nature portfolio

Peer Review File

Cryo-EM Structures Reveal the Molecular Mechanism of SUMO E1-E2 Thioester Transfer

Corresponding Author: Dr Shaun Olsen

Version 0:

Decision Letter:

13th Dec 2024

Dear Dr Olsen,

Thank you again for submitting your manuscript "Cryo-EM Structures Reveal the Molecular Mechanism of SUMO E1-E2 Thioester Transfer". I apologise for the delay in responding, which resulted from the difficulty in timely obtaining and discussing suitable referee reports. Nevertheless, we now have comments (below) from the 3 reviewers who evaluated your paper. In light of these 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 though Reviewers #2 and #3 appreciate the potential novelty of the findings, Reviewer #1 is much more critical. Notably, all the experts raise important concerns that need to be convincingly addressed in a revised manuscript. To increase the chances of success of the manuscript, we ask you to please take heed of all of the reviewers' requests and follow their guidelines both experimentally and textually. More specifically, both Reviewers #1 and #3 note that the molecular contributions of site II are underexplored and need further substantiating with mutants or other experiments. The referees also note that the notable UFD rotation requires mechanistic delineation to identify its triggers and regulation. Moreover, Reviewer #1 requests simulations and clarifications that would confirm and illustrate the relevance of the mimetic, particularly in comparison to the native state. Finally, Reviewer #3 requests, and we editorially agree that this would be a notable improvement, that the effects of some of the newly identified mutants are demonstrated on substrate SUMOylation in cells. 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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https://www.nature.com/documents/nr-reporting-summary.pdf

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 maps, models, and PDB validation reports.

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.

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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,

Dimitris Typas
Senior Editor
Nature Structural & Molecular Biology
ORCID: 0000-0002-8737-1319

Reviewers' Comments:

Reviewer #1 (Remarks to the Author):

Nayak et al. carry out a structural and biochemical analysis of the SUMO E1 activating enzyme. Whilst numerous structures exist for Ub/Ubl E1 enzymes, no structure exists for the complete heterodimeric SUMO E1 in complex with its cognate E2 Ubc9. Using CryoEM, two structures are determined. The first is a singly loaded structure where E2 is engaged and a single SUMO molecule is bound to the initiating adenylation domain, where density revels it is the SUMO-AMP adduct that is trapped at this site. To stabilize the bound E2, a mutant form is used and chemically activated at its catalytic cysteine. This activated E2 can then undergo disfulfide exchange with the E1 catalytic cysteine to form a disulfide mimic of the transthiolation complex. The SUMO at this site is missing, but the disulfide accurately positions the E2 in the catalytically functional location. Key interfaces are identified termed Sites I-III. The functional significance of site I and III interfaces is validated using biochemical assays. Notably, a large rotation and translation of the UFD is required for cysteine juxtaposition.
A second structure obtained is the doubly loaded species, where the SUMO being handed over from E1 cysteine to E2

cysteine (SUMO(t)) is also present. To trap this species, disulfide chemistry is again used but to stably retain the SUMO(t), it is isopeptide linked to an artificial lysine residue introduced adjacent to the E2 catalytic cysteine. Whilst the structures are a very nice addition as they capture the transfer steps in a complete complex, some aspects might have been reasonably assumed by leveraging existing structures and performing modelling. The limited resolution of SUMO(t) and the clunky trapping strategy also dimmish impact. Particularly in light of the more structurally accurate strategy recently used by Lima for the Ub complex. As a result, I would consider this manuscript of borderline suitability for NSMB once the requested revisions are made.

Major points

The structure determination and, biochemical and cellular assays are all performed to a high standard. The notable advance is establishing exactly how the UFD moves relative to current E2-free structures to position the bound E2 Cys in proximity of the E1 Cys. However, there are some concerns over the novelty of the UFD-E2 interaction (site I). There is a reported 2.3 angstrom crystal structure of this reductionist complex (Wang et al PLoS One 2010). The basic E2 patch complementing the acidic UFD patch was characterized in this study. It would have been helpful to discuss how this compares to the cryoEM interface. My feeling is there is not much novelty here so its description in the “Determinants of SUMO E1 specificity for Ubc9” and “Molecular recognition of Ubc9 by SUMO E1” sections might be unwarranted. These two sections come across as laboured and should be consolidated and condensed. The novel aspects should be discussed (UFD rotation/translation, Cys cap reorganisation and its binding to Ubc9) and anything else consistent with prior work should be stated as such and moved to supplementary.

The site II interface is described but not functionally tested. If should be tested for an active role in catalysis and discussion paired back/emphasised accordingly.
Structure determination of the doubly loaded complex is an impressive achievement. However, the caveats relating to how the complex was engineered for stability must be made clearer in the main section. Although the approach has been used before, a schematic showing the chemical differences between the mimetic and the native tetrahedral intermediate should be displayed in the main text (similar to the graphic for the singly loaded complex in Figure 2b). The native tetrahedral chemistry should be modelled in (supplementary data) so it can be understood if the bona fide intermediate is positionally plasusible with the cryoEM model. If not, the implications should be discussed.

Other major points
• Inclusion of sequences alignments across orthologues illustrating conservation of key identified residues would support/counter the conclusions
• The use of charge swapped single and triple/quadruple alanine mutations for validation purposes is unusual. Whilst I appreciate the interfaces may depend on cumulative interactions, the potential perturbation from these extensive disruptions is a concern. This should be addressed by, for example, melting temperature analyses.
• To assess the importance of the hinge region between AAD and UFD mutations to glycine are chosen. A more intuitive substitution would be to rigidifying proline mutations, rather than more flexible glycines. Could the authors comment?
• Show sequence alignment of the AAD-UFD for SUMO and Ub E1 orthologues.

Minor
Define SUMO(a) and SUMO(t) in the main text. E.g. at line 60 for SUMO(a)
Figure 2a draw direct line between cysteines if that is the measurement taken.
Figure 2d. For the reader to appreciate the nature of the thioester transfer assay it would help if a representative gel was inserted.
Figure 3c, d – why not match the bar colours to the colours in the structure figure panels?
Line 225 – define hC (helix C presumably)
Figure 5 a. The Beta12 beta13 loop of the UFD does not look red in the figure.
Figure 5b. Show axis of rotation and direction of translation
Figure 6b-e. It would helpful if the E2 was coloured in slightly different shade of grey so its position can be clearly seen.
The authors define the distinct interfaces as SiteI, II and III. Going forward, they do not stick to this nomenclature, (even within figure 3), making it difficult to follow what specific interfaces are being disrupted in biochemical and cellular assays.
Source data should include all replicate gels, not just representatives

Reviewer #2 (Remarks to the Author):

In this manuscript Nayak et al. reports the functional and structural characterization of two cryoEM structures: the SUMO E1-E2 trans-thioester complex in the presence of one SUMO (adenylate); and a similar complex with an additional SUMO thioester intermediate. The structural details of the trans-thioester transfer from SUMO E1 to E2 are nicely revealed from these structures, with the formation of an extensive interaction network between Ubc9 (E2) and different regions of the E1 multidomain protein The results presented in the manuscript are truly compelling and shed light on several previously unknown aspects of the trans-thioester reaction between E1 and E2 in the SUMO system. The structural characterization is exceptionally well-executed and is complemented by rigorous biochemical and mutagenesis analyses. The manuscript is well-organized and easy to follow. I recommend publication, subject to addressing a few minor issues.

