nature portfolio

Peer Review File

Cryo-EM structures of UBA6 reveal mechanisms of E1–E2 specificity and dual FAT10/ubiquitin thioester transfer

Corresponding Author: Professor Shaun Olsen

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript by Nayak et al. explore the structural specificity of E1 and E2 enzymes. Specifically, the authors ask which structural elements give UBA6 or UBA1 the ability to work with particular E2 enzymes, and how the E1 UBA6 allows transfer of both ubiquitin and FAT10. To address this, they solve the cryo EM structure of UBE2Z in complex with UBA6 loaded with FAT10. Using elegant swap experiments, they show that both the UFD and the SCCH domains of UBA6 are responsible for transfer of FAT10 from UBA6 to UBE2Z. They also clearly show the clashes between this E2 and UBA1, which explains why UBA1 cannot work with UBE2Z. Overall, this manuscript provides novel insights into the structural basis of E1 and E2 specificity and into the dual function of UBA6 with ubiquitin and FAT10, and therefore justifies publication in Nature Communications.
Below are a few points that the authors should address.
1. Recent work on UBA6 specificity for E2 enzymes(PMID 41350950), also addresses the key questions in this manuscript. The authors should refer to this paper.
2. The authors support their structural data with functional assays. However, direct binding experiments would further strengthen the mechanistic conclusions. In particular, such experiments would clarify whether mutations that impair transfer also disrupt binding, or whether binding is retained but transfer is lost.
3. In Figure 3A the authors show that the R575D or R615D mutation does not affect the overall activity, although R615 is close to UBE2Z D157. Can the authors test the effect of a D157R mutation on activity. In addition, the double mutant R575D R615D shows a strong defect in activity. It is not clear whether this defect arises from disruption of the AAD structure or from altered interactions with UBE2Z. Please address this point.
4. In Figure 4 the authors perform swap experiments that highlight the importance of the SCCH and UFD domains for specificity. Interestingly, in the context of UBA6, introduction of the UFD and SCCH domains from UBA1 does not affect charging of UBCH5B. In contrast, in the context of UBA1, introduction of the SCCH domain from UBA6, either alone or together with the UFD, significantly reduces activity. The authors may elaborate on this difference. In addition, it would be helpful to know whether the yield of UBCH5B charging with ubiquitin is similar when catalyzed by UBA1 versus UBA6, and if not whether it is due to differences in the UFD or SCCH domains.

Reviewer #2

(Remarks to the Author)
Review of Cryo-EM structures of UBA6 reveal mechanisms of E1-E2 specificity and dual FAT10/ubiquitin thioester transfer
This manuscript addresses the issue of E1-E2 specificity through a combination of structural and biochemical data. The authors describe a series of Uba6-Ube2Z structures determined in complex with FAT10 or ubiquitin. The cryo-EM complexes range from 2.73 to 3.86 A resolution with reasonable local electron density quality where shown. A thorough description of the observed domain interactions was complemented by clear and well-constructed figures. This structural analysis was followed up by extensive mutagenesis of residues at protein interfaces and thioester charging assays to assess impact on function.
The extensive interactions of Ube2Z with Uba6 provide the basis for observed specificity when compared with other E1-E2 complexes. Conformational changes occur in Uba6 and Ube2Z upon complex formation with extensive contact surfaces aligning the active sites (Fig 2a-b). For example, the LA and LC loop regions of Ube2Z makes interactions with the SCCH domain of Uba6 that would clash with other E1 enzymes (Fig.5f). Differences in the UFD domain of Uba6 and Uba1 also

contribute to E2 specificity. Specific UFD-Ube2Z interactions are contrasted with clashes for superimposed E2s that do not function with Uba6 (Fig 6a,d). These structural interpretations are rationally consistent with the results of domain-swapped chimeric E1 enzymes assayed to charge a panel of E2 enzymes with ubiquitin (Figure 4).
Equally intriguing is the basis for dual FAT10 and Ub functionality of Ube2Z. Complexes determined of dual-loaded Uba6-Ube2Z with either Fat10 or Ub in both adenylation and thioester sites represents an impressive technical feat. A unique hydrophobic patch (LB loop) on Ube2Z similarly captures the C-terminal tail of FAT10 and Ubiquitin (Fig 7b-g). Additional interactions between Ube2Z and Uba6 with the N-terminal Ubl of FAT10 were less well resolved structurally but not conserved with other E2s beyond BIRC6 and Ube2O.
Overall, this manuscript provides a significant advancement in understanding the basis of Uba6 function and I highly recommend it for publication in its current form.

