, 2024) on the 'KOGG' complex made of GLT25D1/ColGalT1 and LH3/PLOD3 should be cited and the relationship between the two works made clear (the focus of the two papers is clearly different).

Response: We thank the reviewer for this insightful suggestion. This point is also related to Reviewer #3 point #1. We have revised accordingly, positioning our work in the context of recent findings and clarifying our unique contributions (Lines 476-497 in "Discussions" section) as briefly mentioned below:

During the preparation or review of our manuscript, two related studies on GLT25D1 were published [1-2]:

[1]. Peng et al., *Nat Commun*. 2025, 16(1):2436, published during our manuscript preparation;

[2]. De Marco et al., *Nat Commun*. 2025, 16(1):3624, published during the review of

our manuscript.

Specifically, Peng et al. described cryo-EM structures of the GLT25D1–LH3 complex bound to UDP or UDP-galactose, while De Marco et al. used crystallography method to determine the structures of GLT25D1 bound to UDP or UDP-galactose. As also noted by this reviewer and reviewer #4, three studies—though differing in methodological emphasis (cryo-EM vs. crystallography) and research focus (GLT25D1-LH3 complex vs. GLT25D1 alone)—complement and enhance one another. In particular, all three works independently reported the dimeric architecture of GLT25D1 and the unexpected binding of UDP-galactose in the NGT domain.

Beyond these consensus findings, our study provides several unique contributions. Most importantly, we uniquely present GLT25D1 in a ternary complex with UDP/UDP-galactose and a designed hydroxylysine-containing acceptor substrate, supported by detailed structure-based functional characterization. Such a ternary complex is essential for a deeper understanding of a protein-targeting glycosyltransferase like GLT25D1, yet was not available in the other two studies. This approach has enabled us to uniquely define the precise catalytic mechanism and elucidate the molecular determinants governing collagen recognition. Furthermore, in addition to the dimeric form consistently reported across all three studies, we have identified a hexameric assembly of GLT25D1, an architectural feature not previously described in this enzyme family. We also performed a detailed functional analysis of disease-related mutations, clarifying their mechanistic impact on GLT25D1 dysfunction—an aspect not explored in the other studies.

References

[1] Peng, J., Li, W., Yao, D. et al. The structural basis for the human procollagen lysine hydroxylation and dual-glycosylation. ***Nat Commun*** 16, 2436 (2025).

[2] De Marco, M., Rai, S.R., Scietti, L. et al. Molecular structure and enzymatic mechanism of the human collagen hydroxylysine galactosyltransferase GLT25D1/COLGALT1. ***Nat Commun*** 16, 3624 (2025).

The energy window for the GIF should be reported.

Response: We thank the reviewer for this comment. The energy window used for the GIF (20ev) has now been specified in the Methods section (Line 641).

The map resolution range in Table in Suppl Table 1 looks weird. I've never really known what the Nature family of journals mean with this value in their standard table but I typically read it as the range in the local resolution metric. If so, then the smallest value should match (or be slightly better than) the nominal map resolution above. But assuming that is the case, then it's not clear what the lower value should be, as the value will depend a lot on input parameters and nominally the lowest resolution feature

will be set by box size, which is completely arbitrary. Rest of the stats look good.

Response: We thank the reviewer for this professional comment. We agree that the reported resolution range can be influenced by multiple parameters and its interpretation may vary. We also note that some publications have omitted this value. Accordingly, we have removed the resolution range entry from **Supplementary Table 1** in the revised manuscript. The rest of the table remains unchanged.

MINOR COMMENTS

92- Put expression system in main text so its clear to the reader.

Response: We thank the reviewer for this suggestion. In response, we have now included this information in the main text (Lines 96–97).

175- in vitro should be in italices

Response: We have corrected this and have italicized "*in vitro*" throughout the revised manuscript.

323 & 328. Not clear why these words are bolded and underlined.

Response: We apology for the confusion. The bold and underlined formatting was originally used to indicate conceptual hierarchy during the drafting stage. We have now reviewed the entire manuscript and removed all such formatting.

534- GIF quantum energy filter. Quantum should be capitalised. This makes it sounds like it relies on a quantum effect.

Response: We apology for this confusion. We have corrected this.

539- this should be fluence

Response: We have corrected this.

Reviewer #3 (Remarks to the Author):

This manuscript presents cryo-EM structures of human GLT25D1 and its ternary complex with substrates, elucidating the enzyme's role in collagen galactosylation. While the structural and biochemical data are rigorously obtained and technically sound, major concerns regarding novelty arise due to substantial overlap with two recently published Nature Communications articles (Nat Commun. 2025, 16(1):3624 and Nat Commun. 2025, 16(1):2436).

1. The Introduction/Discussion must explicitly position this work against these 2025 publications to clarify its unique contributions.

Response: We thank the reviewer for acknowledging the technical soundness of our

work. As recommended, we have revised the manuscript to explicitly compare our study with the recently published works and to clarify our unique contributions (see Lines 476-497 in the “Discussion” section) as briefly mentioned below:

During the preparation or review of our manuscript, two related studies on GLT25D1 were published [1-2]:

[1]. Peng et al., Nat Commun. 2025, 16(1):2436, published during our manuscript preparation;

[2]. De Marco et al., Nat Commun. 2025, 16(1):3624, published during the review of our manuscript.

Specifically, Peng et al. described cryo-EM structures of the GLT25D1–LH3 complex bound to UDP or UDP-galactose, while De Marco et al. used crystallography method to determine the structures of GLT25D1 bound to UDP or UDP-galactose. As also noted by Reviewers #1 and #4, these three studies—though differing in methodological emphasis (cryo-EM vs. crystallography) and research focus (GLT25D1-LH3 complex vs. GLT25D1 alone)—complement and enhance one another. In particular, all three works independently reported the dimeric architecture of GLT25D1 and the unexpected binding of UDP-galactose in the NGT domain.

Beyond these consensus findings, our study provides several unique contributions. Most importantly, we uniquely present GLT25D1 in a ternary complex with UDP/UDP-galactose and a designed hydroxylysine-containing acceptor substrate, supported by detailed structure-based functional characterization. Such a ternary complex is essential for a deeper understanding of a protein-targeting glycosyltransferase like GLT25D1, yet was not available in the other two studies. This approach has enabled us to uniquely define the precise catalytic mechanism and elucidate the molecular determinants governing collagen recognition. Furthermore, in addition to the dimeric form consistently reported across all three studies, we have identified a hexameric assembly of GLT25D1, an architectural feature not previously described in this enzyme family. We also performed a detailed functional analysis of disease-related mutations, clarifying their mechanistic impact on GLT25D1 dysfunction—an aspect not explored in the other studies.

References

[1] Peng, J., Li, W., Yao, D. et al. The structural basis for the human procollagen lysine hydroxylation and dual-glycosylation. ***Nat Commun*** 16, 2436 (2025).

[2] De Marco, M., Rai, S.R., Scietti, L. et al. Molecular structure and enzymatic mechanism of the human collagen hydroxylysine galactosyltransferase GLT25D1/COLGALT1. ***Nat Commun*** 16, 3624 (2025).

2. The quality of Figure 1 requires improvement

-Figure 1A: Abbreviations "LH/PLODS" require definition in the legend or main text.