Comments:

In several instances, the manuscript refers to the E1 structure without E2 as "apo." While this term is often used in the ubiquitin/SUMO field, its traditional meaning in enzymology refers to an inactive enzyme lacking a cofactor or prosthetic group. To avoid potential confusion, I suggest considering an alternative term. Perhaps "E1-only" or "E1-unbound" would be more accurate.

The 175° rotation of the UFD domain, while striking, is somewhat expected if the two active site cysteines must come together. An open question remains: What triggers the dramatic UFD conformational change during trans-thioester transfer? Is E2 binding alone sufficient to induce this conformational shift? Or are additional factors, such as subtle conformational changes near the crossover loop or Zn site, necessary to initiate the UFD movement from its inactive to active state?

Figure 6, panel b, two of the structures have the same PDB Id. Is it correct ?

Regarding the double-loaded SUMO complex, I understand that the resolution is relatively low, particularly in the SUMO(t) region, which may indicate flexibility.
When compared to other E2-UbL(t) or E2-SUMO(t) structures, the orientation of SUMO(t) appears completely opposite. In the current structure, Ubc9 binds the helical face of SUMO(t), while in all other structures, E2 binds the beta surface. How confident are you in the orientation of SUMO(t) in the current structure? Do the maps truly support this orientation?

Which is the role of the FCCH domain? It is not very clear in the main text. Why is this related to the open-close SUMO(t) conformation.

In Figure 7e and Supplementary Figure 11b, the Ubc9-FLAG mutants and WT exhibit apparent differences in molecular weight on Western blot. Could you please provide an explanation for this observation?

Reviewer #3 (Remarks to the Author):

In this manuscript, Nayak et al. present the cryo-EM structure of SUMO-E1 singly loaded with SUMO, bound to the E2 enzyme UBC9. This structure significantly advances our understanding of the transthioesterification reaction, where SUMO is transferred from E1 to E2. The structure clearly demonstrates the unique elements in this system that allow the enzyme specificity. The authors also present the EM structure of E1 -E2 complex doubly loaded with SUMO, albeit at a lower resolution. Together with the biochemical data, the reviewer highly supports the publication of this work in NSMB.

Below are a few points that have to be addressed:

The gel in Figure 1b suggests that some UBA2 remains unbound to UBC9 in the purified lane. Is this the case for the complex used in structure determination, or does this reflect its migration pattern on the gel

Could you indicate the residues corresponding to the crossover loop in Figure 1d (and in other panels where possible)?

In Supplementary Figure 4a, there are mutants not mentioned in the main text that also affect activation. Could you refer to these mutants and discuss any insights they provide regarding activation and transfer.

The 445-ggg-447 mutant is highly flexible but shows no activity. Is this due to the requirement of side chains in that position for UFD movement?

In many cases the authors used mutations that reverse the amino acid charge, showing a defect in activity. Could a double swap mutagenesis be used to test for rescuing these mutations? This could strongly support the structural data and the proposed interactions.

Did the authors find mutations in the crossover loop that prevent interaction with UBC9 (site II), affecting transfer but not activation? The authors provide mutation data for both UFD and SCCH; do similar data exist for the crossover loop?

Figures 2a and 2b (left panels) are similar to Figures 5a and 5b. Is it necessary to include Figures 5a and 5b, or would referring to Figures 2a and 2b suffice?

In Figures 5E and 5F, it would be helpful to label the pink color as referring to the AAD.

In the doubly loaded E1, are there any interactions between SUMO A and SUMO T

The term 'doubly loaded E1' is confusing. In lines 372–375, the authors present this term as ubiquitin/UBL that is covalently attached to the E1. However, in the structure presented in this work (sup fig. 8 a), the authors have covalently linked SUMO to the E2 enzyme, which more closely resembles a post-transfer complex. The differences between these complexes should be explained. A discussion on whether these different complexes involve distinct interactions could be included. Also, how linking SUMO to UBC9 A129K and not to active site cys can affect its interactions has to be discussed. Have the authors attempted to obtain a doubly loaded complex with SUMO covalently linked to the E1?

In Figure 7E, why does the migration of FLAG-SUMO WT differ from that of the FLAG mutants? Also, any idea why charged UBC9 is undetectable in the Y134A mutant in Figure 7e, yet it shows colony growing in fig 7d?

It would be nice to show the effect of the suggested mutations on specific substrates undergoing SUMOylation in cells.

Version 1:

Decision Letter:

Our ref: NSMB-A49870A

23rd Jun 2025

Dear Dr. Olsen,

Thank you for submitting your revised manuscript "Cryo-EM Structures Reveal the Molecular Mechanism of SUMO E1-E2 Thioester Transfer" (NSMB-A49870A). I apologise for the delay in responding which came to be due to an inability to timely obtain and discuss referees' reports. Nevertheless, we now have the comments of the original three reviewers. All reviewers find that the paper has improved in revision and two of them explicitly sign off finding it ready for publication. Reviewer #1 retains certain concerns which can be textually addressed to ensure balance and that certain findings are not overintrepreted or oversimplified. Therefore, we are happy to accept the manuscript in principle 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 a week. 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,

Dimitris Typas
Senior Editor
Nature Structural & Molecular Biology
ORCID: 0000-0002-8737-1319

Reviewer #1 (Remarks to the Author):

Some revisions have improved the clarity and contextual grounding of the work. However, several points remain insufficiently addressed and warrant further consideration before the manuscript is suitable for publication.

The revised text still does not adequately compare the observed E1–E2 conformation to previously reported E1–E2 complex structures in other ubiquitin-like modifier systems (e.g., ubiquitin, NEDD8). This omission is problematic, as it may contribute to an overstatement of the novelty of the conformational rearrangement observed in the SUMO E1–UBC9/SUMO1 complex. While it is appreciated that this specific conformation has not previously been reported for the SUMO E1, the structural and mechanistic conservation across E1s for Ub/Ubls is well established. Therefore, the manuscript should include direct comparisons with homologous E1–E2 structures from other systems, both in the figures and in the text.
A more appropriate framing would be to state:
“In alignment with the homologous ubiquitin system E1–E2 complex [CITATION], the conformational rearrangement observed in the SUMO pathway appears to similarly position the E1 and E2 active sites to facilitate thioester transfer.”