Minor points:
1. While the FAT10-Uba6 complex was modelled in the authors previous 2022 Nature Communications publication, a structure-based comparison of the FAT10 vs ubiquitin interactions in the adenylation pocket was not adequately commented upon to rationalize the dual Ubl specificity of Uba6 relative to other E1s. The authors should consider elaborating on this point if they think it warranted.
2. The UbcH5b~Ub band in the Fig.4b charging assay is rather difficult to see relative to the other E2s profiled especially compared to the UbcH5b results shown in Fig.1b. Perhaps the authors can highlight the band with an arrow?
3. Comparison of FAT10 vs ubiquitin tail interactions with the LB-loop region of E2 surfaces are difficult to appreciate in Figure 7d. Perhaps a better contrasting colour for the Cdc34~Ub tail would help (from gray to yellow for example).

Reviewer #3

(Remarks to the Author)
The manuscript by Nayak et al., provides a very comprehensive analysis of the interactions that underpin E2 selection and charging by UBA6. The manuscript is underpinned by structural studies that is complemented be careful analysis, as well as mutagenesis and activity assays. The analysis appears thorough and for the most part the claims are well substantiated by the data. Together this work helps identify the elements in E1 and E2 enzymes of the ubiquitin-like signalling cascades that lead to specific or promiscuous interactions. This is an important topic of study as the fidelity of the Ub-like conjugation network relies on charging of E2 enzymes with the appropriate Ub-like molecule.

The manuscript is very well written, with no typos! and for the most part the writing is very clear. My major concern is that the text is quite dense – in part this is because the analysis is very thorough – and, as a result, the paper will not be accessible to a general audience. I have made some specific comments below but the authors might have some ideas about how to ensure the paper is more accessible to a broad readership.

Minor points:
Line 74, define RGS
Line 76, rephrase for clarity
Line 78 on, introduction of E2 enzymes needs to include the E2 classes as ‘class iv’ currently first appears on line 103.
Line 101, remove redundancy from ‘unique to UBA6 and absent from UBA1’
The first results section might benefit from being reordered? If not introduced in the introduction the E2 classes could lead this section i.e. a version of lines 118-123. It might be helpful to tabulate the classes and their selectivity?
Line 127, the resolution is 3.2 Å - it would be helpful to provide some context about the areas of high confidence, especially as the authors go on to analyse a number of contacts.
Line 130, ‘the IAD and AAD’ needs some explanation! Perhaps an additional sentence that lays out the domain structure of UBA6 as while familiar to the authors the reader might benefit from guidance.
Figure 1a, the domain architecture panel should be to scale and maybe a separate panel that is first.
Line 156, label ‘a5 and a6’ in figure 2.
Figure 2a, hA helix overlay might be better if a ribbon is shown in the second panel.
Figure 3 and related text, it might be helpful to the reader to integrate the mutations (and data) with the structural description as it is quite hard to track the various mutations to the related structure panel. This would also allow more specific panel references.
Line 217/218, not sure the conclusion is justified?
Line 224, it is not clear that the reciprocal mutations in Ube2z caused any loss (also include aa number as this will help the reader locate the data).
Line 291, Figure 7g is referred to out of order?
Line 363, replace ‘poorly’ with ‘not’?

The Discussion might benefit form a model/schematic that integrates the conclusions for the reader. It would also be interesting to expand more widely and discuss whether there are implications from this study for E2s generally.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors addressed my concerns, and I support publication in Nature Communications.