-Figure 1B: Claims regarding "dimer" and "hexamer" oligomeric states lack validation. Either add size-exclusion chromatography molecular weight standards to calibrate elution peaks, or remove speculative oligomer labels ("dimer," "hexamer") as these

states are not biochemically verified.

-Figure 1C vs. 1D: Content duplication is apparent. Recommend moving Figure 1D to Supplementary Information.

Response: We thank the reviewer for these valuable suggestions. In response, the abbreviations "LH/PLODs" have now been clearly defined in the revised legend of Figure 1A. The oligomer labels in Figure 1B have been removed. Regarding Figure 1C and 1D, we have carefully considered the reviewer's suggestion and would like to explain our rationale for this organization. Figure 1C and 1D represent two independent assay methods, enabling cross-validation of the findings. Moreover, the use of our designed pure peptide in Figure 1D–F allows direct mass spectrometry-based detection of modifications, which is essential for confirming GLT25D1-mediated galactosylation (Fig. 1D), illustrating donor specificity (Fig. 1D vs. 1E), and demonstrating the enzyme's inability to elongate galactosylated substrates (Fig. 1D vs. 1F). Therefore, presenting these results together would provide more comprehensive insights into the function of GLT25D1, which was also mentioned by Reviewer #2. We wish the reviewer finds this rationale acceptable, but we remain open to further discussion and adjustments should the reviewer still have concerns or suggestions regarding the figure organization.

3. Mutant purification (Lines 216–217): The assertion that failed purification of R61A/V143A/D168A/H235A mutants "might be the effect of UDP-galactose" is insufficiently supported. Alternative explanations (e.g., protein misfolding, solubility issues) must be considered or experimentally addressed.

Response: We thank the reviewer for this insightful suggestion. Following the comment, we have revised the statement to incorporate alternative explanations. It now reads: "a result that may be influenced by the stabilizing effect of UDP-galactose, though other factors such as protein misfolding could also contribute." In addition, to further tone down this claim, we have relocated the statement to the "Discussion" section (Lines 416-417).

4. Thermal stability assays (Lines 223–226): Reduced Tm values for mutants only indicate global destabilization, not specifically impaired ligand binding. Direct binding assays (e.g., ITC, SPR) are needed to substantiate claims about UDP-galactose's role in structural stability.

W135 validation: The rationale for including W135A (not part of initial screening) requires clarification.

Response: We thank the reviewer for this insightful comment and we fully agree with the reviewer. In response, we have significantly toned down our claim and have moved all relevant interpretations from "Results" to "Discussion" section (Lines 432-435). There, we present this as a possible interpretation, integrated with the new molecular

dynamics (MD) simulation data performed at the suggestion of Reviewer #1. Below is our explanation for these changes:

We initially designed this thermal stability assays to investigate the potential regulatory function of non-catalytic NGT domain. As the reviewer rightly noted, direct binding assays can substantiate this claim. However, since the GLT25D1 is purified with endogenous UDP-galactose tightly bound to its NGT domain, we could not obtain ligand-free protein for binding analysis. As reviewer #1 pointed out (point #1), the effects of NGT mutants suggest a possible mechanism of allosteric regulation. Following this suggestion, we have performed molecular dynamics (MD) simulations of GLT25D1 in the apo state and in complex with UDP-galactose bound to the NGT domain (**Supplementary Fig. S11C-E**). From the MD results, the apo form showed elevated flexibility across the protein, as reflected by broadly increased root-mean-square fluctuation (RMSF) values (**Supplementary Fig. S11C**), indicating structural destabilization in the absence of ligand. This aligns with our experimental observation that ligand-binding mutations in the NGT domain generally cause more pronounced destabilization than those in the CGT domain (**Supplementary Fig. 12**).

Regarding the W135A mutant, we apologize for the oversight in not clearly stating its rationale in the initial submission. W135 stacks against the galactose moiety of the donor substrate, as shown in Fig. 3B, and was indeed included in our initial mutant screening. We have now corrected this omission in the revised manuscript (Line 224).

5. No significance testing (e.g., p-values, error bars denoting SD/SEM) is reported for any quantitative data in the whole text (activity assays, thermal shifts, etc.).

Response: We thank the reviewer for this valuable suggestion. In response, we have now incorporated p-values into all relevant activity assay figures. For clarity, error bars representing SD were already included in the original submission, and we have reconfirmed their presence during revision. Additionally, as suggested, we have now also provided both p-values and error bars for the thermal shift data (**Supplementary Fig. 12**).

6. Lines 306–308, the importance of Mn^{2+} for enzymatic activity should be contextualized with prior literature citations.

Response: We thank the reviewer for the suggestion. We have cited relevant literatures [1-3] (Line 314 in the revised text) to emphasize the importance of Mn^{2+} for enzymatic activity in revised manuscript.

References

[1] Spiro, M.J. & Spiro, R.G. Studies on the biosynthesis of the hydroxylsine-linked disaccharide unit of basement membranes and collagens. II. Kidney galactosyltransferase. ***The Journal of biological chemistry*** 246, 4910-4918 (1971).

[2] Myllylä, R., Risteli, L. & Kivirikko, K.I. Assay of collagen-galactosyltransferase and collagen-glucosyltransferase activities and preliminary characterization of enzymic reactions with transferases from chick-embryo cartilage. ***European journal of biochemistry*** 52, 401-410 (1975).
[3] Kivirikko, K.I. & Myllylä, R. Collagen glycosyltransferases. ***International review of connective tissue research*** 8, 23-72 (1979)

7. Alternate use of "hydroxylysine" and "hydroxylated lysine" is confusing.

Response: We thank the reviewer for this helpful suggestion. We have revised to consistently use the term "hydroxylysine" instead of “hydroxylated lysine” throughout the manuscript.

Reviewer #4 (Remarks to the Author):

Relevant Expertise of Reviewer

I am a biochemistry and structural biologist who works on carbohydrate-processing enzymes, while I can comment on these aspects, I cannot pass comment on the mass spectrometry nor collagen-specific biology as I am not an expert in these.

Overview

The paper details the structure and mechanism of GLT25D1, an important enzyme in the modification of hydroxylysine residues in collagen. Overall, the paper is of high quality with excellent written and visual descriptions of the data. It is a very important piece of work that complements and enhances the recent papers by De Marco et al., (https://doi.org/10.1038/s41467-025-59017-5) and Peng et al., (https://doi.org/10.1038/ s41467-025-57768-9). The work is generally robust, and evidence provided is of high quality. It also provides essential new insights including a enzyme UDP/peptide complex and proposed catalytic mechanism. However, I have a few concerns that would warrant consideration and adjustment prior to publication.

Response: We thank the reviewer for the support and the important comments to improve the manuscript. We have revised our manuscript accordingly.

[1] Enzyme Kinetics

To assess enzyme kinetics, the authors have chosen to compare the activities of GLT25D1 domains and their mutants relative to the WT enzyme at fixed substrate concentrations. While this approach provides a basic comparison, a more rigorous and informative analysis would ideally to have been for authors to have undertaken full Michaelis-Menten kinetic experiments for all constructs, including the mutants. At a minimum, Michaelis-Menten kinetic experiments should be conducted for the full-

length, CGT, and NGT constructs—although the latter may exhibit little to no activity. Given the resources available to the authors, incorporating these experiments should be feasible and would significantly strengthen the study's conclusions.

