THE EMBO JOURNAL

Functional conservation and divergence of the helix-turn-helix motif of E2 ubiquitin-conjugating enzymes

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DOI: 10.15252/embj.2021108823

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Review Timeline:

Submission Date:	26th May 21
Editorial Decision:	9th Jul 21
Revision Received:	7th Oct 21
Editorial Decision:	17th Nov 21
Revision Received:	22nd Nov 21
Accepted:	25th Nov 21

Editor: Hartmut Vodermaier

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Thank you for submitting your manuscript on UBC helix-turn-helix roles for our consideration. I sent it to three expert referees, who have now returned their below-copied reports. As you will see, the referees' opinion are quite divided, with referees 1 and 2 generally supportive but referee 3 rather critical. In light of additional feedback from the referees on each other's comments, I feel that the study would still be a promising candidate for an EMBO Journal article, pending adequate revision in response to the referees comments. In particular, it would be important to complement the binding studies taking into account the additional ubiquitin binding sites(s) of UBE2R (see refs 1 and 2), to clarify key methodological and result details (esp. NMR, MD, etc.), and to generally improve aspects of presentation and interpretation.

Given that it is our policy to consider only a single round of major revision, it may be helpful to discuss how the various raised concerns might be addressed already during the early stages of your revision work. I would therefore invite you to carefully consider the reports together with your co-workers, and to send me a tentative point-by-point response via email, which could serve as the basis for further discussion via email or online call. It would be particularly interesting to hear whether there may be ways of tackling major point 3 of referee 3, regarding reciprocal mutational interrogation of APC/C binding surfaces.

Detailed information on preparing, formatting and uploading a revised manuscript can be found below and in our Guide to Authors. I should add that we could also offer extension of the default three-months revision period if needed, with our 'scooping protection' (meaning that competing work appearing elsewhere in the meantime will not affect our considerations of your study) remaining valid also throughout this extension.

Referee #1:

In this manuscript, the authors study mechanisms of E2 enzymes to study ubiquitin chain formation, and use biochemical, biophysical and NMR data to identify a different mechanistic role for the HTH region in UBE2S, where it is important for the donor ubiquitin, and other E2s, where it affects the acceptor interaction. They then place this in the context of the E3 ligase, APC and use simulation to explain how APC could accelerate the UBE2S-dependent ubiquitin chain formation.

The experiments are sophisticated, but some questions remain. Also the writing could be improved considerably to clarify to the reader what the paper is about. The manuscript makes transitions from E2/E3 to E2 alone to a different E2 and then back to E2/E3 without explaining these steps. By this lack of clarity the main message of the paper is in danger of getting lost.

Major questions and issues

- The role of the APC is completely dependent on the assumption that the know 'closed' state is the only catalytically competent state. It would be good to clarify this underlying assumption and discuss it a bit

- The BLI curves are not saturating and suggest substantial non-specific binding to the surface; in addition the WT is different in this respect to the mutants. This could be due to a difference in affinity (as suggested here) or to a difference in non-specific binding. This could be checked by a stability measurement such as differential scanning fluorimetry or CD melting experiment; in addition the quantification needs a comment on this aspect, as the current errors do not take this aspect into account.

- The Metris experiment is very interesting and this is certainly a good place to use it. However, it is not clear why this would measures specifically the acceptor binding, so this needs rephrasing. In principle you could specifically measure that by using the E2~Ub interaction with *Ub, but that may not be feasible.

- You could presumably validate the increase/decrease in closed state further by doing E2-Ub NMR with labelled ubiquitin, rather than E2?

Other issues
- It would be helpful to make explicit that Ube2R2 is UBCH3/cdc34
- Fig 1A: orientation changes are confusing ; at very least indicate what rotations take place among them, but easier if you stick to one orientation for the whole figure
- Fig 1e: securin changes are very mild; could this be quantified; clearer for cyclin b, is there an explanation why this is different between substrates?
- Fig 2G please explain (and label) the extra band above *Ub UbdGG
- Fig 2J this can surely not be 2000 milliseconds?
- Fig 3C and D: these different experiments read out something else, but this is not clear from the figure, please add MW marker and indication of what is read out to explain what we are looking at.
- Figure S3 B/C is this equilibrium fitting? Is kinetic fitting possible? There is a clear difference in kinetics, could be worth mentioning

- Fig 4G: is there a reason to havee it in another orientaton than 4C and 4
- Fig 4H : *s are not explicit which residues are highlighted, could this be replaced by more explicit labelling? ; it is bit confusing to have the longer logo UBE2R2, is that useful? Do explain in legend then.
- Fig S4G : please reiterate the set of logo's from 4H as it is annoying to have to go back and forth, please label the most important residue
- Fig 5; please show donor and acceptor Ub in both panels, highlighting the one that is affected by the charged residues.
- Materials and Methods: -
o Line 781 Replace 'Express fluorescently labelled ubiquitin' by: ubiquitin for fluorescent labelling was expressed....
o In these experiments it is not clear where the single cys is relative to Ub sequence: position -1? And what other residues are there?
- Line 799 UB change to Ub

Referee #2:

The manuscript by Welsh and colleagues describes an in-depth structural and biochemical analysis of the helix-turn-helix motif found on many ubiquitin-conjugating enzymes (E2s). Particular focus was applied towards understanding the E2 UBE2S which functions with the APC ubiquitin ligase. These results were compared and contrasted with additional E2s known to function with the APC (UBE2C and UBE2D), as well as UBE2R which is known to function with a totally distinct ubiquitin ligase called SCF. An impressive variety of techniques were employed, spanning structural biology (NMR and computation simulations), biochemistry (ubiquitylation reactions and quantitative protein-protein interaction assays), as well as cell biological protein degradation assays. These results collectively demonstrate that the helix-turn-helix motif is an important functional determinant of E2 function. However, it appears that the helix-turn-helix may differentially affect E2 molecular function. For instance, for UBE2S, it appears to control the formation of a closed complex between the donor ubiquitin and the E2, whereas in UBE2R, it may affect the ability of the acceptor Ubiquitin to bind in a productive manner.

Overall, this manuscript was an absolute pleasure to read. The topic of the paper, the molecular functions of helix-turn-helix motifs in E2s, is timely. Indeed, this reviewer cannot recall any papers on the topic, and as such, this manuscript and the accompanying results are a breath of fresh air. I only have minor comments and one suggestion for the binding experiments (please note that I do not deem this experiment as essential to publishing the paper). I fully endorse publishing the paper in EMBO journal.

In Figures 4E and 4G, the authors estimate the affinity of free Ubiquitin for various UBE2R proteins. The authors assume that they are measuring the binding of free Ub to the acceptor Ub binding site. While this is very likely the case (since the D143K mutation is known to be directly involved in acceptor Ub binding and does show a significant decrease in affinity in both assays), it remains a possibility that the binding measurement is also affected by the presence of additional Ub binding sites, such as the donor site, and perhaps to a lesser extent, the possibility for a back-side Ub binding site. Residues are known that would perturb binding of the donor Ub to UBE2R (see Saha and Deshaies, Mol Cell, 2011), and residues that affect backside binding can be inferred by structural similarity to UBE2D. It would certainly be comforting to the reader if binding to these sites were excluded as potentially confounding the results.