Reviewer #2

(Remarks to the Author)
The authors have fully addressed the issues I raised in my first review.

I now support publication of the manuscript in its current form.

Reviewer #3

(Remarks to the Author)
The authors have satisfactorily addressed all concerns. No further amendments are required.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The manuscript by Nayak et al. explore the structural specificity of E1 and E2 enzymes. Specifically, the authors ask which structural elements give UBA6 or UBA1 the ability to work with particular E2 enzymes, and how the E1 UBA6 allows transfer of both ubiquitin and FAT10. To address this, they solve the cryo EM structure of UBE2Z in complex with UBA6 loaded with FAT10. Using elegant swap experiments, they show that both the UFD and the SCCH domains of UBA6 are responsible for transfer of FAT10 from UBA6 to UBE2Z. They also clearly show the clashes between this E2 and UBA1, which explains why UBA1 cannot work with UBE2Z. Overall, this manuscript provides novel insights into the structural basis of E1 and E2 specificity and into the dual function of UBA6 with ubiquitin and FAT10, and therefore justifies publication in Nature Communications.

We thank the reviewer for their thoughtful and encouraging assessment of our work.

Recent work on UBA6 specificity for E2 enzymes (PMID 41350950), also addresses the key questions in this manuscript. The authors should refer to this paper.

Thank for pointing this out. We have appropriately cited this paper in the Discussion of the revised manuscript (Line 507-508).

The authors support their structural data with functional assays. However, direct binding experiments would further strengthen the mechanistic conclusions. In particular, such experiments would clarify whether mutations that impair transfer also disrupt binding, or whether binding is retained but transfer is lost.

We thank the reviewer for this thoughtful suggestion and agree that direct binding measurements can, in principle, help distinguish defects in complex formation from defects in the chemical transfer step itself. In the present study, our mutagenesis strategy was guided by the cryo-EM structures and designed to perturb well-defined interfaces within the UBA6–UBE2Z and UBE2Z–FAT10 assemblies. The corresponding mutations consistently resulted in reduced thioester transfer activity, with residual activity (~7–10% of wild type) even in the most impaired cases, arguing against complete loss of productive engagement and instead supporting partial destabilization or misalignment of the catalytic configuration.

We did not pursue direct binding measurements for these interactions because E1–E2 complexes are inherently weak and transient, particularly outside the context of nucleotide- and modifier-bound states. This makes rigorous quantitative binding measurements technically challenging and difficult to interpret. Consistent with this, our work and the related study on BIRC6 noted above (Riechmann et al., 2025) have demonstrated that the UBA6–UBE2Z interaction does not yield a discernible complex by size-exclusion chromatography and that binding was not detectable by fluorescence polarization assays.

Importantly, the structural data presented here directly visualize the interfaces disrupted by mutation and reveal how these contacts contribute to catalytic alignment during thioester transfer. As such, the mechanistic conclusions regarding the roles of the UFD and SCCH domains in E2 specificity are supported by a combination of structural, mutational, and functional evidence, even in the absence of standalone binding assays. While the development of quantitative binding platforms to systematically assess E1–E2 interactions (including the use of the chimeric E1 framework developed here) would be of considerable interest, we believe such efforts fall beyond the scope of the present study.

In Figure 3A the authors show that the R575D or R615D mutation does not affect the overall activity, although R615 is close to UBE2Z D157. Can the authors test the effect of a D157R mutation on activity? In addition, the double mutant R575D R615D shows a strong defect in activity. It is not clear whether this defect arises from disruption of the AAD structure or from altered interactions with UBE2Z. Please address this point.

We thank the reviewer for this insightful question regarding the contribution of the AAD–E2 interface. We agree that assessing the role of UBE2Z D157 could further inform interpretation of this contact. While we attempted to pursue additional mutagenesis at this position, we were unable to complete the requested D157R experiment within the revision timeframe.