Response: We thank for this insightful comment. We fully agree that kinetic analysis can strengthen our study. As recommended, we have performed kinetic experiments for the full length, the isolated CGT domain, and NGT domain (Supplementary Fig. 11A). The kinetic parameters obtained for the wild-type (WT) GLT25D1 (Supplementary Fig. S11B) were consistent with other studies [1-2]. Moreover, the NGT domain indeed showed negligible activity, as anticipated.

Additionally, following a related suggestion from Reviewer #1 (point #1), we have extended our kinetic analysis to include the NGT mutant D166A and conducted molecular dynamics (MD) simulations to explore the potential mechanism of allosteric regulation. These new kinetic and computational data are now included as Supplementary Figure 11 and collectively provide deeper insights into the function of GLT25D1 (please see related contents in "Discussion" section Lines 419-448).

References

[1] Perrin-Tricaud, C., Rutschmann, C. & Hennet, T. Identification of domains and amino acids essential to the collagen galactosyltransferase activity of GLT25D1. PLoS ONE 6, e29390 (2011).

[2] De Marco, M., Rai, S.R., Scietti, L. et al. Molecular structure and enzymatic mechanism of the human collagen hydroxylysine galactosyltransferase GLT25D1/COLGALT1. Nat Commun 16, 3624 (2025).

Also, in relation to the kinetics, it is also unclear how the data has been normalised relative to the WT protein, e.g. in Figure 3 the WT activity against gelatin is 40% of the activity of WT. This is a contradiction, and it is likely authors have normalised activity to WT against Hyl peptide - however this needs clarification. The methods do indicate 'The final activity was normalized to the GLT25D1 amount quantified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels.' However, this seems like a very unusual way of normalising activity given protein concentrations have been determined. It is also unclear how the SDSPAGE normalisation has occurred; did authors quantify band intensities? If so, these values should be provided in the source data files. Still this is a very unusual way to normalise kinetics, and is possibly incorrect, given authors should be using the same quantity of protein in each reaction mix (which they do suggest they are doing within the methods). Please could authors better clarify the mechanism of data collection and normalisation.

Response: We thank the reviewer for raising this important point. The reviewer is correct in noting the wild-type (WT) activity against gelatin is normalized to WT

activity against the hydroxylysine peptide. We have now clarified this in the Methods section (Lines 592-594 in "Methods" section).

Regarding the use of SDS-PAGE for quantification, this was implemented because initial purity assessments (Supplementary Fig. 5) revealed impurities for certain mutants, and we also observed some inaccuracy in protein concentration determinations of certain mutants. To ensure accurate activity comparisons across variants, we therefore used SDS-PAGE band intensity quantification to normalize the enzyme amounts for WT GLT25D1 and its mutants, rather than relying solely on pre-assay concentration measurements. As helpfully suggested, we have clarified this in the "Methods" section (Lines 588-590) and provided the original SDS-PAGE data in the source data file.

It is also unclear what the three independent replicates are, and this should be clarified e.g. is this from three entirely separate experimental setups? If so, how many technical replicates in each?

Response: We apologize for the confusion. We have now clarified the experimental details. For each enzyme or variant, we independently prepared three separate enzymatic reactions. Each reaction was then individually measured using the UDP-Glo™ Glycosyltransferase Assay kit (Promega).

[2] Bound ion

In the NGT domain, authors have modelled a Mn2+ ion, the recent paper by De Marco et al., has indicated by ICP-MS that this is a Ca2+ ion. Please could authors comment on this, the main text suggests this hasn't been investigated.

Also, an important note, the De Marco paper have modelled the galactose differently in their NGT domain, in my opinion the modelling in this paper under review is much more likely to be correct given the highly unlikely puckering and orientation of the galactose in De Marco et al., however this discrepancy should be discussed within the text given its importance. The Peng et al.,modelling more closely aligns to the paper under review. However, the UDP in CGT is modelled differently in the Peng et al., paper. Given the large discrepancy, there should be a discussion of the modelling of the UDP-Gal and UDP in this paper against the other papers to resolve the discrepancy - a supplementary figure displaying densities and modelling of the UDP and UDP-Gal from all papers would be particularly useful.

Response: We thank the reviewer for these valuable observations and suggestions. We have accordingly incorporated a detailed discussion about the discrepancies in metal ion identity and substrate modeling among these independently determined structures (Lines 497-515 in the "Discussions" section) as briefly mentioned below:

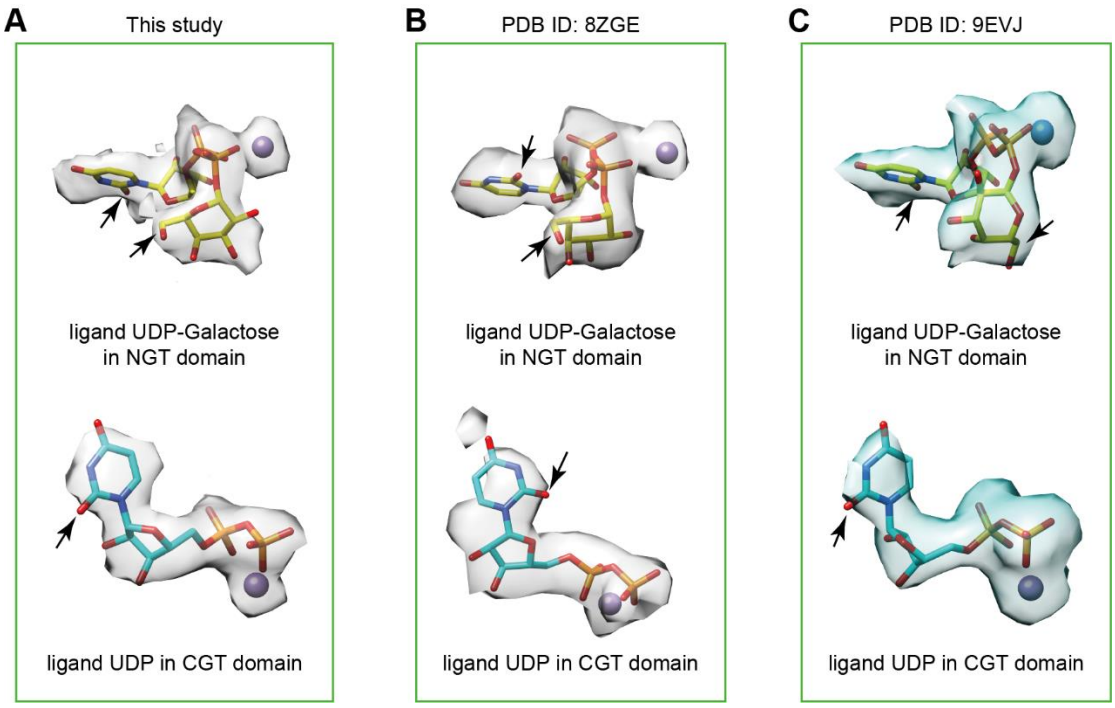
Regarding the metal ion, our cryo-EM sample for the ternary complex was prepared in the presence of 1 mM $MnCl_2$, and accordingly, we modelled Mn^{2+} ions in the ligand binding sites of both the NGT and CGT domains. De Marco et al. reported a

Ca^{2+} ion at the equivalent site of the NGT domain and a Mn^{2+} ion in the CGT domain. It is possible that metal ion occupancy may be influenced by cellular or experimental conditions, and further investigation will be needed to determine the physiologically relevant cofactor.