Of the conformation is distinct, then the divergence should be discussed.
This framing acknowledges precedent, avoids overstating novelty, and properly situates the current findings within the

broader landscape of ubiquitin-like conjugation mechanisms.

The authors state that " Importantly, the mutants generated for UBC9 and SUMO E1 were well-folded exhibiting comparable melting temperatures to WT proteins in thermal shift assays ", yet the thermal shift data suggest otherwise. Specifically, the T166A/Q167A double mutant shows a ~10°C decrease in melting temperature, and the E160A/P163A mutant is reduced by ~5°C compared to WT. Discrepancies are observed for the majority of mutants, including single point mutations in UBC9. These are significant differences and do not support the claim of comparable stability.
This reinforces my initial concern that the observed loss of activity in these mutants may be due to reduced protein stability or global conformational effects, rather than specific disruption of functional interfaces.

At minimum, the manuscript should be revised to accurately describe the melting temperature results and acknowledge the potential confounding effect of protein destabilization on functional assays. As it stands, the interpretation appears overly confident and insufficiently supported by the data presented – particularly in relation to the the Site II interface.

Additional points:

The Results section still contains numerous statements that would benefit from appropriate citation, especially where previous structural or mechanistic work is relevant. More citations would help clarify what aspects of the findings are novel and where they build on existing studies.

The term FSC is not defined in the manuscript. Please define this term (Fourier Shell Correlation) at its first use, especially for readers less familiar with cryo-EM methods.

In summary, while the revised manuscript has improved in several respects, key issues around comparative structural context, interpretation of mutant data, and the significance of specific interfaces remain only partially addressed.

Reviewer #2 (Remarks to the Author):

I don't have any further comments on the manuscript. I am satisfied with the provided response.

Reviewer #3 (Remarks to the Author):

The authors have thoroughly addressed all of my concerns. I support publication in Nature Structural & Molecular Biology

Version 2:

Decision Letter:

20th Aug 2025

Dear Dr. Olsen,

We are now happy to accept your revised paper "Cryo-EM Structures Reveal the Molecular Mechanism of SUMO E1-E2 Thioester Transfer" 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,

Dimitris Typas
Senior Editor
Nature Structural & Molecular Biology
ORCID: 0000-0002-8737-1319

REVIEWER #1

Major:

- ***However, there are some concerns over the novelty of the UFD-E2 interaction (site I). There is a reported 2.3 angstrom crystal structure of this reductionist complex (Wang et al PLoS One 2010). The basic E2 patch complementing the acidic UFD patch was characterized in this study. It would have been helpful to discuss how this compares to the cryoEM interface. My feeling is there is not much novelty here so its description in the "Determinants of SUMO E1 specificity for UBC9" and "Molecular recognition of UBC9 by SUMO E1" sections might be unwarranted. These two sections come across as laboured and should be consolidated and condensed. The novel aspects should be discussed (UFD rotation/translation, Cys cap reorganisation and its binding to UBC9) and anything else consistent with prior work should be stated as such and moved to supplementary.***

  We appreciate the reviewer's suggestion and agree that direct comparison to the previously reported of S. cerevisiae UBC9-UFD fragment structure (Wang et al., 2010) is essential for contextualizing our findings. We have now incorporated this comparison into the revised Results section (Line: 223-237) and expanded the discussion accordingly (Line: 541-555).

  While the general architecture of the UFD–UBC9 interaction is conserved, our full-length human SUMO E1–UBC9 cryo-EM structure reveals several novel and functionally relevant differences that could not be captured in the reductionist fragment. First, structural alignment of the UFD domains shows a ~23° rotation in UBC9 orientation between the human and yeast complexes (**Supplementary Fig. 6c**). This shift alters the positioning of UBC9 helix A and its interactions with the acidic surface of the UFD. While part of this difference likely reflects divergence in primary sequence between human and yeast proteins, it is also plausible that these changes reflect dynamic rearrangements as the E2 progresses through different stages of the E1–E2 reaction cycle. Specifically, the yeast UFD–E2 fragment may represent an initial recognition state, whereas our full-length human E1–E2 complex captures a conformation poised for thioester transfer. The differences in helix A positioning support this interpretation and highlight the value of studying the full-length complex.

  Importantly, our cryo-EM structure captures additional features absent in the previous UFD–E2 fragment, including conformational reorganization of the catalytic Cys domain, particularly the Cys cap, and its direct interaction with UBC9. Notably, in all other Ubl E1–E2 structures, the Cys cap is disordered. In contrast, the SUMO E1–E2 complex shows that the Cys cap undergoes a defined conformational change and contributes to the interface with UBC9. These elements, which were not accessible in fragment-based structural studies, provide key mechanistic insights into the coordination of E2 by the full E1 assembly and its transitions during activation. Lastly, comparative analysis of the UFD surfaces reveals key differences in the composition and spatial distribution of acidic residues (**Supplementary Fig. 6d**), which in turn influence how UBC9 residues Lys14, Arg17, and Lys18 engage the interface. In the human structure, the UBC9-binding surface is more distributed, involving additional contact points that are not present in the yeast complex (**Supplementary Fig. 6e**). These observations further underscore functional divergence and offer insight into species-specific adaptations in E1–E2 engagement.

  We have also revised the "*Determinants of SUMO E1 specificity for UBC9*" section to better emphasize how our structure-guided sequence comparisons across UFD domains (including E1s not examined in Wang et al.) illuminate unique specificity determinants of SUMO E1 versus other Ubl E1s. In response to the reviewer's suggestion, we have consolidated and streamlined overlapping text, and relocated portions that directly recapitulate prior observations to the Supplementary section. The main text now focuses on newly revealed and functionally important features.

- ***The site II interface is described but not functionally tested. If should be tested for an active role in catalysis and discussion paired back/emphasised accordingly.***

  We thank the reviewer for this important suggestion. In response, we generated targeted mutations in both UBC9 and SUMO E1 to assess the functional relevance of the site II interface. Specifically, we mutated

UBC9 residue Ile4 (I4A) and several residues in SUMO E1 (E160A/P163A and T166A/Q167A) that form key contacts at this interface. Each of these mutants exhibited marked defects in thioester transfer activity, consistent with a critical role for site II in SUMO E1-E2 thioester transfer. These new functional data strongly support the structural interpretation and demonstrate that site II is not merely a passive contact surface, but contributes actively to E2 engagement and thioester transfer. The results have been incorporated into **Fig. 3d** and **Supplementary Fig. 4e**, and line: 239-249. The source data have been updated accordingly. We have also revised the corresponding discussion to reflect these findings and clarify the significance of this interface.