Importantly, however, we had already examined a more stringent perturbation of this region by combining D157A with Q99A (Q99A/D157A), two residues that together form the putative interaction surface with UBA6 Arg575 and Arg615. This double mutant resulted in only a modest reduction in thioester transfer activity (~20%; Fig. 3e), suggesting that this interface contributes weakly and cooperatively rather than serving as a dominant determinant of activity. On this basis, we do not anticipate that a single D157R substitution would produce a substantial or readily detectable defect under our assay conditions.

With respect to the strong defect observed for the UBA6 R575D/R615D double mutant, our data indicate that this effect is not solely attributable to disruption of the E2 interface. Rather, analysis of the UBA6–FAT10 thioester formation control assays (now presented in Supplementary Fig. 4a) indicates that this double mutation partially impairs formation of the upstream E1~FAT10 thioester intermediate, consistent with a broader contribution of these residues to AAD function and active-site organization. We have clarified this point explicitly in the revised manuscript (Line 236–239), and we thank the reviewer for prompting this clarification.

In Figure 4 the authors perform swap experiments that highlight the importance of the SCCH and UFD domains for specificity. Interestingly, in the context of UBA6, introduction of the UFD and SCCH domains from UBA1 does not affect charging of UBCH5B. In contrast, in the context of UBA1, introduction of the SCCH domain from UBA6, either alone or together with the UFD, significantly reduces activity. The authors may elaborate on this difference. In addition, it would be helpful to know whether the yield of UBCH5B charging with ubiquitin is similar when catalyzed by UBA1 versus UBA6, and if not whether it is due to differences in the UFD or SCCH domains.

Thank you for highlighting this asymmetry in the domain-swap behavior. We agree that this difference is informative. Quantitatively, we note that under our assay conditions WT UBA1 exhibits a modestly lower baseline efficiency for charging UBCH5B with ubiquitin compared to WT UBA6 (~85% of WT UBA6 activity; Fig. 4c,d, first panels). Thus, domain swaps introduced into the UBA1 background further reduce activity relative to the corresponding UBA6-based constructs.

More importantly, however, we interpret this behavior in mechanistic terms. UBCH5B is a promiscuous E2 whose productive engagement relies primarily on canonical UFD-mediated docking and does not require precise SCCH–E2 alignment. In the context of UBA6, introduction of UBA1-derived UFD or SCCH domains does not perturb UBCH5B charging, consistent with its relaxed interface requirements. In contrast, insertion of the UBA6 SCCH into the UBA1 scaffold appears to be mildly disruptive, likely reflecting imperfect compatibility between the UBA6 SCCH geometry and the surrounding UBA1 domain architecture rather than a specific requirement of UBCH5B for SCCH-mediated contacts.

Thus, while UBCH5B remains largely insensitive to domain identity per se, the UBA1 scaffold is more sensitive to domain perturbation than UBA6, which we propose reflects differences in overall conformational tuning between the two E1 enzymes. We have clarified this interpretation in the revised manuscript (Line 279–288).

Reviewer #2 (Remarks to the Author):

Review of Cryo-EM structures of UBA6 reveal mechanisms of E1-E2 specificity and dual FAT10/ubiquitin thioester transfer

This manuscript addresses the issue of E1-E2 specificity through a combination of structural and biochemical data. The authors describe a series of Uba6-Ube2Z structures determined in complex with FAT10 or ubiquitin. The cryo-EM complexes range from 2.73 to 3.86 A resolution with reasonable local electron density quality where shown. A thorough description of the observed domain interactions was complemented by clear and well-constructed figures. This structural analysis was followed up by

extensive mutagenesis of residues at protein interfaces and thioester charging assays to assess impact on function.

The extensive interactions of Ube2Z with Uba6 provide the basis for observed specificity when compared with other E1-E2 complexes. Conformational changes occur in Uba6 and Ube2Z upon complex formation with extensive contact surfaces aligning the active sites (Fig 2a-b). For example, the LA and LC loop regions of Ube2Z makes interactions with the SCCH domain of Uba6 that would clash with other E1 enzymes (Fig.5f). Differences in the UFD domain of Uba6 and Uba1 also contribute to E2 specificity. Specific UFD-Ube2Z interactions are contrasted with clashes for superimposed E2s that do not function with Uba6 (Fig 6a,d). These structural interpretations are rationally consistent with the results of domain-swapped chimeric E1 enzymes assayed to charge a panel of E2 enzymes with ubiquitin (Figure 4).