Regarding ligand modeling, there are also variations as mentioned by the reviewer. As suggested, we have added a new **Supplementary Figure 13** that directly compares the cryo-EM densities and ligand models across these studies. This provides a clear visual reference to help readers evaluate the different structural interpretations:

(1) In the NGT domain, the galactose moiety of UDP-galactose adopts a favorable conformation in our structure, consistent with that reported by Peng et al., but distinct from the conformation reported by De Marco et al. (**Supplementary Fig. 13A-C**). The UDP moiety, however, is consistent between our model and that of De Marco et al., whereas Peng et al. modeled the uracil group in a flipped orientation (**Supplementary Fig. 13A-C**).

(2) In the CGT domain, the UDP orientation is consistent between our study and De Marco et al., but again differs from the flipped uracil conformation reported by Peng et al. (**Supplementary Fig. 13A-C**).



Supplementary Figure 13. Comparison of nucleotide-ligand modeling and corresponding cryo-EM densities among three recent independent structural studies on GLT25D1.

A, GLT25D1 ternary complex with UDP/UDP-Galactose and a hydroxylysine-containing acceptor peptide (this study).

B, GLT25D1/LH3 bound to UDP/UDP-galactose (Peng et al. ***Nat Commun***, 16, 2436 (2025)).

C, GLT25D1 bound to UDP/UDP-Galactose (De Marco et al. ***Nat Commun***, 16: 3624-

3624 (2025)).
For each panel, the top section depicts the UDP-galactose bound to the NGT domain, and the bottom section shows the UDP bound to the CGT domain. Arrows highlight key modeling discrepancies among the corresponding ligands.

[3] Cancer Mutants

Lines 340-351, I'm not sure what this passage of text adds to the narrative. The cancer-associated mutations shown in Supp Fig 11 are located across the protein and I think it is ambitious to suggest that mutations within the circled regions all share a mechanistic basis with the other disease mutants. It would have been more useful to have summarised how each cancer-associated mutation could affect structure/activity in table format. I think a more informative narrative is simply stating that mutations in both domains are associated with cancer, indicating the importance of the NGT domain.

Response: We thank the reviewer for this insightful suggestion. We fully agree and have revised the text accordingly (Lines 352-357 in the section "Molecular insights into pathogenic mutations of GLT25D1"). As suggested, we now emphasize that cancer-associated mutations are distributed across both domains, and to strengthen this point, we specifically highlight four cancer-associated mutations, which locate to key ligand-binding interfaces in both domains: R147H, R147C, and D168H in the NGT domain, and G377D in the CGT domain. The revised text now reads:

"These mutations are distributed across both the NGT and CGT domains (Supplementary Fig. 10), consistent with the functional importance of each domain. Notably, four cancer-associated mutations (R147H, R147C, D168H, and G377D) are situated at key ligand-binding interfaces and are likely to impair GLT25D1 function: R147H, R147C, and D168H in the NGT domain (Fig. 3B), and G377D in the CGT domain (Fig. 4D)."

[4] Catalytic mechanism

Lines 284-288. Authors shown convincing evidence for D522 acting as catalytic base in the mechanism, however regarding "Furthermore, the invariant hydroxylysine (Hyl) is stabilized within this cleft by the "YW" motif (Y494 and W495), with its ε-amino group anchored by E523 (Fig. 5B). This configuration positions the 5-hydroxyl group of hydroxylysine in close proximity to the β-phosphate of UDP in CGT active site, appropriate for galactose transfer." can authors comment on the predicted distance between the proposed nucleophile (Hyl-O) and the predicted position of the anomeric carbon of the galactose, and the suitability of this distance for a concerted Sn2 mechanism. Would authors suggest in slight contradiction to Figure 5F that the D523-Hyl bond is perhaps broken prior to attack? Importantly, the E523 sidechain is not

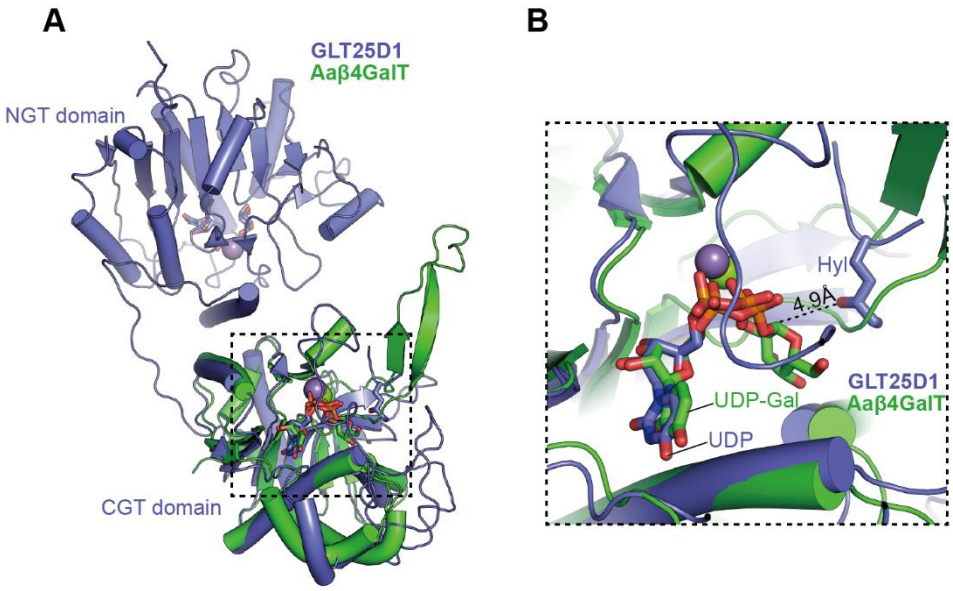
evident in the density map so it is unclear if it is even forming the proposed interaction with the Hyl. While perhaps beyond the scope of the paper, given it is unclear how easily resources can be accessed, MD and QM/MM simulations would have considerably enhanced the manuscript in this regard.

Response: We thank the reviewer for these insightful comments and constructive suggestions regarding the catalytic mechanism.

To address the question of the reaction distance and mechanism, we performed a DALI search using the CTD domain of GLT25D1, which identified a structural homolog: the bacterial galactosyltransferase Aaβ4GalT complexed with UDP-galactose (PDB ID: 8XOC). Although the sequence identity is low (<20%), GLT25D1 CTD domain and Aaβ4GalT can be superimposed with an RMSD of 3.2 Å over 171 aligned residues (**Response Fig. 2A**), revealing a well-conserved architecture of their active sites (**Response Fig. 2B**). Based on this structural superposition, we generated a composite model of the GLT25D1 CGT domain with UDP-galactose (**Response Fig. 2B**). In this composite model, the nucleophile (Hyl-O) is positioned for an in-line attack on the anomeric carbon (C1) of the galactose moiety, with modeled distance of 4.9 Å (**Response Fig. 2B**). While we acknowledge that this value is a homology-based estimate and local conformational dynamics may occur during catalysis process in reality, the overall geometry appears to support the catalytic mechanism proposed in **Fig. 5F**.