I also request that the authors perform quantification of the ubiquitylation reactions in Fig 1 C. It would be informative to see how substrate consumption tracks with the identity of the UBE2S mutant. In particular, the author's model suggests that substrate consumption is not affected by the helix-turn-helix mutations, whereas chain elongation is. Similarly, some quantification of the degree of chain elongation would be helpful. I find this a particularly important point, because while the authors do perform quantitative Ub discharge assays to estimate the effects of these mutations on the rate of Ub transfer, the results from the E3-independent assays are somewhat harder to interpret for a variety of reasons. In summary, quantifying the results in Fig 1 C will enable the reader to get a fix in terms of how important the helix-turn-helix really is towards the overall mechanism of Ub transfer within the context of the fully reconstituted system with APC.

Similarly, while the author's measure approximately a 3-fold defect using their pulse-chase assay and comparing WT and helix-turn-helix mutant UBE2S, it is probably worth mentioning, perhaps in the Discussion section, that while this may not seem like a massive defect, the reader should consider that this will apply for every Ub transfer event, and thus the total defect is likely greatly magnified when considering the generation of the entire poly-ubiquitin chain. It would also be worthy to mention the caveat that the 3-fold rate may be different within the context of the APC, since, for instance, the Ub concentration will undoubtedly be much higher in the fully reconstituted reaction than in the pulse-chase setup.

Minor points:

Figure 1 E: it is probably not fair to say that Securin degradation is enhanced for the helix-turn-helix mutant compared to WT UBE2S. Perhaps the Cyclin B data stand alone and are sufficient to make the author's point and the Securin results may be left out of the revision?

There are several gels in the results where it would be informative to the reader to know whether the SDS-PAGE was under reducing or non-reducing conditions (e.g. Figs 1 D and F).

Finally, I suggest that the authors consider tamping down the supposition that a dogmatic view dominates the field that E2s share a common mechanism (e.g. see lines 92 and 103 from the introduction). This feels a bit like a strawman argument to me, and I would still see the work as a major contribution to the field without it.

Referee #3:

The core UBC domain of E2 enzymes is surprisingly complex. In addition to active sites and regions that help position donor ubiquitin for nucleophilic attack, E2s contain regions that confer productive interactions with specific E3s and have additional faces that, when engaged, can modulate function. Also, some UBCs encode information that results in ubiquitin chain specificity, presumably as a consequence of positioning of acceptor ubiquitin. In this study Welsh, Bolhuis and colleagues explore roles of the C terminal most part of the UBC, the helix turn helix (HTH). Central to this manuscript is UBE2S, this E2 functions with the anaphase promoting complex/cyclosome (APC/C) E3 ubiquitin ligase, and results in K11-specific chain formation. Other E2s assessed include UBE2R2 (CDC34 ortholog) and, to a lesser extent, UBE2D2 and UBE2C. The central conclusions are that negative charges in the HTH can limit E2 activity through different mechanisms and that the authors' findings can explain the activation of UBE2S by the APC/C. This study asserts conclusions that would, if validated experimentally, have the potential to enhance our understanding of the UBE2S HTH and the function of the APC/C with UBE2S. Unfortunately, experimental validation is either cursory or absent. Regarding the analysis of other E2s, the findings are also primarily based on modeling. For these other E2s, however, it is not evident that even validation of modeling would provide meaningful insights.

For the K11 ubiquitin-chain specific E2, UBE2S, biochemical evidence is shown demonstrating that naturally-occurring Glu residues in the HTH negatively impact both APC/C dependent ubiquitylation and E3-independent ubiquitylation when compared to a double Arg mutation (E139R/E143R) or individual E139R and E143R mutants. Discharge assays support the idea that this is due to decreased reactivity of the thioester-linked donor ubiquitin and not a consequence of effects on acceptor nucleophiles. Molecular Dynamics simulations are asserted as providing evidence that the E139R/E143R double mutant has diminished interactions with the HTH compared to the WT E2. Modeling suggests that the WT interacts with ubiquitin through hydrogen bonding between E139 and Ub R54. As this interaction is lacking in the double mutant, it might have a greater propensity to assume closed conformations, where it could interact with the cross-over helix 2 of the E2, which would favor transfer of the bound ubiquitin. NMR studies (HSQC shifts and changes in dynamics) looking at the WT and mutant E2 bound to ubiquitin are presented that are interpreted as being consistent with this. The HTH seems to show greater dynamics in the double mutant and decreased dynamics in the cross-over helix. Changes in linewidth data are consistent with the changes in dynamics suggesting that the HTH shows greater backbone flexibility in the E139R/E143R mutant compared to the WT with the opposite being the case for the cross-over helix. Based on these findings, the authors suggest that APC2, which binds to the UBE2S HTH, would clash with E2-bound ubiquitin (mimicking E139R) and thereby favor closed conformations in the context of the WT E2. The authors state that their Rosetta modeling is consistent with this. The reason why this is interesting is, unlike other RING-E2 interactions, binding of the RING domain of the APC (APC11) to UBE2S~Ub does not by itself efficiently activate ubiquitination. However, absolutely no experiments that utilize APC2 are carried out to support their modeling.

For UBE2D2, UBE2C, and UBE2R2 the corresponding, naturally-occurring, HTH residues are either Lys or Arg. Replacement of these with Glu results in a decrease in ubiquitin transfer. This is further explored for UBE2R2 where biochemical analyses indicate that the R149E substitution (residue 149 is comparable to residue 139 of UBE2S) results in decreased access by acceptor ubiquitin. Consistent with this, Arg to Glu substitutions result in a diminution in the already low affinity of ubiquitin for UBE2R2 from 650uM to >4000uM. Based on existing structures and Molecular Dynamics simulations, it is hypothesized that R149E results in a new hydrogen bonding pattern in the 'gate loop,' which is on one side of the active site Cys of UBE2R2. This change in bonding is predicted by the authors to alter the acceptor ubiquitin binding site and thereby provide a molecular explanation for the impact of this mutation.

Major Points:
1. The findings with UBE2R2, and the other E2s containing basic charges in key HTH residues, are of unclear significance. The authors' own modeling (without supporting empirical data) suggests that the R149E mutation in UBE2R2 diminishes activity through the creation of an unnatural hydrogen bonding pattern between the HTH and the gate loop. Thus, the overall theme of the paper, that conservation and divergence of specific amino acids within the HTH is important, is not established. Further, it would not be established even if experiments that support the proposed new interaction are presented. Additionally, based on binding data and modeling, the authors state that "our results strongly demonstrate that the UBE2 HTH is used for interaction with the acceptor Ub during chain synthesis." There is no experimental data to support this conclusion. There is no direct analysis of HTH interactions with acceptor ubiquitin and the effect of the R149E mutation on the binding affinity between acceptor ubiquitin and E2-Ub conjugates are not assessed. This is important as the position and affinity of acceptor ubiquitin can only be defined in the context of a potential donor. For the authors to make a statement about the role of the HTH direct evidence is required, not findings that, from the authors' own modeling, can be attributed to allosteric effects on adjacent regions of the E2.