- ***Structure determination of the doubly loaded complex is an impressive achievement. However, the caveats relating to how the complex was engineered for stability must be made clearer in the main section. 1.Although the approach has been used before, a schematic showing the chemical differences between the mimetic and the native tetrahedral intermediate should be displayed in the main text (similar to the graphic for the singly loaded complex in Figure 2b). 2.The native tetrahedral chemistry should be modelled in (supplementary data) so it can be understood if the bona fide intermediate is positionally plasusible with the cryoEM model. If not, the implications should be discussed.***

  We thank the reviewer for the thoughtful and constructive feedback. We agree that the engineered nature of the SUMO E1–Ubc9–SUMO1(a)/SUMO1(t) complex and the distinction between the mimetic and native intermediate should be made clearer in the main text. To address this, we have added a schematic in **Fig. 6a** (left panel) illustrating the architecture of the trapped SUMO E1–Ubc9–SUMO1(a)/SUMO1(t) complex. In addition, we now include a panel in **Supplementary Fig. 9a** that compares the chemical structure of the isopeptide-linked mimetic used in this study to the native tetrahedral intermediate formed during E1–E2 thioester transfer. These additions are intended to help the reader visualize the differences between the mimetic and the *bona fide* catalytic intermediate.

  To further evaluate the relevance of our structure to the native thioester transfer step, we analyzed the geometry of the active site in our cryo-EM model. We measured the distances between the catalytic cysteine of SUMO E1 and the C-terminus of SUMO1(t), as well as between the Ubc9 active site (Lys129 in our mimetic) and the C-terminus of SUMO1 (**Fig.6a, right inset**). These distances are consistent with those observed in related pre- and post-transfer complexes and suggest that only minor conformational adjustments would be required to achieve a catalytically competent geometry. This supports the interpretation that the structure captured in our study closely approximates a *bona fide* transfer intermediate.

  We also note that the mimetic strategy employed here has been previously validated in several high-profile studies, including Streich et al. (Nature 2016), Yuan et al. (Nat. Commun. 2021), Varejão et al. (Nat. Commun. 2021), Afsar et al. (Nat. Commun. 2023), and Goffinont et al. (JBC 2023). These studies, which span ubiquitin, ISG15, and SUMO systems, demonstrate that lysine-linked mimetics preserve the geometry and interaction network of native E2~Ubl intermediates and provide reliable structural insights into both E1–E2 and E2-E3 transfer mechanisms.

  Lastly, we would like to note that throughout the manuscript, we have been careful to avoid overinterpreting or overstating the results of the double-loaded mimetic structure and have limited our conclusions to features related to overall architecture and domain organization. We have revised the main text to clearly distinguish between native and engineered complexes and to transparently convey the limitations and interpretive scope of the structural data.

- ***Inclusion of sequences alignments across orthologues illustrating conservation of key identified residues would support/counter the conclusions***

  We thank the reviewer for this helpful suggestion. In response, we generated a new sequence alignment (now included as **Supplementary Fig. 5c,d and Supplementary Fig. 6e**) highlighting all the key residues involved in the SUMO E1–UBC9 interface. This alignment includes orthologs from multiple species and

demonstrates that the majority of the interacting residues identified in our structure are highly conserved. These observations reinforce the functional significance of the interface and support our structural and mechanistic conclusions.

- ***The use of charge swapped single and triple/quadruple alanine mutations for validation purposes is unusual. Whilst I appreciate the interfaces may depend on cumulative interactions, the potential perturbation from these extensive disruptions is a concern. This should be addressed by, for example, melting temperature analyses.***

  We appreciate the reviewer's concern regarding the potential structural perturbations introduced by charge-swapped and multi-residue alanine substitutions. To address this directly, we performed thermal stability assays on several of the key charge-swap and triple/quadruple alanine mutants. The results, now included in **Supplementary Fig. 5a, b**, indicate that these mutations do not compromise global protein stability. These data support the interpretation that the observed defects in thioester transfer reflect disruption of specific interface contacts rather than nonspecific protein destabilization. The Methods and source data file have also been updated accordingly.

- ***To assess the importance of the hinge region between AAD and UFD mutations to glycine are chosen. A more intuitive substitution would be to rigidifying proline mutations, rather than more flexible glycines. Could the authors comment?***

  We thank the reviewer for this insightful comment. Our initial strategy was indeed to test both extremes of conformational flexibility at the AAD–UFD hinge region (residues 445–447), reasoning that hinge function might depend on dynamic mobility. To this end, we introduced two sets of mutations: a rigidifying triple-proline substitution (445-PPP-447) and a flexibility-enhancing triple-glycine substitution (445-GGG-447). Biochemical analysis revealed that the 445-PPP-447 mutant was completely defective in SUMO E1 thioester formation (**Supplementary Fig. 4a, Lane 4**), suggesting that excessive rigidity at the hinge compromises E1 activation of SUMO (e.g. SUMO E1~SUMO thioester formation). Consequently, this mutant was not used for further functional studies. In contrast, the 445-GGG-447 mutant formed the SUMO E1~SUMO thioester efficiently but was defective in transfer to UBC9 (**Fig. 2d; Supplementary Fig. 4a, Lane 5**), indicating that hinge flexibility may play a specific role in enabling conformational transitions required for E2 engagement or thioester transfer.

  In response to the reviewer's suggestion, we further explored intermediate levels of hinge rigidity by introducing single and double proline substitutions. Importantly, both the 445-PSK-447 (single proline) and 445-APP-447 (double proline) mutants retained normal SUMO E1~SUMO thioester formation, yet were defective in SUMO1 transfer to UBC9, suggesting that even moderate rigidification of the hinge region selectively impairs the E2 transfer step. These findings reinforce the notion that fine-tuned flexibility at the AAD–UFD hinge is critical for proper function, and that even modest rigidification can compromise catalytic efficiency. The new results have been incorporated in **Supplementary Fig. 4e and 4i**, and the source data have been updated accordingly.

- ***Show sequence alignment of the AAD-UFD for SUMO and Ub E1 orthologues.***

  We thank the reviewer for this helpful suggestion. In response, we have included a sequence alignment of the AAD–UFD regions from representative SUMO and ubiquitin E1 orthologs (now presented as **Supplementary Fig. 6f**). This alignment highlights conserved and divergent features across E1 families and supports our interpretation of the structural and functional specialization of the SUMO E1 hinge region.