Regarding the mechanism illustrated in **Fig. 5F**, we apologize for confusion due to the lack of explanation. The figure legend has been revised to clarify the proposed catalytic step as follows: "Specifically, D522 deprotonates the 5-hydroxyl group of hydroxylysine, activating it as a nucleophile to attack the anomeric carbon (C1) of the galactose moiety." As for E523, although our structural and biochemical evidence suggest its potential role in interacting with the hydroxylysine side chain, we acknowledge that the corresponding electron density is not clearly resolved. Therefore, we have revised the main text to clearly state that E523 is "potentially" involved in this interaction (Line 292).

We fully agree with the reviewer that molecular dynamics (MD) and QM/MM simulations would provide deeper mechanistic insights. While such comprehensive computational studies lie beyond the scope of the current structural and biochemical work, we believe the structural framework and mechanistic hypotheses established here will serve as a solid foundation for future in-depth investigations.



Response Figure 2. Structural comparison between GLT25D1 CGT domain (with UDP in CGT domain) and bacterial galactosyltransferase Aaβ4GalT (complexed with UDP-galactose).

A, Structural alignment between GLT25D1 CGT domain (this study; in blue; with UDP and Hyl shown as sticks) and Aaβ4GalT (PDB ID: 8XOC; in green; with UDP-galactose shown as sticks).

B, Close-up view of the ligand binding sites between GLT25D1 CGT domain and Aaβ4GalT, as marked by the dashed box in (**A**). In this estimated composite model, the nucleophile (Hyl-O) is positioned for an in-line attack on the anomeric carbon (C1) of the galactose moiety, with modeled distance of 4.9 Å.

[5] Terminology

Cryo-EM does not produce 'electron density' maps, this term needs updating throughout the text (see: DOI: 10.1002/pro.3060 and DOI: 10.1016/j.sbi.2019.04.006). Also, when density maps are shown in figures the thresholds should be indicated.

Response: We thank the reviewer for this correction and for providing the relevant references. We fully agree and have therefore replaced all instances of "electron density map" with "EM map" throughout the manuscript. Furthermore, as suggested, we have now specified the display thresholds for all density maps in their respective figure legends.

[6] Multimeric state

Given the Marco et al., paper proposes that the enzyme is dimeric, there needs to be discussion in the main text acknowledging this work, and a discussion of the

discrepancies.

Response: We thank the reviewer for this constructive suggestion. We have revised the manuscript accordingly to acknowledge recent related works and to discuss discrepancies regarding the oligomeric assembly of GLT25D1 (Line 492-495 in "Discussion" section). While all three independent studies—including ours, De Marco et al.[1], and Peng et al.[2]—consistently report the dimeric form of GLT25D1, our study further identifies a hexameric assembly, an architectural feature not previously described in this enzyme family. These findings have now been incorporated into the "Discussion" section (Lines 476-497), where we position our work in the context of recent structural studies and clarify the unique contributions of our findings.

References

[1] De Marco, M., Rai, S.R., Scietti, L. et al. Molecular structure and enzymatic mechanism of the human collagen hydroxylysine galactosyltransferase GLT25D1/COLGALT1. ***Nat Commun*** 16, 3624 (2025).

[2] Peng, J., Li, W., Yao, D. et al. The structural basis for the human procollagen lysine hydroxylation and dual-glycosylation. ***Nat Commun*** 16, 2436 (2025).

Other Revisions

[1] Remove the bold and underlining at the start of sentences e.g. 'Additionally', 'Further Analysis'

Response: We have removed all the bold and underlining labels throughout the manuscript.

[2] It would be good to see a discussion regarding why NGT is not active from a structural viewpoint.

Response: We thank for this suggestion. In response, we have added a discussion about the reason why NGT is not active (Lines 402-410 in "Discussions" section). It reads as follows:

"However, multiple lines of evidence indicate that the NGT domains lacks catalytic activity (Fig. 3D, Figure 4A, Supplementary Fig. 5H). Consistently, our cryo-EM analysis of the ternary complex further reveals that the acceptor peptide substrate binds specifically to the CGT domain rather than the NGT domain (Fig. 4B). Additionally, although a negatively charged residue (D265) is located near the anomeric carbon (Fig. 3B)—a position often associated with catalytic base function in glycosyltransferases—alanine substitution at this site only mildly impaired enzymatic activity (Fig. 3D), ruling out its role in catalysis."

[3] Could authors clarify if MS experiments were run once per sample or were there repeats.

Response: The MS experiments were run once per sample. We have clarified this in the "Methods" section (Line 625). The objective of MS experiments was qualitative analysis and we initially cross-validated MS findings with independent biochemical assays—for example, the consistent donor substrate specificity identified both in the MS data (Fig. 1D-1E) and in the biochemical assays (Fig. 1C).

[4] Lines 71-74: This part could better describe the motif e.g. presence of Hyp.

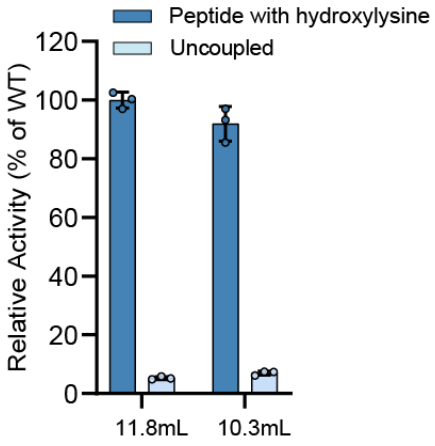
Response: We appreciate the reviewer's suggestion. We have rephrased this part (Lines 78-79 in the revised text) to better describe the motif, including the presence of hydroxylysine Hyl and hydroxyproline Hyp. It now reads as follows:

"GLT25D1 is responsible for transferring galactose from UDP-galactose to hydroxylysine (Hyl) at specific Yaa-positions within the characteristic Gly-Xaa-Yaa motifs of the collagen helical domain, where Yaa is often occupied by Hyl or Hyp (hydroxyproline)."

[5] Line 96: It is unclear from the text which of the two elution peaks were used to determine that GLT25D1 was active.

Response: The activity assay was conducted with the protein sample obtained after affinity chromatography and desalting, without size-exclusion chromatography. We have clarified this in the "Methods" section (Lines 563-567).

To directly address the reviewer's query, we measured the enzymatic activities of both elution peaks, and found that both peaks exhibit comparable levels of enzymatic activity (**Response Figure 3**).



Response Figure 3. Enzymatic activity of GLT25D1 from two elution peaks.
Enzymatic activity of GLT25D1 samples from two SEC size-exclusion chromatography peaks (10.3ml-peak and 11.8ml-peak, respectively).

[6] Line 100: 'In presence of Mn2+' needs adding.

Response: We appreciate the reviewer's suggestion and have revised accordingly (Lines 104-105 in the revised text).

[7] Line122-126: Unclear if the protein used was a mix of the dimer/tetramer SE peak, or an isolated peak.

Response: Thank you for raising this important point. This comment is related to your point #25. We have now supplemented the relevant details to address both issues.