2. The data supporting the conclusion that UBE2S E139R/E143R is favoring open conformations through interactions with donor ubiquitin is incomplete and thin. Minor effects on the E2 core are shown, which are really quite sparse given that this is the only major point in the manuscript where there is any attempt at experimental validation.
a. The premise for carrying out the NMR analysis is based on Molecular Dynamics simulations, yet no MD data is shown.
b. In lines 343-348 referring to the NMR the authors state that the spectra indicate the ubiquitin is in an open state. But there is no assignment on the spectra and no references to what peaks shift. This is uninformative.
c. Similarly, for both the dynamics and linewidth data there is no indication of what residues shift and no discussion of how the specific residues correlate with what is known in the literature about effects on the crossover helix.
d. Since modeling implicates hydrogen bonding between UBE2S E139 and Ub R54 as being critical (Fig 4C) this should be more rigorously tested by mutating R54 and assessing this mutation both biochemically and through NMR.
e. Another obvious part of this analysis should be an assessment of whether E139R is sufficient to cause the changes in dynamics and flexibility induced by the double mutant. Conversely, the role of E143 and its mutation to Arg needs to be addressed. It is striking that, despite the significance attributed to E143 this residue it isn't even shown in Fig 4C where the E139-R54 interaction is modeled.
f. Related to this, why in Fig 2F, does E139R activate diubiquitin formation, while both E139R/E143R and E143 more closely resemble WT? This data might suggest more dramatic changes with E139R alone when analyzed by NMR. On the other hand, the data shown in Fig 1 and Fig 4A suggests that all three mutants are similar in their activation.

3. A major conclusion of this study, based only on modeling, is that the binding of APC2 will essentially mimic the UBE2S E139R/E143R double mutation by creating steric clashes that would mimic the E139R substitution and favor closed conformations. By extension, this would provide a molecular basis for the activation of UBE2S~Ub specifically when engaged with the APC/C. This hypothesis requires rigorously experimental testing - there is none. Additionally, in Fig 1, increased activity is observed with Glu to Arg mutations in the presence of the APC/C as well as in its absence. What significance should be ascribed to this? Is this expected, and can this be explained in the context of APC2 interaction with the HTH region of the E2?

4. The finding that UBE2S favors K11 chains makes this E2 of particular interest. It is quite surprising that there is no attempt to assess or at least discuss what role the HTH might play in this specificity given that, for UBE2R, the authors implicate the HTH in binding the acceptor ubiquitin, which is where chain specificity presumably arises. The statement on lines 409 to 412 in the Discussion that "By combining NMR, MD simulations, and detailed biochemical assays, we propose that the APC2 interaction limits the conformational space of the donor Ub, enriching the closed_E2~Ub conformation and facilitating nucleophilic attack of the thioester bond by K11 of Ub." is misleading to someone who has not carefully read the manuscript. There is nothing that addresses why K11, in particular, would be the favored nucleophile.

5. No experimental data is provided to backs up the significance of the phylogenetic analysis. As there is nothing to suggest that there is an evolutionary significance attributed to the Arg HTH residues in UBE2R2, the intellectual basis for having this in the paper is questionable.

Other comments:

6. The methods are lacking detail in a number of places. The authors are encouraged to reassess this part of the manuscript.

7. The quality of the biotin-LRLRGG assays shown in Fig 3D and Fig 4B are difficult to interpret due to backgrounds and diffuse bands. It might also be helpful here to show the input biotinylated E2 conjugate.

8. Key residues in Fig. 1B should be labeled.

9. The text referring to Fig. 2K is uninformative.

10. In addition to what is mentioned above, there are assertions in the text that are either overstatements or simply not backed up by data.
"Taken together, this region is capable of functional diversification that can provide specialization for E2 activity." (page 15 lines 388-390)
"Overall, we show that the E2 HTH impacts the intrinsic activity of multiple E2s, can be modulated by the E3, and reused for different steps in chain elongation for cell cycle regulation and countless other signaling pathways." (page 6 line 144-147).

11. CTP and WHB should be defined.

12. On page 11 line 268, Km should be Kd.

13. Fig 3H is uninformative as presented.

Point-by-point responses in red*:*

General response to Reviewers: We thank the Reviewers for their careful assessment of our paper and helpful suggestions for improving our manuscript. We tried to address all your suggestions, both in terms of experiments and text.

Referee #1:

In this manuscript, the authors study mechanisms of E2 enzymes to study ubiquitin chain formation, and use biochemical, biophysical and NMR data to identify a different mechanistic role for the HTH region in UBE2S, where it is important for the donor ubiquitin, and other E2s, where it affects the acceptor interaction. They then place this in the context of the E3 ligase, APC and use simulation to explain how APC could accelerate the UBE2S-dependent ubiquitin chain formation.

The experiments are sophisticated, but some questions remain. Also the writing could be improved considerably to clarify to the reader what the paper is about. The manuscript makes transitions from E2/E3 to E2 alone to a different E2 and then back to E2/E3 without explaining these steps. By this lack of clarity the main message of the paper is in danger of getting lost.

We appreciate the reviewer's careful consideration of our manuscript. To improve the overall flow of the manuscript, we changed the order that we describe the experiments to: finding the mutations in UBE2S (without the APC/C) > testing the helix-turn-helix (HTH) mutations in multiple E2s alone > comparing UBE2R and UBE2S to show the differences in HTH function > detailed characterization of the UBE2R^{HTH} > mechanistic explanations of the UBE2S^{HTH} substitutions > APC/C-dependent UBE2S activity. We also attempted to improve these transitions for accessibility and clarity of the manuscript and to help guide the reader through several complicated biochemical assays. We have also increased the number of figures to improve the readability of the manuscript.

Major questions and issues

- The role of the APC is completely dependent on the assumption that the know 'closed' state is the only catalytically competent state. It would be good to clarify this underlying assumption and discuss it a bit

We have clarified this underlying assumption in the text (Lines 131-134, 395-396, 510-511) and provided more experimental evidence that the "closed" state is critical for UBE2S-dependent Ub transfer. First, we took a new line of experimental investigation to create a FRET system of the UBE2S~Ub conjugate, similar to the UBC13~Ub and RNF4 system from (Branigan *et al*, 2020). However, despite ~20 constructs and ~150 liters of bacterial cultures, we were unsuccessful in our attempt to adapt this system for UBE2S~Ub during the revision process. Instead, we were able to include more mutational data with a previously described mutant (C118A) that impairs the formation of the "closed" state of UBE2S (Wickliffe *et al*, 2011). When this substitution is added to the wild-type enzyme, ubiquitination is dramatically reduced, demonstrating the utility of the "closed" state. In support of our hypothesis, the E139R and E143R substitutions partially rescued this defect with and without the APC/C but the enhanced activity as a result of these mutations were reduced (Figures 5F and 7E). Together, this demonstrates that the "closed" state is needed but we also cannot claim that it is the only catalytically competent state.

- The BLI curves are not saturating and suggest substantial non-specific binding to the surface;

in addition the WT is different in this respect to the mutants. This could be due to a difference in affinity (as suggested here) or to a difference in non-specific binding. This could be checked by a stability measurement such as differential scanning fluorimetry or CD melting experiment; in addition the quantification needs a comment on this aspect, as the current errors do not take this aspect into account.

We agree that BLI has limitations, which we comment on in revised manuscript (Lines 291-293). At high concentrations needed to saturate the binding curves, Ub displays thermodynamic non-ideality, and therefore, we used the METRIS assay as a secondary approach. However, the fact that the K_d is similar to the K_m of the acceptor Ub observed in (Liwocha *et al*, 2021) and in our manuscript suggests that the BLI data are relatively reliable to measure the acceptor Ub. Furthermore, we obtained K_m estimates for UBE2R-mediated diUb synthesis that agree with the BLI and METRIS measurements, giving us good confidence in the values obtained with BLI (Figure 4G-I).