MINOR

- ***Define SUMO(a) and SUMO(t) in the main text. E.g. at line 60 for SUMO(a)***

  Thank you for pointing this out. We have now defined SUMO(a) (SUMO adenylate) and SUMO(t) (thioester-linked SUMO) in the main text at lines 59-63, respectively.

- ***Figure 2a draw direct line between cysteines if that is the measurement taken.***

  We have updated **Fig. 2a** to show a direct line connecting the cysteines, reflecting the actual distance measured.

- ***Figure 2d. For the reader to appreciate the nature of the thioester transfer assay it would help if a representative gel was inserted.***

  We have added a representative gel for the thioester transfer assay in **Fig. 2f** (*right panel*), using the WT and ΔCys cap variant of SUMO E1 to illustrate the assay.

- ***Figure 3c, d – why not match the bar colours to the colours in the structure figure panels?***
  Line 225 – define hC (helix C presumably)

  The bar colors in new **Fig. 3b, 3d and 3e** now match the colors used in the corresponding structure figure panels. We have also defined "hC" (helix C) in the main text on line 257.

- ***Figure 5 a. The Beta12 beta13 loop of the UFD does not look red in the figure.***

  We have recolored the β12–β13 loop in red and boxed it for clarity in **Fig. 5a**.

- ***Figure 5b. Show axis of rotation and direction of translation***

  We have updated **Fig. 5b** to include both the axis of rotation and the direction of translation. The figure legend has also been revised accordingly. We also added this for UBA1 in **Fig. 5d** to allow for comparison.

- ***Figure 6b-e. It would helpful if the E2 was coloured in slightly different shade of grey so its position can be clearly seen.***

  We have modified **Fig. 6** to display the E2 in a darker shade of grey, improving visibility and contrast.

- ***The authors define the distinct interfaces as SiteI, II and III. Going forward, they do not stick to this nomenclature, (even within figure 3), making it difficult to follow what specific interfaces are being disrupted in biochemical and cellular assays. Source data should include all replicate gels, not just representatives.***

  We have added additional labels in **Fig. 3** to clarify interface assignments and now refer explicitly to Site I, II, and III throughout the text. In the Results section, we also specify the interface being tested in each biochemical and cellular assay. Regarding the source data, to ensure completeness and transparency we have now included all replicate gel and blot images for all experiments presented in the manuscript.

REVIEWER #2

- ***In several instances, the manuscript refers to the E1 structure without E2 as "apo." While this term is often used in the ubiquitin/SUMO field, its traditional meaning in enzymology refers to an inactive enzyme lacking a cofactor or prosthetic group. To avoid potential confusion, I suggest considering an alternative term. Perhaps "E1-only" or "E1-unbound" would be more accurate.***

  We appreciate the reviewer's thoughtful comment. We agree that the term may cause confusion due to its traditional enzymological definition. To improve clarity and precision, we have replaced "apo" with "E2-free" throughout the manuscript, figures, and legends. We believe "E2-free" accurately and unambiguously reflects the absence of the E2 enzyme (UBC9) in these structural states, without implying absence of cofactors such as SUMO or ATP. We believe this will improve readability for both field specialists and broader audiences.

- ***The 175° rotation of the UFD domain, while striking, is somewhat expected if the two active site cysteines must come together. An open question remains: What triggers the dramatic UFD conformational change during trans-thioester transfer? Is E2 binding alone sufficient to induce this conformational shift? Or are additional factors, such as subtle conformational changes near the crossover loop or Zn site, necessary to initiate the UFD movement from its inactive to active state?***

  We thank the reviewer for raising this important mechanistic question. While the 175° rotation of the UFD is indeed consistent with the need to juxtapose the catalytic cysteines for trans-thioester transfer, the structural trigger for this dramatic conformational shift remains unresolved. Our structural data show that UBC9 binding is correlated with the repositioning of the UFD into the active conformation, but whether E2 engagement alone is sufficient to drive this transition, or whether additional local rearrangements, such as in the crossover loop or Zn-binding subdomain, contribute to the process, is not fully captured in the cryo-EM snapshots we have obtained.

  We have revised the Discussion to highlight this open question and now explicitly note that the UFD rotation may involve a combination of E2-induced allostery and local conformational priming within the AAD or crossover loop (see lines 533-539). Our current structures provide a framework for addressing these possibilities using future dynamic studies to more precisely define the sequence of conformational changes that guide the transition from an inactive to catalytically competent E1–E2 complex.

- ***Figure 6, panel b, two of the structures have the same PDB Id. Is it correct?***

  Yes, this is correct. The PDB entry in question contains eight copies of the UBA1–CDC34–Ub(t) complex within a single asymmetric unit. Four of these copies adopt the open conformation for Ub(t), while the other four adopt the closed conformation.

- ***Regarding the double-loaded SUMO complex, I understand that the resolution is relatively low, particularly in the SUMO(t) region, which may indicate flexibility.***
  When compared to other E2-UbL(t) or E2-SUMO(t) structures, the orientation of SUMO(t) appears completely opposite. In the current structure, UBC9 binds the helical face of SUMO(t), while in all other structures, E2 binds the beta surface. How confident are you in the orientation of SUMO(t) in the current structure? Do the maps truly support this orientation?

  We thank the reviewer for this thoughtful and important question. We acknowledge that the resolution in the SUMO(t) region is lower than in the core of the complex, likely reflecting inherent flexibility of this portion of the assembly. In light of this, we were careful not to overinterpret side-chain interactions and have focused our conclusions on the overall domain architecture and relative positioning of structured elements. Despite the moderate local resolution, the SUMO1 α-helix, in particular, fits clearly and unambiguously into well-defined cryo-EM density only in the configuration we have modeled. While we do not claim side-chain level precision in this region, the clear secondary structure alignment, particularly of the α-helix and the C-terminus of SUMO(t), supports the orientation presented in our model.

  Notably, the orientation of SUMO(t) in our structure differs from the canonical E2–Ubl interaction, where the E2 typically binds the β-sheet surface of the Ubl. In our model, Ubc9 engages the helical face of SUMO(t). This noncanonical interaction may reflect the unique architecture of the post-transfer mimic in the context of the E1–E2–SUMO complex and suggests that E2–SUMO(t) orientation may vary depending on structural context and the stage of the catalytic cycle. Importantly, this conformation was the dominant form observed across all 3D classifications and particle populations in our cryo-EM dataset. We did not detect alternative conformations corresponding to the “open” and “closed” states described for Ub(t) in canonical E1–E2 complexes. This further supports the idea that the orientation captured here reflects a stable intermediate relevant to the SUMO system.