Equally intriguing is the basis for dual FAT10 and Ub functionality of Ube2Z. Complexes determined of dual-loaded Uba6-Ube2Z with either Fat10 or Ub in both adenylation and thioester sites represents an impressive technical feat. A unique hydrophobic patch (LB loop) on Ube2Z similarly captures the C-terminal tail of FAT10 and Ubiquitin (Fig 7b-g). Additional interactions between Ube2Z and Uba6 with the N-terminal Ubl of FAT10 were less well resolved structurally but not conserved with other E2s beyond BIRC6 and Ube2O.

Overall, this manuscript provides a significant advancement in understanding the basis of Uba6 function and I highly recommend it for publication in its current form.

We thank the reviewer for their careful and thorough evaluation of our study and for their detailed summary of both the structural and biochemical findings. We are pleased that the reviewer found the analyses compelling and the conclusions well supported.

While the FAT10-Uba6 complex was modelled in the authors previous 2022 Nature Communications publication, a structure-based comparison of the FAT10 vs ubiquitin interactions in the adenylation pocket was not adequately commented upon to rationalize the dual Ubl specificity of Uba6 relative to other E1s. The authors should consider elaborating on this point if they think it warranted.

Thank you for this suggestion. We agree that explicitly contrasting FAT10 and ubiquitin interactions is important for understanding the basis of UBA6 dual-Ubl specificity. While the structural determinants governing FAT10 and ubiquitin engagement by the UBA6 adenylation domains were examined in detail in our prior work and that of Schindelin and colleagues (Yuan et. al, Nature Communications, 2022; Truongvan et al., Nature Communications, 2022), we have now expanded the Discussion to more clearly integrate these earlier findings with the new E1–E2 thioester-transfer structures presented here.

We now describe how UBE2Z employs a shared catalytic scaffold but remodels its hB region and LB loop to accommodate distinct surface chemistries of FAT10 and ubiquitin, polar complementarity for FAT10 and hydrophobic packing for ubiquitin, thereby enabling dual-Ubl reactivity without loss of specificity (Discussion; Line 541-545). In addition, we highlight the role of the FAT10 N-terminal domain, which engages UBA6-specific and UBE2Z-specific acidic surfaces during activation and thioester transfer, respectively, a feature absent in ubiquitin. We believe these additions clarify how UBA6 achieves dual-Ubl specificity across both activation and transfer steps while keeping the primary focus on the newly resolved E1–E2 mechanisms.

The UbcH5b~Ub band in the Fig.4b charging assay is rather difficult to see relative to the other E2s profiled especially compared to the UbcH5b results shown in Fig.1b. Perhaps the authors can highlight the band with an arrow?

Thank you for this suggestion. We have revised Fig. 4b to improve the visibility of the UbcH5B~Ub band by modestly adjusting brightness and contrast uniformly across the panel, without altering the underlying data or affecting any conclusions. In addition, we have explicitly labeled the positions of all E2s and their corresponding E2~Ub species in the gel, including UbcH5B~Ub, to aid visual identification and comparison with Fig. 1b.

Comparison of FAT10 vs ubiquitin tail interactions with the LB-loop region of E2 surfaces are difficult to appreciate in Figure 7d. Perhaps a better contrasting colour for the Cdc34~Ub tail would help (from gray to yellow for example).

Thank you for this helpful suggestion. We have revised Fig. 7d by changing the color of the CDC34~Ub tail to yellow, which provides clearer visual contrast and facilitates comparison with the FAT10 and UBE2Z interactions in the LB-loop region.