- The Metris experiment is very interesting and this is certainly a good place to use it. However, it is not clear why this would measures specifically the acceptor binding, so this needs rephrasing. In principle you could specifically measure that by using the E2~Ub interaction with *Ub, but that may not be feasible.

We really liked the idea of testing the UBE2R~Ub interaction with Ub and these data are now included in Figure 4F. By including the donor Ub in the METRIS experiment, we are more directly measuring the acceptor Ub binding site. The incorporation of the R149E substitution in the UBE2R~Ub conjugate resulted in a ~2-fold decrease in acceptor Ub binding. This result provided additional support for our hypothesis that the conformational changes in the gating loop are responsible for the defect that we observed with the R149E mutation. We rationalize that the addition of the conjugated Ub, which is known to make direct contacts with the gating loop, helps to stabilize the acceptor Ub binding site.

Additionally, we have rephrased certain parts of the text to clarify the limitations of METRIS and BLI (Lines 291-302).

- You could presumably validate the increase/decrease in closed state further by doing E2-Ub NMR with labelled ubiquitin, rather than E2?

The suggested experiment is a good idea and was attempted. However, the amount of E2 needed to add to the ^{15}N-labeled Ub were problematic, as the UBE2S variants were not stable at those high concentrations. Instead, additional NMR studies were conducted with the individual UBE2S variants, E139R and E143R (Figure EV4). The resulting NMR data from both individual mutations displayed intermediate changes in UBE2S linewidth upon Ub binding compared to the E139R/E143R double mutant (Figure 5C-D), suggesting that both substitutions contribute to changes in UBE2S dynamics and the increased function (Lines 396-401).

Other issues
- It would be helpful to make explicit that UBE2R2 is UBCH3/cdc34

This correction is now made in the introduction, Line 97.

- Fig 1A: orientation changes are confusing ; at very least indicate what rotations take place among them, but easier if you stick to one orientation for the whole figure

We have attempted to standardize the orientations throughout the manuscript, including Figure 1A-B.

- Fig 1e: securin changes are very mild; could this be quantified; clearer for cyclin b, is there an explanation why this is different between substrates?

As suggested by Reviewer 2, the securin blot has been removed. Overall, there is no clear explanation for these differences yet. However, we often see this finding that some substrates are more sensitive to UBE2S activity than others. This result likely has to do with the chains formed on the substrate and proteasome activity, which are outside the scope of this manuscript.

- Fig 2G please explain (and label) the extra band above *Ub UbdGG

The band is a contaminant from fluorescent labeling and is now marked in Figure 2F.

- Fig 2J this can surely not be 2000 milliseconds?

We thank the reviewer for catching this mistake. The previous plot mistakenly contained frames and we have adjusted it to contain the correct time of 20ns.

- Fig 3C and D: these different experiments read out something else, but this is not clear from the figure, please add MW marker and indication of what is read out to explain what we are looking at.

The molecular weights of the products have now been indicated, and we have attempted to clarify the readouts in Figure 3.

- Figure S3 B/C is this equilibrium fitting? Is kinetic fitting possible? There is a clear difference in kinetics, could be worth mentioning

We used equilibrium fitting to determine the K_d in graphs S3B/C (now Figure EV3C-D). Kinetic fitting would be possible for the WT, but this was not practical for the mutants. We do notice a faster association rate in the WT that is not seen in the mutants, which is potentially interesting given the idea that long-range electrostatics contribute to protein association. However, this concept was beyond the scope of our study.

- Fig 4G: is there a reason to have it in another orientation than 4C and 4

We have synchronized all of our E2 orientations except for Figure 4D, where the E2 is rotated 180 degrees to better show the acceptor Ub binding site.

- Fig 4H : *s are not explicit which residues are highlighted, could this be replaced by more explicit labelling? ; it is bit confusing to have the longer logo for UBE2R2, is that useful? Do explain in legend then.

We have removed the residue highlights and have instead labeled specific residues of interest in Figure 2I, Figure EV2C, and its legend, which now contain the weblogos. The UBE2R weblogo was elongated to show the conservation in position D143K.

- Fig S4G : please reiterate the set of logo's from 4H as it is annoying to have to go back and forth, please label the most important residue

We apologize for this oversight. The full set of logos are shown in Figure EV2C.

- Fig 5; please show donor and acceptor Ub in both panels, highlighting the one that is affected by the charged residues.

Both donor and acceptor Ub are now added in both panels of the Synopsis figure (formerly Figure 5).

- Materials and Methods: -
o Line 781 Replace 'Express fluorescently labelled ubiquitin' by: ubiquitin for fluorescent labelling was expressed....

This correction has been made in Line 1021-1022.

o In these experiments it is not clear where the single cys is relative to Ub sequence: position -1? And what other residues are there?

The N-terminus of the Ub sequence used in this study is now added to the methods, Lines 1023-1025.

- Line 799 UB change to Ub

Thank you for noticing this error. It is now corrected on Line 1099.

Referee #2:

The manuscript by Welsh and colleagues describes an in-depth structural and biochemical analysis of the helix-turn-helix motif found on many ubiquitin-conjugating enzymes (E2s). Particular focus was applied towards understanding the E2 UBE2S which functions with the APC ubiquitin ligase. These results were compared and contrasted with additional E2s known to function with the APC (UBE2C and UBE2D), as well as UBE2R which is known to function with a totally distinct ubiquitin ligase called SCF. An impressive variety of techniques were employed, spanning structural biology (NMR and computation simulations), biochemistry (ubiquitination reactions and quantitative protein-protein interaction assays), as well as cell biological protein degradation assays. These results collectively demonstrate that the helix-turn-helix motif is an important functional determinant of E2 function. However, it appears that the helix-turn-helix may differentially affect E2 molecular function. For instance, for UBE2S, it appears to control the formation of a closed complex between the donor ubiquitin and the E2, whereas in UBE2R, it may affect the ability of the acceptor Ubiquitin to bind in a productive manner.

Overall, this manuscript was an absolute pleasure to read. The topic of the paper, the molecular functions of helix-turn-helix motifs in E2s, is timely. Indeed, this reviewer cannot recall any papers on the topic, and as such, this manuscript and the accompanying results are a breath of fresh air. I only have minor comments and one suggestion for the binding experiments (please note that I do not deem this experiment as essential to publishing the paper). I fully endorse publishing the paper in EMBO journal.

We appreciate the reviewer's kind words regarding our manuscript!

In Figures 4E and 4G, the authors estimate the affinity of free Ubiquitin for various UBE2R proteins. The authors assume that they are measuring the binding of free Ub to the acceptor Ub binding site. While this is very likely the case (since the D143K mutation is known to be directly involved in acceptor Ub binding and does show a significant decrease in affinity in both assays), it remains a possibility that the binding measurement is also affected by the presence of additional Ub binding sites, such as the donor site, and perhaps to a lesser extent, the possibility for a back-side Ub binding site. Residues are known that would perturb binding of the donor Ub to UBE2R (see Saha and Deshaies, Mol Cell, 2011), and residues that affect backside binding can be inferred by structural similarity to UBE2D. It would certainly be comforting to the reader if binding to these sites were excluded as potentially confounding the results.