  We have revised the manuscript to clearly describe the distinct orientation of SUMO(t), highlight its potential functional implications, and acknowledge the limits of resolution in this region. We agree that determining whether this conformation reflects a transient intermediate, a SUMO-specific adaptation, or a feature of the engineered system will be an important focus of future structural and biochemical studies.

- ***Which is the role of the FCCH domain? It is not very clear in the main text. Why is this related to the open-close SUMO(t) conformation.***

  We thank the reviewer for this insightful comment. In canonical E1 enzymes such as UBA1, UBA6, and UBA7, the FCCH domain is a well-structured globular domain (~100 amino acids) that packs against the SCCH domain to stabilize its open conformation. In adenylation-competent conformations involving Ub, the FCCH also forms a small network of contacts with the Ub-adenylate. In pre-transfer states of UBA1 (PDB: 7K5J^{OPEN} and 9B5N), the FCCH further stabilizes the thioester-linked Ub(t) in an open conformation relative to the E2. This positioning prevents steric clashes with the incoming E2 and facilitates productive thioester transfer. After transfer, Ub(t) becomes covalently linked to the E2 active-site cysteine and adopts a closed conformation (PDB: 7K5J^{CLOSE} and 9B5O), promoting dissociation of the E2–Ub(t) product by reducing its affinity for the E1.

  By contrast, the FCCH domain of SUMO E1 is significantly shorter (~42 amino acids), lacks predicted secondary structure, and is disordered in all available SUMO E1 structures, including the present study. These structural features as well as our cryo-EM data in which we failed to observe any classes of the SUMO E1-E2 complex with SUMO(t) in the open conformation raise the possibility that the open-to-closed Ubl(t) transition seen in ubiquitin E1 systems may not occur, or may occur differently, in the SUMO system, potentially due to the absence of a structured FCCH domain. We have revised the main text to clarify these distinctions and to explain their relevance to the observed SUMO(t) conformation in **Fig. 6b and 6c** (Line 473-479). We agree that resolving the mechanistic basis of Ubl(t) positioning and FCCH function in SUMO E1 will be an important area for future investigation.

- ***In Figure 7e and Supplementary Figure 11b, the UBC9-FLAG mutants and WT exhibit apparent differences in molecular weight on Western blot. Could you please provide an explanation for this observation?***

  We thank the reviewer for noting this. The differences in apparent molecular weight between UBC9-WT and mutant constructs are due to variations in the number of FLAG tags incorporated into each construct for the original set of experiments. To ensure consistency and facilitate direct comparison, we have regenerated all UBC9 constructs with a standardized 1×FLAG tag and repeated all of the experiments using these matched constructs. The updated results, which are now included in revised **Fig. 7d, e, f, and Supplementary Fig. 12b, c,** are fully consistent with our original findings and support the same conclusions.

REVIEWER #3

- ***The gel in Figure 1b suggests that some UBA2 remains unbound to UBC9 in the purified lane. Is this the case for the complex used in structure determination, or does this reflect its migration pattern on the gel***

  We thank the reviewer for this observation. The sample shown in **Fig. 1b** is the same preparation used for cryo-EM grid preparation and structure determination. While some free UBA2 is visible on the SDS-PAGE gel, this does not reflect the particle population used for reconstruction. During cryo-EM data processing, in silico 2D-classification and refinement allowed us to isolate and reconstruct particles corresponding to the fully assembled SUMO E1–UBC9–SUMO(a) complex. This approach yielded a high-resolution 2.7 Å structure of the intact complex, as shown in the manuscript.

- ***Could you indicate the residues corresponding to the crossover loop in Figure 1d (and in other panels where possible)?***

  Thank you for this helpful suggestion. We have now annotated the crossover loop with its corresponding residue numbers in **Fig. 1d** to improve clarity. In addition, we have included the residue range for the crossover loop in the main text at Line 67. Where relevant, we have added similar annotations in other

figure panels to aid interpretation.

- ***In Supplementary Figure 4a, there are mutants not mentioned in the main text that also affect activation. Could you refer to these mutants and discuss any insights they provide regarding activation and transfer.***

  We thank the reviewer for this helpful suggestion. The purpose of **Supplementary Fig. 4a** was to investigate contacts made by the UFD domain in both the thioester transfer–active and –inactive conformations, with a focus on residues within the AAD and the hinge region (residues 445–447) that rotates during this transition. One such residue is Arg127, which forms conserved contacts with the AAD in both conformational states (**Fig. 2a–b**). Based on structural analysis, Arg127 sits at the core of the interface between the UFD and AAD domains, suggesting a role in maintaining structural integrity. Consistent with this, biochemical assays showed that mutation of Arg127, either alone or in combination with nearby residues, severely impaired SUMO activation. We reference this finding explicitly in the main text (Lines 164–167). We also systematically mutated the hinge region (445–ASK–447) to assess how its flexibility affects E1 activity. As shown in **Supplementary Fig. 4a** and discussed in the text, replacing the hinge with 445–GGG–447 (more flexible) preserved SUMO activation but impaired transfer to UBC9, while the 445–PPP–447 mutant (more rigid) was completely defective in activation. This likely reflects disruption of the Zn^{2+}-binding motif, as Cys444, one of the coordinating residues, lies adjacent to the hinge. This observation is consistent with prior findings that the Zn^{2+} motif is critical for E1 activation of SUMO (Wang and Chen, JBC 2010).

  To further probe hinge flexibility, we generated additional variants during this revision, including 445–ASP–447, 445–APP–447, 445–APK–447, and 445–PSK–447. Among these, the APP and PSK mutants showed decreased thioester transfer activity while maintaining SUMO activation, reinforcing the idea that this region must retain a precise balance of flexibility and structural stability. These new results are presented in **Supplementary Fig. 4e and 4i,** and are referenced in the main text (Lines 173–180). Collectively, these findings emphasize that both the AAD–UFD interface and the hinge region are critical not only for structural coordination during the E1 catalytic cycle, but also for discriminating between the activation and transfer steps.

- ***The 445-ggg-447 mutant is highly flexible but shows no activity. Is this due to the requirement of side chains in that position for UFD movement?***

  We thank the reviewer for this thoughtful question. The 445–ASK–447 sequence appears to function as a hinge that provides the appropriate balance of flexibility and structural restraint necessary for UFD repositioning during E2 engagement. Our rationale for generating the 445–GGG–447 mutant was to increase flexibility in this region; however, the resulting loss of thioester transfer activity suggests that excessive flexibility impairs proper orientation of the UFD and/or UBC9 for catalysis.