For cryo-EM analysis of the apo-state (Supplementary Fig. 1A–C), we used a pooled sample containing both major oligomeric forms, corresponding to the 10.3 mL and 11.8 mL SEC peaks shown in Figure 1B.

For cryo-EM analysis of the liganded ternary complex, since our data processing strategy relied heavily on large numbers of dimer particles (Supplementary Fig. 6A–C), we specifically used the later 11.8 mL SEC peak.

We have revised the indicated text (Lines 130–132; Line 245) as well as the "Methods" section (Line 630-631; Lines 634-635) to clearly state this information, ensuring it is unambiguous for readers.

[8] Line 172-174: How was significance determined? Also, by eye, it looks like activity of gelatin doesn't change but does on Hyl containing peptide?

Response: We apologize for this confusion. The intended description was "not dramatically" rather than "not significantly". We have corrected this in the revised manuscript (Line 181 in the revised text).

[9] Line 180-190: Please include surface area for dimer-dimer interactions that comprise the hexamer.

Response: Thank you for this valuable suggestion. We have included this surface area (1924 $Å^2$) in the revised text (Line 190).

[10] Line 242 space needed between 'Fig.' and '1D'.

Response: We have corrected this in the revised manuscript.

[11] Line 251: Please specify in main text how H560 and R351 stabilise loop.

Response: We thank for this suggestion. In our initial submission, we had the description about H560 and R351 in the figure legend of Supplementary Fig. 7C. In the revised manuscript, we have added this description to the main text (Line 255-257) as follows:

"H560 and R351 function to stabilize this ordered loop through backbone stacking at its beginning and a salt bridge with D570 at its middle, respectively (Supplementary Fig. 7C)"

[12] Line 324 "the SVD-associated point mutations L151R and A154P, located in helix α3, play critical roles in stabilizing the domain core fold"
Here it is inferred the point mutations induce stability, which they don't, I would rephrase and be more specific about the effects of point mutants, e.g. L151R is introducing a larger, charged residue into a stabilising hydrophobic core.
Response: We thank the reviewer for this insightful suggestion. We have revised accordingly (Lines 330-335 under section "Molecular insights into pathogenic mutations of GLT25D1"). It now reads as follows:
"The SVD-associated point mutations L151R and A154P, located in helix α3, are expected to disrupt the domain's core fold stability (Fig. 6A). Specifically, the L151R mutation introduces a large, charged residue into a hydrophobic core, while A154P is likely to impair helical structure and packing."

[13] Line 336 "Indeed, neither G377R nor R471W significantly", while this is quite possibly the case, as band intensity has not been calculated and statistical tests not performed, whether the result is 'significant' cannot be determined, the sentence needs to be less absolute.
Response: We appreciate this valuable suggestion and have revised accordingly. We have removed the "significantly" and the new phrasing is: "neither G377R nor R471W appear to markedly affect …" (Line 345 in the revised text).

[14] Line 375, β-pyrophosphate should be β-phosphate
Response: We have corrected "β-pyrophosphate" to "β-phosphate" (Line 385 in the revised manuscript).

[15] Line 456-467: Please provide more details regarding how the SDM was performed including primer sequences.
Response: We thank the reviewer for this suggestion. We have now provided a more detailed description of the site-directed mutagenesis (SDM) procedure in the "Materials and Methods" section (Lines 529-536). Additionally, the corresponding primer sequences have been included as Supplementary Table 3.

[16] Line 464: How were cells transferred to BMMY media.
Response: We thanks for this comment. In the revised manuscript, we have added detailed description of the transferring to BMMY media as follows (Lines 540-542 in "Methods" section):
"cells were harvested by centrifugation at 3,000g for 5 min. The BMGY medium was then removed, and the cell pellet was resuspended in BMMY medium."

[17] Line 466: 'following the manufacturer's protocol' it is unclear from the text which protocol this is, as the sentence doesn't refer to a very specific technique.

Response: We apologize for the confusion. The sentence referred to the standard induction protocol from the Pichia Expression Kit USER GUIDE (Invitrogen, Publication Number MAN0000012). We have revised the manuscript to explicitly cite this manual and include the specific induction conditions for clarity (Lines 542-545 in "Methods" section).

[18] Line 469-470: 'was applied to Ni-NTA affinity chromatography' Ni-NTA is the technique, do you mean Ni-NTA resin? Was this gravity flow purification or FPLC-mediated?

Response: We thank the reviewer for this comment. In response, we have revised the phrasing to "Ni-NTA resin" as suggested and clarified that the purification was performed using an ÄKTA pure chromatography system (Lines 548-550 in "Methods" section).

[19] Line 470: 'After washing', what was the wash buffer and how much was applied?

Response: We thank the reviewer for this comment. The washing procedure has been described in detail in the revised manuscript (Lines 550-552 in "Methods" section), which now reads:

"The column was sequentially washed with 10 column volumes each of buffer A containing 20 mM, 50 mM, and 100 mM imidazole, respectively."

[20] Line 472-472: What ratio of HRV3C to protein did you use? Where was the HRV3C sourced?

Response: We thank for this comment. The HRV3C protease (sourced from Genscript) was used at a ratio of 1:20 (w/w, protease:protein), and this information has been included in the revised manuscript (Lines 553-556 in "Methods" section).

[21] Line 478: How was protein concentration quantified? If by A280 please provide ext.co and Mw used.

Response: Thank you for this comment. Protein concentration was quantified by A280 using NanoDrop Spectrophotometers, with an extinction coefficient of 107635 M^{-1} cm^{-1} and a molecular weight of 68.3 kDa, respectively. These details have been added to the revised manuscript in the "Methods" section (Lines 560-562).

[22] Line 482: How was the hydroxylated peptide synthesised?

Response: We thank the reviewer for raising this question. We have now included the commercial source of the hydroxylated peptide and a corresponding synthesis reference in the "Methods" section (Lines 571-575).

[23] Line 484: I would prefer the concentration of protein to be reported in molarity to enable immediate comparison with the donor and acceptor substrate concentrations.

Response: We thank for this suggestion. As recommended, we have now included the protein concentration expressed in both molarity and mass units (Lines 577-578 in the "Methods" section).

[24] Line 526: How were grids glow discharged?

Response: We thanks for this comment. The detailed glow-discharge conditions have been added to the revised manuscript (Lines 628-629 in "Methods" section) as follows: "The grids (Quantifoil R1.2/1.3 Au, 300 mesh) were glow-discharged using a PELCO EasiGlow unit at (15 mA for 60 s)."

[25] Line 525-532: It is unclear whether the dimer or tetramer peaks from the SE were used for the unliganded and ternary state. The latter cryo-EM data suggest tetramer was used for the unliganded CTD structure and dimer for ternary structure, if this is not the case, can authors comments on why they see dimer in the latter?

Response: Thank you for raising this important point. This comment is related to your point #7. We have now supplemented the relevant details to address both issues.

For cryo-EM analysis of the apo-state (Supplementary Fig. 1A–C), we used a pooled sample containing both major oligomeric forms, corresponding to the 10.3 mL and 11.8 mL SEC peaks shown in Figure 1B.

For cryo-EM analysis of the liganded ternary complex, since our data processing strategy relied heavily on large numbers of dimer particles (Supplementary Fig. 6A–C), we specifically used the later 11.8 mL SEC peak.