Reviewer #3 (Remarks to the Author):

The manuscript by Nayak et al., provides a very comprehensive analysis of the interactions that underpin E2 selection and charging by UBA6. The manuscript is underpinned by structural studies that is complemented be careful analysis, as well as mutagenesis and activity assays. The analysis appears thorough and for the most part the claims are well substantiated by the data. Together this work helps identify the elements in E1 and E2 enzymes of the ubiquitin-like signalling cascades that lead to specific or promiscuous interactions. This is an important topic of study as the fidelity of the Ub-like conjugation network relies on charging of E2 enzymes with the appropriate Ub-like molecule.

The manuscript is very well written, with no typos! and for the most part the writing is very clear. My major concern is that the text is quite dense – in part this is because the analysis is very thorough – and, as a result, the paper will not be accessible to a general audience. I have made some specific comments below but the authors might have some ideas about how to ensure the paper is more accessible to a broad readership.

We thank the reviewer for their careful and balanced evaluation of our manuscript and for highlighting both the strengths of the structural and functional analyses and the broader significance of this work for Ub-like signaling fidelity. We appreciate the concern regarding accessibility for a general readership. In response, we have made targeted revisions throughout the manuscript to improve clarity and readability, including refining phrasing, better integrating structural descriptions with functional data, and adding an integrative model schematic to help guide the reader through the key conclusions. We believe these changes improve accessibility while preserving the depth and rigor of the analysis and thank the reviewer for their suggestions.

Minor points:

Line 74, define RGS

We have defined RGS proteins in Line 74 (now Line 75).

Line 76, rephrase for clarity

Thank you for your suggestion. We have rephrased line 76 (now line 76-78). It has been changed to “but as the only E1 for FAT10, UBA6 promotes proteasomal targeting of substrates upon TNF-α and IFN-γ stimulation, coupling its activity to cytokine-controlled proteostasis”.

Line 78 on, introduction of E2 enzymes needs to include the E2 classes as ‘class iv’ currently first appears on line 103.

Thank you for your suggestion. We have defined class IV E2 enzymes early in the introduction (Line 82-84)

Line 101, remove redundancy from ‘unique to UBA6 and absent from UBA1’

We have rephrased the following line to “unique to UBA6” (Line 105).

The first results section might benefit from being reordered? If not introduced in the introduction the E2 classes could lead this section i.e. a version of lines 118-123. It might be helpful to tabulate the classes and their selectivity?

Thank you for this thoughtful suggestion. We carefully considered reordering the first Results section and leading with E2 class definitions. However, after much consideration we chose to retain the current organization because we felt it most effectively establishes the biological and mechanistic rationale for UBA6-mediated E2 selectivity that frames the remainder of the study and motivates the subsequent structural and biochemical analyses.

The relevant E2 class definitions are now introduced in the Introduction (Lines 82–84), allowing readers to follow the Results without ambiguity. While we agree that a tabulated overview of E2 classes and their selectivity could serve as a useful general reference, several E2s among the tens of Ub E2s remain relatively understudied, making comprehensive classification of their E1 selectivity challenging based on existing literature. We view systematic classification of E2 behavior across the ubiquitin system as an important direction for future work, building on the mechanistic framework established here.

Line 127, the resolution is 3.2 Å - it would be helpful to provide some context about the areas of high confidence, especially as the authors go on to analyse a number of contacts.

Thank you for this suggestion. For all contacts discussed in the manuscript and subsequently tested by mutagenesis, we verified that the corresponding residues are supported by well-resolved cryo-EM map density. To provide additional context, we include a local resolution map for the entire UBA6–UBE2Z complex (Supplementary Fig. 1c), color-coded from lower (red) to higher (blue) resolution. We have also added text to the Results to clarify that the interfaces analyzed are located within regions of high local resolution.

Line 130, 'the IAD and AAD' needs some explanation! Perhaps an additional sentence that lays out the domain structure of UBA6 as while familiar to the authors the reader might benefit from guidance.

We apologize for this omission and fully agree that additional guidance would benefit a broader audience who We have now explicitly defined the adenylation domain and its constituent active and inactive adenylation domains (AAD and IAD) in the Introduction (Lines 54–57), Additional domains are introduced and described in the same paragraph to provide a picture of the multidomain architecture of canonical E1s and the function of each of these domains. We believe this revised description now provides a clearer context before these terms are used in the Results section.