  Importantly, structural analysis shows that the side chains of residues 445–447 do not form direct contacts with other domains or with UBC9, suggesting that the loss of activity is not due to disrupted interactions but rather to a failure to maintain the conformational integrity required for productive UFD movement. These findings support the idea that not just residue identity but mechanical properties at this hinge are critical for function.

- ***In many cases the authors used mutations that reverse the amino acid charge, showing a defect in activity. Could a double swap mutagenesis be used to test for rescuing these mutations? This could strongly support the structural data and the proposed interactions.***

  We thank the reviewer for this excellent suggestion. To directly test the specificity of charge-based interactions, we performed a double-swap mutagenesis experiment targeting the interaction between SUMO E1 Arg237 and UBC9 Glu132, as proposed in our structural model (**Fig. 3c, right**). As expected, the R237E mutation in SUMO E1 abolished thioester transfer activity when paired with wild-type UBC9. However, when this mutant was tested in combination with a charge-reversal mutation in UBC9 (E132K), we observed partial rescue of activity. This result provides functional support for a direct electrostatic

interaction between these residues at the E1–E2 interface. The new data have been incorporated in **Fig. 3f** and **Supplementary Fig. 4h**, and discussed in the manuscript (Line:271-276). The source data file has been updated accordingly. This finding further reinforces the structural interpretation and highlights the specificity of the site III interface.

- ***Did the authors find mutations in the crossover loop that prevent interaction with UBC9 (site II), affecting transfer but not activation? The authors provide mutation data for both UFD and SCCH; do similar data exist for the crossover loop?***

  We thank the reviewer for this insightful question. In response, we generated and tested mutations specifically within the crossover loop of SUMO E1, which contributes to the site II interface with UBC9. The SUMO E1 mutants E160A/P163A and T166A/Q167A, located within the crossover loop, exhibited defective thioester transfer to UBC9, while maintaining normal E1~SUMO thioester formation. This indicates that these crossover loop residues are dispensable for E1 activation but are critical for facilitating transfer to the E2. We also tested the UBC9 I4A mutant, which contacts this region and similarly showed a transfer-specific defect. These new data, included in **Fig. 3d** and **Supplementary Fig. 4e**, support the functional relevance of site II and demonstrate that the crossover loop contributes directly to productive E2 engagement during the thioester transfer step. The source data file has been updated accordingly.

- ***Figures 2a and 2b (left panels) are similar to Figures 5a and 5b. Is it necessary to include Figures 5a and 5b, or would referring to Figures 2a and 2b suffice?***

  We thank the reviewer for this thoughtful suggestion. While we acknowledge that the left panels of **Fig. 5a and 5b** are similar to those in **Fig. 2a and 2b**, we respectfully feel it is important to retain them in **Fig. 5.** Our rationale is that **Fig. 5** presents additional structural comparisons and domain motions (e.g., UFD rotation and E2 translation), and having the reference conformation side-by-side in the same figure facilitates direct visual comparison without requiring the reader to flip back to an earlier panel. This layout enhances clarity and accessibility, particularly when interpreting the conformational transitions illustrated in subsequent panels. To avoid redundancy, we have ensured that figure legends and labels are concise and non-repetitive. We hope the reviewer agrees that keeping these panels contributes to a more coherent and self-contained presentation of the structural analysis.

- ***In Figures 5E and 5F, it would be helpful to label the pink color as referring to the AAD.***

  Thank you for the helpful suggestion. We have updated **Fig. 5E and 5F** to explicitly label the pink region as the AAD to improve clarity for the reader.

- ***In the doubly loaded E1, are there any interactions between SUMO A and SUMO T***

  We thank the reviewer for this insightful question. In our cryo-EM reconstruction, SUMO(a) and SUMO(t) are positioned in relatively close proximity; however, we do not observe any direct or stable contacts between them in the final model. To further investigate potential interactions, we performed 3D classification to assess whether the relative positions of SUMO(a) and SUMO(t) vary across particle populations. In a small number of classes, the two SUMO moieties appeared closer together; however, in these cases, the density corresponding to SUMO(t) was not well resolved and could not be reliably interpreted. Given the limited resolution and ambiguous density in those classes, we chose not to include that analysis in the manuscript. Nevertheless, we agree that the possibility of transient or dynamic interactions between SUMO(a) and SUMO(t) is intriguing and may be better addressed in future studies.

- ***The term 'doubly loaded E1' is confusing. In lines 372–375, the authors present this term as ubiquitin/UBL that is covalently attached to the E1. However, in the structure presented in this work (sup fig. 8 a), the authors have covalently linked SUMO to the E2 enzyme, which more closely resembles a post-transfer complex. The differences between these complexes should be explained. A discussion on whether these different complexes involve distinct interactions could be included. Also, how linking SUMO to UBC9 A129K and not to active site cys can affect its interactions has to be discussed. Have the authors attempted to obtain a doubly loaded complex with SUMO***

covalently linked to the E1?

We thank the reviewer for this important point and the opportunity to clarify our interpretation of the SUMO(t) orientation in the double-loaded complex. As described in our response to Reviewer 1, we have added a schematic (Figure 6a, left panel) and a comparative chemical illustration (Supplementary Figure 9a) to clarify the differences between the isopeptide-linked mimetic used in our study and the native tetrahedral intermediate formed during SUMO transfer. These additions were made specifically to help the reader visualize the nature of the engineered system and how it relates to a bona fide catalytic intermediate.

To evaluate how well the observed structure reflects a catalytically relevant state, we analyzed the geometry of the active site. As noted in our prior response, we measured the distances between the catalytic cysteine of SUMO E1, the active-site residue of Ubc9 (Lys129 in the mimetic), and the C-terminus of SUMO1(t) (**Fig.6a, right inset**). These measurements indicate that only modest structural adjustments would be required to bring the reactive atoms into alignment for thioester transfer. This finding supports the interpretation that our structure closely approximates the thioester transfer intermediate during catalysis. Although the local resolution in the SUMO(t) region is lower—likely due to flexibility—we observed a consistent and dominant orientation across all 3D classes in our cryo-EM dataset. We did not observe alternate conformations resembling the “open” and “closed” Ub(t) configurations seen in other systems. This dominant configuration, along with the proximity of the two catalytic cysteines to the C-terminus of SUMO1(t), provides strong support for the assigned orientation and overall model.