We have revised the indicated text (Lines 130–132; Line 245) as well as the "Methods" section (Line 630-631; Lines 634-635) to clearly state this information, ensuring it is unambiguous for readers.

[26] Line 563: Can the authors check the 275,468 number, a C3-symmetry expansion of 125,162 would suggest 375,486 particles.

Response: We apologize for the typographical error. The number should indeed be 375,486 particles. We have corrected this.

[27] Line 596: 'Interactive' not 'manual' rebuilding

Response: We have replaced 'manual' with 'Interactive'.

[28] Line 802: I think Figure 1A is a little confused as it doesn't specifically show lysine residues as the modified amino acids. I think a chemical diagram showing each step would have been better but is not essential.

Response: Thank you for your valuable suggestion. We have revised Figure 1A accordingly. Chemical diagram showing the detailed modification on lysine has been added (Fig. 1A).

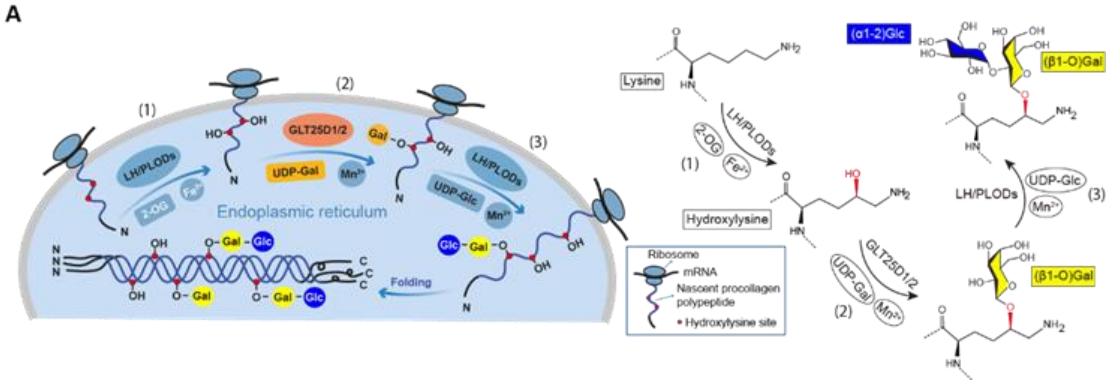


Fig. 1A. Schematic representation of the collagen modification pathway. Hydroxylysine residues formed by lysyl hydroxylases (LH/PLODs) at specific sites of nascent procollagen are galactosylated by GLT25D1/2. Some of this modification undergo further glucosylation by lysyl hydroxylases.

[29] Line 838: Use red or pink to describe asterisk, not salmon as this is confusing.

Response: The term 'salmon' has been replaced with 'pink' to describe asterisk accordingly.

[30] Line 857: Trimer not 'timer'

Response: The spelling mistake has been corrected.

[31] Line 870: The UDP in the diagram is all orange, making the phosphates difficult to ascertain and in contrast to the insert. I would change this to yellow to match insert.

Response: We thank for this suggestion. The UDP in Figure 3A and 3B has been changed to yellow to match inset.

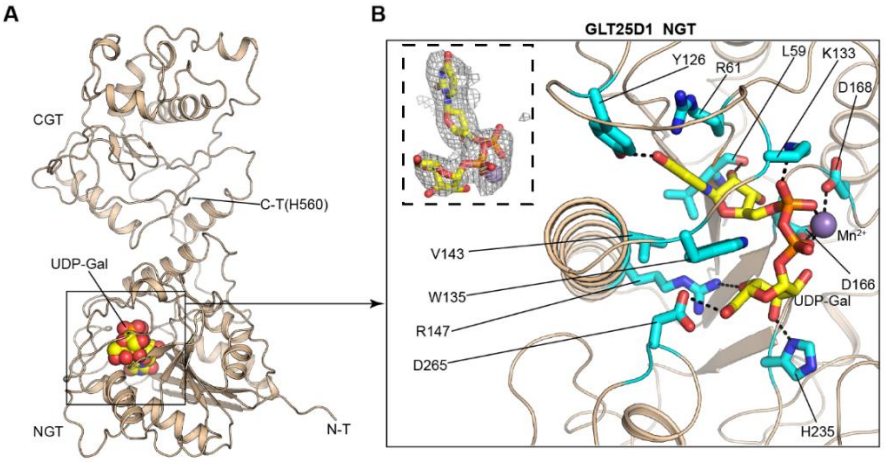


Figure 3. Endogenous UDP-galactose bound to NGT domain of GLT25D1

[32] Line 891: You are missing a few hydrogen bonds in this diagram suggested in main text e.g. K470 to Ala in peptide.

Response: We thank for the suggestion. The hydrogen bonds (yellow dashed lines) between enzyme and peptide residues have been added into the revised Figure 5B.

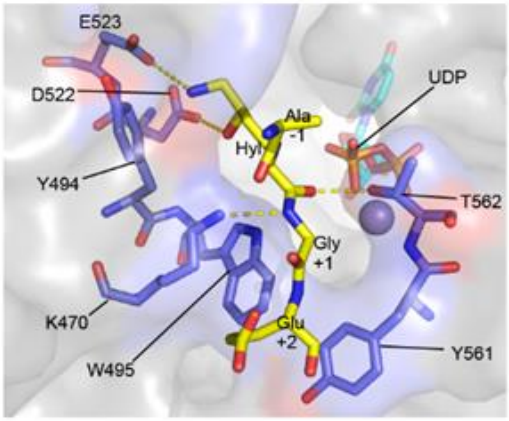


Figure 5B. Close-up view of the CGT active site in the ternary complex structure of GLT25D1 (in grey surface rendering) with collagen acceptor peptide (yellow stick) and UDP (cyan stick).

[33] Line 913: For figure 6 the 'C' and 'D' are mapped incorrectly in legend, C should be B and D should be C.

Response: The panel labels have been corrected as suggested.

[34] Line 929-930 and 1001-1002: For Sup Figure 1D and E and 7D and E, please provide the corrected, unmasked, masked, and randomised FSC curves.

Response: We thank the reviewer for this suggestion. In response, the updated corrected, unmasked, masked, and randomised FSC curves have been provided.

[35] Line 970: Trimer not 'timer'

Response: The spelling mistake has been corrected.

[36] Line 980-986: Can you provide uniport identifiers in parentheses after organism's name in legend.

Response: We thank the reviewer for this suggestion. The corresponding uniport identifiers have now been added in the specified place (legend of Supplementary Fig. 4).

[37] Line 1066: Most and least conserved not 'conservative'.

Response: We thank the reviewer for this comment. "Most/least conservative" has been replaced with "most/least conserved" in the specified place (legend of Supplementary Fig. 9).

Reviewer #5 (Remarks to the Author):

This manuscript presents a rigorous structural and biochemical dissection of GLT25D1, a collagen galactosyltransferase essential for post-translational modification of hydroxylysine residues. The authors employ high-resolution cryo-EM, structure-guided mutagenesis, and enzymatic assays to map the catalytic site, elucidate substrate specificity, and rationalize the effects of disease associated mutations. The work is technically sound and clearly presented, and it provides a valuable resource for understanding the molecular mechanism of collagen glycosylation.