We thank for the reviewer for their insightful comments. As discussed above in our response to Reviewer 1, we used METRIS to test UBE2R2~Ub conjugates and still observed binding to Ub, which suggests we are not measuring the donor Ub binding site. Regarding the classical backside binding of UBE2D, this interaction is typically disrupted by the S22R mutation. In UBE2R2, an Arginine residue is already present at the equivalent position.

Based on the METRIS data of the UBE2R2~Ub conjugate (Figure 4F), R149E still reduced the binding to Ub by ~2-fold. We expected that the addition of the donor Ub to partially rescue the defect because it stabilizes the position of the E2 gating loop. Consistent with these results, we performed kinetic enzyme assays by titrating the acceptor Ub, which revealed both an apparent K_m and V_{max} defect when the R149E-substituted variant was tested (Figure 4G-I). This cross validation between binding and enzyme kinetics further suggests that the R149E mutation reduces acceptor Ub binding and has a catalytic defect due to a change in gating loop dynamics.

I also request that the authors perform quantification of the ubiquitination reactions in Fig 1 C. It would be informative to see how substrate consumption tracks with the identity of the UBE2S mutant. In particular, the author's model suggests that substrate consumption is not affected by the helix-turn-helix mutations, whereas chain elongation is. Similarly, some quantification of the degree of chain elongation would be helpful. I find this a particularly important point, because while the authors do perform quantitative Ub discharge assays to estimate the effects of these mutations on the rate of Ub transfer, the results from the E3-independent assays are somewhat harder to interpret for a variety of reasons. In summary, quantifying the results in Fig 1 C will enable the reader to get a fix in terms of how important the helix-turn-helix really is towards the overall mechanism of Ub transfer within the context of the fully reconstituted system with APC.

These quantifications of reactions in Figure 6E (formerly Figure 1C due to the changes in the order in response to Reviewer 1) have been performed and have been added to the revised manuscript in Figure EV5C. In addition, we have new quantifications of assays with substitutions at the UBE2S^{HTH} and in APC2 to understand how important the HTH is for Ub transfer, both with and without the APC/C (Figure 7B-D). Furthermore, these assays are engineered to only look at Ub chain elongation by using a Ub-fused substrate, bypassing the need for Ub priming (Lines 450-452).

Similarly, while the author's measure approximately a 3-fold defect using their pulse-chase assay and comparing WT and helix-turn-helix mutant UBE2S, it is probably worth mentioning, perhaps in the Discussion section, that while this may not seem like a massive defect, the reader should consider that this will apply for every Ub transfer event, and thus the total defect

is likely greatly magnified when considering the generation of the entire poly-ubiquitin chain. It would also be worthy to mention the caveat that the 3-fold rate may be different within the context of the APC, since, for instance, the Ub concentration will undoubtedly be much higher in the fully reconstituted reaction than in the pulse-chase setup.

We appreciate the reviewer's comment on the significance of the effect, and we elaborated on this point in the discussion, Lines 505-511.

Minor points:

Figure 1 E: it is probably not fair to say that Securin degradation is enhanced for the helix-turn-helix mutant compared to WT UBE2S. Perhaps the Cyclin B data stand alone and are sufficient to make the author's point and the Securin results may be left out of the revision?

The securin result is left out of the revision, as suggested.

There are several gels in the results where it would be informative to the reader to know whether the SDS-PAGE was under reducing or non-reducing conditions (e.g. Figs 1 D and F).

These conditions are added to the figure legends and their corresponding methods sections, when applicable. All gels were ran under non-reducing conditions.

Finally, I suggest that the authors consider tamping down the supposition that a dogmatic view dominates the field that E2s share a common mechanism (e.g. see lines 92 and 103 from the introduction). This feels a bit like a strawman argument to me, and I would still see the work as a major contribution to the field without it.

We thank the reviewer for their response, and we toned down this description, as suggested.

Referee #3:

The core UBC domain of E2 enzymes is surprisingly complex. In addition to active sites and regions that help position donor ubiquitin for nucleophilic attack, E2s contain regions that confer productive interactions with specific E3s and have additional faces that, when engaged, can modulate function. Also, some UBCs encode information that results in ubiquitin chain specificity, presumably as a consequence of positioning of acceptor ubiquitin. In this study Welsh, Bolhuis and colleagues explore roles of the C terminal most part of the UBC, the helix turn helix (HTH). Central to this manuscript is UBE2S, this E2 functions with the anaphase promoting complex/cyclosome (APC/C) E3 ubiquitin ligase, and results in K11-specific chain formation. Other E2s assessed include UBE2R2 (CDC34 ortholog) and, to a lesser extent, UBE2D2 and UBE2C. The central conclusions are that negative charges in the HTH can limit E2 activity through different mechanisms and that the authors' findings can explain the activation of UBE2S by the APC/C. This study asserts conclusions that would, if validated experimentally, have the potential to enhance our understanding of the UBE2S HTH and the function of the APC/C with UBE2S. Unfortunately, experimental validation is either cursory or absent. Regarding the analysis of other E2s, the findings are also primarily based on modeling. For these other E2s, however, it is not evident that even validation of modeling would provide meaningful insights.

For the K11 ubiquitin-chain specific E2, UBE2S, biochemical evidence is shown demonstrating that naturally-occurring Glu residues in the HTH negatively impact both APC/C dependent

ubiquitination and E3-independent ubiquitination when compared to a double Arg mutation (E139R/E143R) or individual E139R and E143R mutants. Discharge assays support the idea that this is due to decreased reactivity of the thioester-linked donor ubiquitin and not a consequence of effects on acceptor nucleophiles. Molecular Dynamics simulations are asserted as providing evidence that the E139R/E143R double mutant has diminished interactions with the HTH compared to the WT E2. Modeling suggests that the WT interacts with ubiquitin through hydrogen bonding between E139 and Ub R54. As this interaction is lacking in the double mutant, it might have a greater propensity to assume closed conformations, where it could interact with the cross-over helix 2 of the E2, which would favor transfer of the bound ubiquitin. NMR studies (HSQC shifts and changes in dynamics) looking at the WT and mutant E2 bound to ubiquitin are presented that are interpreted as being consistent with this. The HTH seems to show greater dynamics in the double mutant and decreased dynamics in the cross-over helix. Changes in linewidth data are consistent with the changes in dynamics suggesting that the HTH shows greater backbone flexibility in the E139R/E143R mutant compared to the WT with the opposite being the case for the cross-over helix. Based on these findings, the authors suggest that APC2, which binds to the UBE2S HTH, would clash with E2-bound ubiquitin (mimicking E139R) and thereby favor closed conformations in the context of the WT E2. The authors state that their Rosetta modeling is consistent with this. The reason why this is interesting is, unlike other RING-E2 interactions, binding of the RING domain of the APC (APC11) to UBE2S~Ub does not by itself efficiently activate ubiquitination. However, absolutely no experiments that utilize APC2 are carried out to support their modeling.

For UBE2D2, UBE2C, and UBE2R2 the corresponding, naturally-occurring, HTH residues are either Lys or Arg. Replacement of these with Glu results in a decrease in ubiquitin transfer. This is further explored for UBE2R2 where biochemical analyses indicate that the R149E substitution (residue 149 is comparable to residue 139 of UBE2S) results in decreased access by acceptor ubiquitin. Consistent with this, Arg to Glu substitutions result in a diminution in the already low affinity of ubiquitin for UBE2R2 from 650uM to >4000uM. Based on existing structures and Molecular Dynamics simulations, it is hypothesized that R149E results in a new hydrogen bonding pattern in the 'gate loop,' which is on one side of the active site Cys of UBE2R2. This change in bonding is predicted by the authors to alter the acceptor ubiquitin binding site and thereby provide a molecular explanation for the impact of this mutation.