We also note, as emphasized in our response to Reviewer 1, that the lysine-based SUMO(t) mimetic strategy has been validated in multiple prior studies across the SUMO and ubiquitin fields (e.g., Streich et al., Nature 2016; Yuan et al., Nat. Commun. 2021; Varejão et al., Nat. Commun. 2021; Goffinont et al., JBC 2023). These studies demonstrate that such mimetics faithfully reproduce E2~Ubl geometry and enable accurate structural analysis of E1–E2–Ubl complexes. That said, we have been cautious in our interpretation and have deliberately limited our conclusions to architectural features that are well supported by the data. As noted in the manuscript, whether additional conformational rearrangements of SUMO(t) occur during catalysis remains an open question, and we agree that further investigation will be needed to determine whether the orientation captured in this structure reflects a transient intermediate or a SUMO-specific adaptation. Resolving this will be a key focus of future structural and biochemical studies. We have not yet attempted to obtain a doubly loaded SUMO E1 where SUMO1(t) is covalently linked to E1.

In Figure 7E, why does the migration of FLAG-SUMO WT differ from that of the FLAG mutants? Also, any idea why charged UBC9 is undetectable in the Y134A mutant in Figure 7e, yet it shows colony growing in fig 7d?

As note in our response to Reviewer 2, the differences in apparent molecular weight between UBC9-WT and mutant constructs are due to variations in the number of FLAG tags incorporated into each construct for the original set of experiments. To ensure consistency and facilitate direct comparison, we have regenerated all UBC9 constructs with a standardized 1×FLAG tag and repeated all of the experiments using these matched constructs. The updated results, which are now included in revised **Fig. 7d, e, f, and Supplementary Fig. 12b, c,** are fully consistent with our original findings and support the same conclusions.

- ***It would be nice to show the effect of the suggested mutations on specific substrates undergoing SUMOylation in cells.***

We appreciate this valuable suggestion. To assess the functional consequences of the UBC9 mutations in a cellular context, we examined the SUMOylation of a well-characterized substrate, cMYC. Using a cell-based SUMOylation assay, we found that cMYC modification comparable to previously reported data in the presence of wild-type UBC9, but substantially reduced or undetectable in cells expressing UBC9 mutant constructs. These results demonstrate that the introduced mutations disrupt UBC9’s ability to catalyze SUMO transfer to a physiologically relevant substrate *in vivo*. The updated data are presented in **Supplementary Fig. 12c, d** and further support the critical role of the identified interface in E2 function.

Reviewer #1 (Remarks to the Author):

Some revisions have improved the clarity and contextual grounding of the work. However, several points remain insufficiently addressed and warrant further consideration before the manuscript is suitable for publication.

1. The revised text still does not adequately compare the observed E1–E2 conformation to previously reported E1–E2 complex structures in other ubiquitin-like modifier systems (e.g., ubiquitin, NEDD8). This omission is problematic, as it may contribute to an overstatement of the novelty of the conformational rearrangement observed in the SUMO E1–UBC9/SUMO1 complex.

We thank the reviewer for this suggestion. We feel we have adequately discussed the comparison of SUMO E1-E2 conformations with other reported E1-E2 structures of Ubls (Ub and NEDD8) under the following sections of the Results: "Determinants of SUMO E1 specificity for UBC9", "Distinct UFD transitions accompany thioester transfer across Ubl systems" and "Architecture of doubly-loaded SUMO E1-E2 mimetic and comparison to other Ubls". We have presented figures highlighting this comparison with other E1-E2 systems in the following figures; Fig. 4, Fig. 5, Extended Data Fig. 7e, Extended Data Fig. 8.

2. While it is appreciated that this specific conformation has not previously been reported for the SUMO E1, the structural and mechanistic conservation across E1s for Ub/Ubls is well established. Therefore, the manuscript should include direct comparisons with homologous E1–E2 structures from other systems, both in the figures and in the text.
A more appropriate framing would be to state:
"In alignment with the homologous ubiquitin system E1–E2 complex [CITATION], the conformational rearrangement observed in the SUMO pathway appears to similarly position the E1 and E2 active sites to facilitate thioester transfer."

We have appropriately added the suggested line under discussion, Line: 396 "Although the general organization of the SUMO E1–UBC9 complex resembles that of canonical Ubl E1–E2 pairs, the path to catalysis is marked by unique domain dynamics". Moreover, we have made direct comparisons with other E1-E2 complexes in Fig. 4, Fig.5 and Extended Data Fig. 8.

3. Of the conformation is distinct, then the divergence should be discussed.
This framing acknowledges precedent, avoids overstating novelty, and properly situates the current findings within the broader landscape of ubiquitin-like conjugation mechanisms.

We thank the reviewer for the suggestion. We have adequately discussed the differences observed between our SUMO E1-E2 structures with other Ubls E1-E2 structures. The differences can be summarized with respect to 1) the distribution of SUMO E1 acidic patch residues as compared to other E1-E2s, 2) the rotation SUMO E1 UFD domain (~ 175°) as compared to other E1s (~20° for UBA1), 3) arrangement of basic residues on UBC9 as compared to other E2s, 3) Distinct structural elements like the β1-β2 loop of E2 and H18/H33 of E1s.

4. The authors state that " Importantly, the mutants generated for UBC9 and SUMO E1 were well-folded exhibiting comparable melting temperatures to WT proteins in thermal shift assays ", yet the thermal shift data suggest otherwise. Specifically, the T166A/Q167A double mutant shows a ~10°C decrease in melting temperature, and the E160A/P163A mutant is reduced by ~5°C compared to WT. Discrepancies are observed for the majority of mutants, including single point mutations in UBC9. These are significant differences and do not support the claim of comparable stability.

This reinforces my initial concern that the observed loss of activity in these mutants may be due to reduced protein stability or global conformational effects, rather than specific disruption of functional interfaces.
At minimum, the manuscript should be revised to accurately describe the melting temperature results and acknowledge the potential confounding effect of protein destabilization on functional assays. As it stands, the interpretation appears overly confident and insufficiently supported by the data presented – particularly in relation to the the Site II interface.

We have added the following sentence to accurately describe the melting curve results in line:171-176 “To exclude loss of activity due to mutation-induced instability, we performed thermal shift assays for several SUMO E1 and UBC9 mutants. Some mutants diverged in terms of stability when compared to the WT SUMO E1 and UBC9 so, though we cannot with absolute certainty exclude potential mitigating effects due to reduced stability, our combined biochemical and structural data indicate that the observed effects arise due to compromised interactions.”

Additional points:

5. The Results section still contains numerous statements that would benefit from appropriate citation, especially where previous structural or mechanistic work is relevant. More citations would help clarify what aspects of the findings are novel and where they build on existing studies.

We have added appropriate citations in lines: 341 and 348.

6. The term FSC is not defined in the manuscript. Please define this term (Fourier Shell Correlation) at its first use, especially for readers less familiar with cryo-EM methods.

We added appropriate added text in lines: 93-95 to define FSC.

In summary, while the revised manuscript has improved in several respects, key issues around comparative structural context, interpretation of mutant data, and the significance of specific interfaces remain only partially addressed.