However, in considering the broader impact expected for publication in Nature Communications, I am uncertain whether the conceptual advance reaches the necessary threshold. While the structural insights into GLT25D1 are new and welcome, they pertain to a single, well-known enzyme, and the study largely confirms or refines prior expectations (e.g., involvement of the CGT domain in catalysis, recognition of the "X-Hyl-Gly" motif). The physiological or translational implications are discussed but not experimentally explored, and the potential connections to disease, though interesting, remain somewhat speculative.

Response: We thank the reviewer for the positive assessment of our study's technical quality and value to the field of collagen glycosylation. We have carefully considered the comment and have revised the Discussion to more clearly present the unique contribution and the broader implications of our findings.

Although GLT25D1 is a well-known important enzyme in collagen biology and is linked to severe human diseases (such as SVDs, VCI, connective tissue disorders), the molecular basis underlying its crucial collagen modifying activity has remained elusive. Our work addresses this key gap by resolving the first ternary complex of GLT25D1 with both UDP/UDP-galactose and a designed hydroxylysine-containing collagen-mimetic substrate. Such a ternary complex is essential for understanding a protein-targeting glycosyltransferase like GLT25D1. This advance, complemented by comprehensive functional analyses, enables us not only to unambiguously delineate the catalytic center within the CGT domain and elucidate the molecular determinants governing collagen recognition, but also to establish an inverting S_N2 catalytic mechanism with Asp522 acting as the catalytic base.

Beyond these insights, our work also reveals several important and unexpected findings. We identified an endogenous UDP-galactose molecule bound to the non-

catalytic NGT domain that contributes to structural stability, suggesting a previously unrecognized mode of enzyme regulation. Furthermore, we uncovered evidence of a higher-order oligomeric assembly (dimer–hexamer), an architectural feature not previously described in this enzyme family. Finally, by systematically characterizing disease-associated mutations linked to SVDs, VCI, and related connective tissue disorders, we provide mechanistic explanations for how disease-associated mutations impair GLT25D1 function through distinct molecular mechanisms. These insights not only advance the molecular understanding of disease etiology but also establish a foundation for developing biochemical diagnostics to detect GLT25D1 dysregulation. In response to Reviewer #1's suggestion, we have further expanded the Discussion to include potential therapeutic strategies targeting GLT25D1 dysfunction.

Collectively, these results establish a comprehensive mechanistic framework for collagen galactosylation that spans donor and acceptor substrate recognition, precise catalytic mechanism, regulation, oligomerization architecture, and pathogenic mechanisms. We hope that this work will stimulate future research to explore the fundamental and translational implications of these findings.

Minor comments:

Line 73: the authors refer to a Gly-X-Y sequence. Y is the symbol for tyrosine, but it seems it is not used to denote tyrosine here.

Response: We thank for this comment. To avoid confusion of symbol Y, 'Gly-X-Y' have been replaced with three-letter symbol 'Gly-Xaa-Yaa' (Line 78 in the revised text).

There are several instances where "timer" has been written in place of "trimer" by mistake.

Response: We thank for this suggestion. The spelling mistakes have been corrected.

Point-to-point responses to the reviewers' comments

We greatly thank the constructive suggestions from reviewers. Our responses below are in blue.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors have done a great job and have thoroughly addressed my previous comments. I have only two minor points for further clarification:

Response: We thank the reviewer for the support and for the valuable help to improve our manuscript.

a.-) Could the authors explain why the CGT domain exhibits a better Km towards gelatin than the wild-type enzyme? It seems the NGT domain may negatively affect binding to gelatin.

Response: We thank the reviewer for this insightful comment. We agree with the reviewer that CGT domain exhibits a lower Km than the WT enzyme, suggesting that NGT domain may negatively affect the binding of gelatin to CGT domain. This interpretation is consistent with our kinetic data and molecular dynamics simulations, which indicate allosteric communication between the NGT and CGT domains (Supplementary Fig. 11). To clarify this point, we have added the following statement in the revised 'Discussion' section (fourth paragraph):

"Consistent with this notion, kinetic analysis also indicates that the NGT domain influences the CGT domain, as evidenced by a lower Km of the CGT domain toward gelatin compared to that of the wild-type enzyme (Supplementary Fig. 11A)."

b.-) How many replicates were performed for the molecular dynamics simulations? I have not found this in Methods.

Response: We thank the reviewer for pointing this out. The molecular dynamics simulations were conducted with four replicates. This missing information has now been added to the "Molecular dynamics simulations" subsection (second paragraph) within the Methods section of the revised manuscript.

Reviewer #2(Remarks to the Author):

Dear authors, fellow reviewers, and editorial team,

The authors have addressed the concerns I had in my original review. I'm particularly glad to see that Tyr flipping into the density in a visually pleasing fashion.

I lack the subject specific knowledge to comment on the other reviewers comments.

Yours sincerely,

Jamie Blaza

University of York

Response: We thank the reviewer for the support and for the valuable help to improve our manuscript.

Reviewer #3(Remarks to the Author):

The authors have answered all my concerns. I am now recommend the publication of this work.

Response: We thank the reviewer for the support and for the valuable help to improve our manuscript.

Reviewer #4 (Remarks to the Author):

The authors have made considerable improvements to the manuscript since the initial review improving its overall quality. The results support the conclusions, and the results are of significance to the field. The methodology is sound and meets the expectations of the field and is sufficiently detailed to be able to be reproduced. My points raised within the initial review have been addressed and the work is now of high quality.

Response: We thank the reviewer for the support and for the valuable help to improve our manuscript.

Although, I have three very minor edit suggestions, linked to the new edits made:

[1] Line 181 in revised manuscript needs to say "against gelatin" as the peptide with Hyl is significantly less.

Response: We thank the reviewer for this valuable suggestion and we have revised accordingly by adding ‘against gelatin’ as suggested.

*[2] Line 1050 p value number is same for***and****, they should be different.*

Response: We apologize for the typo. We have corrected this (in the figure legend of Figure 2I) and it now reads: “***$p < 0.001$; ****$p < 0.0001$”.

[3] Line 1094 the hydrogen bonds differ between "B” and "D" even though they are simply rotated images, this likely results from incorrect H-bonds in "B”. Tied to this

Line 298-299"its interaction with acceptor peptide only relies on the B-carbon of this residue" while in fact H-bonding suggests involvement of T562 hydroxyl. Did the authors mean "sidechain"?
These edits are minor and do not detract from my enthusiasm for the work.

Response: We thank the reviewer for the support and for this insightful comment. We would like to clarify that panels "B" and "D" illustrate different aspects of the interaction. While panel "B" highlights specific hydrogen bonds, panel "D" displays distances between selected groups to depict the narrowest constriction within the binding cleft. We have revised the sentence noted by the reviewer to avoid confusion (located in the first paragraph under section 'Acceptor peptide binding mode and catalytic mechanism'). The revised sentence now reads as follows:
"Interestingly, alanine substitution of T562 preserved activity (Fig. 5E), suggesting that its interaction with acceptor peptide may more rely on the β-carbon of this residue (Fig. 5D)."

Reviewer #5(Remarks to the Author):

All of my comments have been addressed in the revision.
Response: We thank the reviewer for the support and for the valuable help to improve our manuscript.