Major Points:
1. The findings with UBE2R2, and the other E2s containing basic charges in key HTH residues, are of unclear significance. The authors' own modeling (without supporting empirical data) suggests that the R149E mutation in UBE2R2 diminishes activity through the creation of an unnatural hydrogen bonding pattern between the HTH and the gate loop. Thus, the overall theme of the paper, that conservation and divergence of specific amino acids within the HTH is important, is not established. Further, it would not be established even if experiments that support the proposed new interaction are presented.

We apologize for this misconception about the point of our study that might arise from the title “Functional conservation and divergence of the helix-turn-helix of E2 ubiquitin conjugating enzymes”. Our title is referring to different functions that the HTH region has in different E2s during ubiquitination and not specifically to the amino acid conservation and divergence, which we do offer as supporting evidence. We clarify that we meant conservation of specific resides within an E2 family member indicates functional importance and that divergence of amino acids and structural motifs of the HTH may lead to new functions within the broader E2 family. We have attempted to clarify our stance through numerous text edits.

Additionally, based on binding data and modeling, the authors state that "our results strongly demonstrate that the UBE2 HTH is used for interaction with the acceptor Ub during chain synthesis." There is no experimental data to support this conclusion. There is no direct analysis of HTH interactions with acceptor ubiquitin and the effect of the R149E mutation on the binding affinity between acceptor ubiquitin and E2-Ub conjugates are not assessed. This is important as the position and affinity of acceptor ubiquitin can only be defined in the context of a potential donor. For the authors to make a statement about the role of the HTH direct evidence is required, not findings that, from the authors' own modeling, can be attributed to allosteric effects on adjacent regions of the E2.

We appreciate this comment and have tried to address this comment with biochemical assays. First, we performed METRIS assays with an isopeptide-linked E2~Ub mimic (see response to Reviewers 1&2), mimicking the presence of a donor Ub. Furthermore, we still observed reduced binding for the R149E variant in the context of the conjugated Ub, albeit a lesser reduction than in the E2 alone (Figure 4F). This finding is consistent with the R149E defect being dependent on the gating loop since the donor Ub also interacts with this region. Second, we determined the kinetic parameters for UBE2R2 wild-type and the D143K and R149E variants. As expected, both the apparent K_m had increased for HTH-substituted variants, indicating a defect in acceptor Ub binding, and a large decrease in the apparent V_{max}, supporting the role of the gating loop (Figure 4G-I). Furthermore, the statement that the helix-turn-helix is used for the interaction with the acceptor will be expanded to suggest an indirect role of the HTH (Lines 328-335). Finally, the position of the acceptor model was validated with charge-swapped mutations in a previous study (Hill et al 2016) and the mutational data for UBE2R that we cite in the manuscript. Overall, we feel that this is the most direct binding data that we have that the HTH, which also includes D143K, binds the acceptor Ub.

2. The data supporting the conclusion that UBE2S E139R/E143R is favoring open conformations through interactions with donor ubiquitin is incomplete and thin. Minor effects on the E2 core are shown, which are really quite sparse given that this is the only major point in the manuscript where there is any attempt at experimental validation.

We agree with the reviewer that more support for the model that UBE2S E139R/E143R is favoring the open conformation is beneficial. However, this finding is difficult to directly observe. We attempted to establish a FRET system to directly monitor the “open”-“closed” transitions but were unable to establish this system in the timeframe of the revision (see Response to Reviewer 1). Instead, we did include additional mutational analysis that shows 1) the “closed” state is the catalytically competent state (Figure 5F and 7E), 2) the activating mutations to the HTH partially rescue these mutations (Figure 5F and 7E), and 3) the R54E mutation reduces the activation of the E139R (Figure 5B). While these effects may be small, they are statistically significant and translate into other E2s. Since Ub chain formation involves multiple turnover events (as discussed by Reviewer 2), even these small affects will lead to greater defects during polyubiquitination. Furthermore, given the proposed role of the HTH, by reducing the population or lifetime of inactive “open” states in a conformational ensemble of E2~Ub, we would expect the effects to be relatively small. However, many crystal structures of E2~Ub exist that show the Ub interacting with the HTH and are discussed as more evidence that this “open” state can both exist and would likely be present in some fraction of the E2~Ub population (Lines 532-535). Ultimately, our results help the Ub field to better understand the complex aspects of Ub transfer and reporting this work will allow others to test this concept in their system.

a. The premise for carrying out the NMR analysis is based on Molecular Dynamics simulations, yet no MD data is shown.

We have clarified the text regarding the MD of UBE2S by removing some of the text associated with MD simulations we do not show (Lines 365-372). Now, we only included 1 state from the UBE2S WT MD run that suggests a potential interaction between the donor Ub and HTH, which we previously showed and is now Figure 5A. We also follow up the MD with the reporting of the R54E mutant reducing the activity of the E139R variant and then use NMR to better understand the mutations (Figure 5B-D).

b. In lines 343-348 referring to the NMR the authors state that the spectra indicate the ubiquitin is in an open state. But there is no assignment on the spectra and no references to what peaks shift. This is uninformative.

A close-up view of some of the assignments was included in former Figure 4D (now Figure 5C) and the analysis of chemical shift perturbation that was shown in former Supplemental Figure 4C (now Figure EV4) could not have been performed without the assignments. We now include the NMR spectral overlay with assignments for four constructs, wild type, E139R, E143R, and ER/ER mutants, in their corresponding free and Ub bound states in Figure EV4 and the Appendix Figures 2-5. Furthermore, we have clarified that we don't see the "open" state, but rather we see a more "closed" state for the substituted variants.

c. Similarly, for both the dynamics and linewidth data there is no indication of what residues shift and no discussion of how the specific residues correlate with what is known in the literature about effects on the crossover helix.

We thank the reviewer for this comment, and we have provided more information about which residues change in response to Ub binding (Lines 385-396). In particular, we note residues 118-122 at the C-terminal end of the cross-over helix, which from the known literature interacts with the hydrophobic patch of the donor Ub during the formation of a closed E2~Ub, are stabilized (specific residues now noted in Figure 5D). We also included more specific remarks about which regions appear more dynamic, including the active site and HTH. A model of the closed UBE2S~Ub is also added to Figure 5E for more context of the "closed" configuration. Furthermore, experiments are performed with a mutation that disrupts the closed conformation, C118A discussed further below, to demonstrate that the increased activity of both the ER/ER variant and the APC/C are sensitive to this substitution and therefore the "closed" conformation of the E2~Ub (Figures 5F and 7E).

d. Since modeling implicates hydrogen bonding between UBE2S E139 and Ub R54 as being critical (Fig 4C) this should be more rigorously tested by mutating R54 and assessing this mutation both biochemically and through NMR.

We directly tested the impact of the R54E mutation in UBE2S autoubiquitination assays. Specifically, the R54E substitution reduced the effects of the E139R mutant (Figure 5B). Furthermore, we don't envision a single "open" state, but instead an ensemble of states where the many positively charged residues on Ub can interact with the negatively charged HTH. We have expanded this point in the text, Lines 424-426, and Figure 5G.

e. Another obvious part of this analysis should be an assessment of whether E139R is sufficient to cause the changes in dynamics and flexibility induced by the double mutant. Conversely, the role of E143 and its mutation to Arg needs to be addressed. It is striking that, despite the significance attributed to E143 this residue it isn't even shown in Fig 4C where the E139-R54 interaction is modeled.