Figure 1a, the domain architecture panel should be to scale and maybe a separate panel that is first.

Thank you for this suggestion. We have rescaled the UBE2Z domain architecture schematic to match the scale used for UBA6. In addition, following the reviewer's recommendation, we have repositioned this schematic as panel a in Figure 1.

Line 156, label 'a5 and a6' in figure 2.

Thank you for pointing this out. To ensure consistent helix nomenclature throughout the manuscript, we have renamed α5 and α6 as hE and hF, in line with the hA, hB, … convention used for E2 helices elsewhere in the text (Line 160). These helices are now labeled accordingly in Figure 2a (middle panel). We have also carefully reviewed the manuscript and figures to ensure that helix nomenclature is used consistently throughout.

Figure 2a, hA helix overlay might be better if a ribbon is shown in the second panel.

Thank you for this suggestion. We agree that a ribbon representation provides a more detailed view of helix geometry compared to cylinders. To maintain visual consistency across the main figures, we have retained the cylinder representation for hA in Figure 2a. However, to more clearly illustrate the relative positioning and geometry of this helix, as suggested, we have added a new panel (Supplementary Fig. 1f) showing ribbon representations of helix hA from UBE2Z in our structure alongside the corresponding helix from PDB: 5A4P. This supplementary panel provides a clearer structural comparison while preserving consistency in the main figures.

Figure 3 and related text, it might be helpful to the reader to integrate the mutations (and data) with the structural description as it is quite hard to track the various mutations to the related structure panel. This would also allow more specific panel references.

We appreciate this suggestion and have revised the text to more explicitly integrate mutational analyses with the corresponding structural interfaces, with mutations discussed immediately following their structural context and accompanied by specific panel references (Fig. 3a–e)

Line 217/218, not sure the conclusion is justified?

Thank you for the suggestion. We agree that this was an overinterpretation and have removed the conclusion from this sentence in the revised manuscript (Line 224-225).

Line 224, it is not clear that the reciprocal mutations in Ube2z caused any loss (also include aa number as this will help the reader locate the data).

Thank you for this helpful suggestion. We have now explicitly included the amino acid identities of the mutated residues on the UBE2Z surface in the manuscript text (Line 229-231) to facilitate comparison with the structural panels. The reciprocal UBE2Z mutant (E225A/F228A/R232D) exhibits a minor but reproducible reduction in thioester transfer activity, retaining approximately 75–80% of wild-type activity, as shown in Fig. 3d. To better reflect the magnitude of this effect, we have revised the text to replace “partial loss” with “only a modest loss” (Line 231).

Line 291, Figure 7g is referred to out of order?
Thank you for pointing this out. We have removed the out-of-sequence reference to Fig. 7g at this location, as the LB-loop interactions depicted in that panel are discussed in detail later in the manuscript.

Line 363, replace ‘poorly’ with ‘not’?

Thank you for your suggestion. We have replaced ‘poorly’ with ‘not’ in Line 382-384 and agree that this wording is more accurate.

The Discussion might benefit form a model/schematic that integrates the conclusions for the reader. It would also be interesting to expand more widely and discuss whether there are implications from this study for E2s generally.

We appreciate this helpful suggestion. We have now included an integrative model schematic summarizing the key conclusions of this study in Supplementary Fig. 4e,f, which depicts how UBA6 and UBA1 employ distinct SCCH domain architectures to encode E2 specificity. In addition, we have expanded the final two paragraphs of the Discussion to explicitly address the broader implications of our findings for E2 recognition across Ub and Ubl conjugation systems. In this revised text, we discuss how E2 specificity can arise from different architectural strategies across E1 enzymes, rather than from a single conserved mechanism, and how the dual-domain framework revealed here for UBA6 may provide a generalizable approach for dissecting E1–E2 pairing principles in other Ub/Ubl pathways.