In our modeling assays, we did not see close interactions between E143 and the donor Ub, and we think that it is difficult for the tethered Ub to reach this far. However, we agree that the role of E139R and E143R could be slightly different. To further examine contributions from these individual mutations to the dynamics and flexibility of UBE2S, we performed additional NMR studies on E139R and E143R constructs in the presence and absence of Ub, which are shown in the new Figure EV4C-H (with the assigned spectra in Appendix Figure 3-4). The resulting NMR data from both individual mutations displayed intermediate changes in UBE2S linewidth upon Ub binding compared to the ER/ER, suggesting that both substitutions contribute to changes in UBE2S dynamics. Overall, comparisons of the NMR data on all four UBE2S constructs suggest that both E139 and E143 play a role in the enhanced formation of the "closed" state of E2~Ub.

f. Related to this, why in Fig 2F, does E139R activate diubiquitin formation, while both E139R/E143R and E143 more closely resemble WT? This data might suggest more dramatic changes with E139R alone when analyzed by NMR. On the other hand, the data shown in Fig 1 and Fig 4A suggests that all three mutants are similar in their activation.

In addition to the NMR with the individual mutants, as mentioned above, we also performed several biochemical assays with either E139R or E143R in both APC/C and E3-independent assays to deconvolute the effects of E139R or E143R (Figure 5-7). While the overall results are similar for both mutants, we did notice that E139R has a more pronounced effect in diUb formation while E143R seems to have a greater effect in APC/C dependent assays. This does suggest that these may have slightly different roles in the context APC/C, but overall their effects are largely the same (Figure 7C and D). This concept is discussed further below.

3. A major conclusion of this study, based only on modeling, is that the binding of APC2 will essentially mimic the UBE2S E139R/E143R double mutation by creating steric clashes that would mimic the E139R substitution and favor closed conformations. By extension, this would provide a molecular basis for the activation of UBE2S~Ub specifically when engaged with the APC/C. This hypothesis requires rigorously experimental testing - there is none. Additionally, in Fig 1, increased activity is observed with Glu to Arg mutations in the presence of the APC/C as well as in its absence. What significance should be ascribed to this? Is this expected, and can this be explained in the context of APC2 interaction with the HTH region of the E2?

To address this concern, we performed multiple experiments demonstrating that APC2 is responsible for UBE2S activation and to delineate the role of the E139R and E143R mutations. Together, we provide a quantitative assessment of the contributions of each interaction and their dependence on the "closed" state of UBE2S~Ub.

First, we attempted to develop a FRET-based system similar to (Branigan *et al.*, 2020) to monitor the dynamics of the E2~Ub interaction. Despite ~20 constructs and ~150 liters of bacterial cultures, we were unsuccessful in our attempt during the revision process.

Second, individual and double mutants of UBE2S were tested in substrate ubiquitination and diUb synthesis assays in the context of the APC/C or the APC/C Platform (which lacks the substrate recruitment module) harboring known APC2 mutations (V542A/E543A and K562D) that disrupt its interaction with UBE2S (Figure 7A). Similar to our previous studies, these versions of the APC/C were defective in their ability to stimulate UBE2S-dependent diUb synthesis and substrate ubiquitination (Brown *et al*, 2016; Brown *et al*, 2014). We also tested these mutants in the context of the E139R and E143R backgrounds. As expected, the UBE2S-

activating mutations partially rescued the loss of APC2-dependent activation, providing more evidence to support our claim that the E139R/E143R mutants perform a similar function as APC2 (Figure 7B-D).

Lastly, a UBE2S cross-over helix mutation (e.g., C118A) was introduced in these backgrounds and demonstrated that the “closed” form of the E2~Ub conjugate is needed for the enhanced activity of these mutants in an APC/C-dependent and -independent manner (Figures 5F and 7E). Furthermore, the fact that the E139R and E143R substitutions partially rescue the defect of C118A also supports the concept that the increased activation is through driving the “closed” state.

4. The finding that UBE2S favors K11 chains makes this E2 of particular interest. It is quite surprising that there is no attempt to assess or at least discuss what role the HTH might play in this specificity given that, for UBE2R, the authors implicate the HTH in binding the acceptor ubiquitin, which is where chain specificity presumably arises. The statement on lines 409 to 412 in the Discussion that "By combining NMR, MD simulations, and detailed biochemical assays, we propose that the APC2 interaction limits the conformational space of the donor Ub, enriching the closed_E2~Ub conformation and facilitating nucleophilic attack of the thioester bond by K11 of Ub." is misleading to someone who has not carefully read the manuscript. There is nothing that addresses why K11, in particular, would be the favored nucleophile.

The later part of the statement regarding K11 as a nucleophile is pulling from the existing literature, as UBE2S is a well-known K11-specific E2, and the mass spectrometry data (Figure 1E)(Bremm *et al*, 2010; Garnett *et al*, 2009; Wickliffe *et al.*, 2011; Williamson *et al*, 2009; Wu *et al*, 2010). However, this statement is adjusted to summarize our work (Lines 503-511). While we agree with the reviewer that there are some outstanding questions regarding the molecular basis for the specificity of UBE2S, we feel that looking at UBE2S specificity for K11 is out of the scope of our manuscript.

5. No experimental data is provided to backs up the significance of the phylogenetic analysis. As there is nothing to suggest that there is an evolutionary significance attributed to the Arg HTH residues in UBE2R2, the intellectual basis for having this in the paper is questionable.

With the reorientation of the text, we were able to expand our discussion of the deep sequence alignment and phylogenetic data (Lines 218-240). These data are important because 1) it shows an overall exclusion of negatively charged residues in the HTH from all but a few E2s at positions analogous to 139 and 143 (Figure 2I and EV2C). 2) Using Shannon Entropy (Fig EV2B), we show there is structural diversification of these regions and that they occur in E2s with unusual functions, providing evidence that the HTH region is important to study in other E2s (Lines 539-554). 3) The phylogeny shows we are testing E2s broadly across the E2 family tree and not narrowly, and it acts as a good platform to display the HTH charge coloring (Figure EV2A). Similar statements were added to the text.

Also, we have expanded our discussion of conservation of individual residues in specific E2 HTHs throughout the text. Our data, depicted in the form of Weblogos, shows that highly conserved charges in the HTH of individual family members have some critical functions. For example, D143 and R149 are highly conserved in UBE2R, and other residues that we mutated in this region that were less conserved did not show a phenotype, like residue 150. We include reactions of these mutants to help illustrate these points (Figure EV1D and EV3B). These findings are also true of UBE2S, UBE2C, and UBE2D. Furthermore, we provide more direct statements that link amino acid conservation in the HTH to E2 functionality (Lines 230-233).

Other comments:

6. The methods are lacking detail in a number of places. The authors are encouraged to reassess this part of the manuscript.

The methods have been updated to include more details with a specific focus on the NMR and MD experiments.

7. The quality of the biotin-LRLRGG assays shown in Fig 3D and Fig 4B are difficult to interpret due to backgrounds and diffuse bands. It might also be helpful here to show the input biotinylated E2 conjugate.

To improve the quality of these assays, we changed to monitoring a fluorescent-LRLRGG peptide. Even though the peptide was changed, the results remained the largely the same and the data are cleaner (Figure 3E, F). The E2 conjugate band is also presented in the Source Data.

8. Key residues in Fig. 1B should be labeled.

As suggested, the residues are now labeled.

9. The text referring to Fig. 2K is uninformative.

The text for Fig 2J (formerly Fig. 2K) has been expanded for clarity (Lines 233-234).

10. In addition to what is mentioned above, there are assertions in the text that are either overstatements or simply not backed up by data.
"Taken together, this region is capable of functional diversification that can provide specialization for E2 activity." (page 15 lines 388-390)

During the restructuring of the manuscript, this line was removed.

"Overall, we show that the E2 HTH impacts the intrinsic activity of multiple E2s, can be modulated by the E3, and reused for different steps in chain elongation for cell cycle regulation and countless other signaling pathways." (page 6 line 144-147).

This sentence has been toned down as suggested (Lines 151-153).

11. CTP and WHB should be defined.

These acronyms are now defined (Lines 168-9 and 121-123).

12. On page 11 line 268, Km should be Kd.

This specific reference was referring to the kinetic parameters from a previous publication to show that the K_m value of the acceptor Ub from (Liwocha *et al.*, 2021) and the K_D values determined from the BLI from our current study are similar.

13. Fig 3H is uninformative as presented.

Previous Fig. 3H, now Fig. 4D, has been updated for clarity with improved orientation and labeling.

REFERENCES:

Branigan E, Carlos Penedo J, Hay RT (2020) Ubiquitin transfer by a RING E3 ligase occurs from a closed E2~ubiquitin conformation. *Nat Commun* 11: 2846

Bremm A, Freund SM, Komander D (2010) Lys11-linked ubiquitin chains adopt compact conformations and are preferentially hydrolyzed by the deubiquitinase Cezanne. *Nat Struct Mol Biol* 17: 939-947

Brown NG, VanderLinden R, Watson ER, Weissmann F, Ordureau A, Wu KP, Zhang W, Yu S, Mercredi PY, Harrison JS *et al* (2016) Dual RING E3 Architectures Regulate Multiubiquitination and Ubiquitin Chain Elongation by APC/C. *Cell* 165: 1440-1453

Brown NG, Watson ER, Weissmann F, Jarvis MA, VanderLinden R, Grace CRR, Frye JJ, Qiao R, Dube P, Petzold G *et al* (2014) Mechanism of polyubiquitination by human anaphase-promoting complex: RING repurposing for ubiquitin chain assembly. *Mol Cell* 56: 246-260

Garnett MJ, Mansfeld J, Godwin C, Matsusaka T, Wu J, Russell P, Pines J, Venkitaraman AR (2009) UBE2S elongates ubiquitin chains on APC/C substrates to promote mitotic exit. *Nat Cell Biol* 11: 1363-1369

Liwocha J, Krist DT, van der Heden van Noort GJ, Hansen FM, Truong VH, Karayel O, Purser N, Houston D, Burton N, Bostock MJ *et al* (2021) Linkage-specific ubiquitin chain formation depends on a lysine hydrocarbon ruler. *Nat Chem Biol* 17: 272-279

Wickliffe KE, Lorenz S, Wemmer DE, Kuriyan J, Rape M (2011) The mechanism of linkage-specific ubiquitin chain elongation by a single-subunit E2. *Cell* 144: 769-781

Williamson A, Wickliffe KE, Mellone BG, Song L, Karpen GH, Rape M (2009) Identification of a physiological E2 module for the human anaphase-promoting complex. *Proc Natl Acad Sci U S A* 106: 18213-18218

Wu T, Merbl Y, Huo Y, Gallop JL, Tzur A, Kirschner MW (2010) UBE2S drives elongation of K11-linked ubiquitin chains by the anaphase-promoting complex. *Proc Natl Acad Sci U S A* 107: 1355-1360

Thank you for submitting your revised manuscript for our consideration. It has now been assessed once more by original referees 1 and 2, whose comments are copied below. With both of them being satisfied with the revisions and fully supportive of publication, we shall be happy to accept the study after a final round of minor revision, to incorporate the remaining comments/ suggestions of the referees as appropriate.

In addition, I would ask you to address a few editorial points during this final revision:

REFEREE REPORTS

Referee #1:

This manuscript has much improved with interesting added analysis and substantial rewriting, which clarifies the ideas. I would be happy to see this published with the following minor adjustments;

Although the manuscript is much clearer, the abstract does not manage to make the main idea, that HTH can have differential regulatory roles in different E2s, with two possible options worked out in more detail completely clear and it could be good to see if this could be yet more explicitly formulated, rather than going into the detail of a and that particular E2, without mentioning its name.

also please reformulate line 234-235 as they now seem to suggest that Ube2a has been tested.

Referee #2:

In the reviewer's opinion, the authors have done an outstanding job not only addressing my concerns but those for all three reviewers. This paper is well-organized and filled with interesting findings that are relevant not only to members of the ubiquitin field but to anyone who cares deeply about quantitative enzyme mechanism elucidation. I wholeheartedly endorse publication in EMBO and only have one very minor suggestion that I leave to the authors to decide whether to incorporate in their final draft.

In lines 507-511, it is stated " However, why would UBE2SHTH have residues that slow down catalysis? We propose that the APC/C overcomes this defect
as the UBE2SHTH -APC2 interaction limits the conformational space of the donor Ub,enriching the "closed" E2~Ub conformation, the only known active conformation of UBE2S~Ub."

In my mind, this may also represent a mechanism towards keeping E2 activity at bay in the absence of E3 activator. This would prevent the wasteful discharge of E2~UB thioesters to acceptors other than E3-bound substrate. Indeed, CDC34 is greatly activated in the presence of CRLs, albeit through a distinct mechanism.

Point-by-point responses in red:

Referee #1:
This manuscript has much improved with interesting added analysis and substantial rewriting, which clarifies the ideas. I would be happy to see this published with the following minor adjustments;

We would like to thank the reviewer for their careful consideration of our manuscript, and their support for its publication!

Although the manuscript is much clearer, the abstract does not manage to make the main idea, that HTH can have differential regulatory roles in different E2s, with two possible options worked out in more detail completely clear and it could be good to see if this could be yet more explicitly formulated, rather than going into the detail of a and that particular E2, without mentioning its name.

We thank the reviewer for their helpful comments and have rewritten the abstract to accommodate these changes.

also please reformulate line 234-235 as they now seem to suggest that Ube2a has been tested.

We thank the reviewer for pointing out the confusion created by that sentence. It has been edited for clarity, lines 279-281.

Referee #2:
In the reviewer's opinion, the authors have done an outstanding job not only addressing my concerns but those for all three reviewers. This paper is well-organized and filled with interesting findings that are relevant not only to members of the ubiquitin field but to anyone who cares deeply about quantitative enzyme mechanism elucidation. I wholeheartedly endorse publication in EMBO and only have one very minor suggestion that I leave to the authors to decide whether to incorporate in their final draft.

We thank the reviewer for their enthusiastic response!

In lines 507-511, it is stated " However, why would UBE2SHTH have residues that slow down catalysis? We propose that the APC/C overcomes this defect
as the UBE2SHTH -APC2 interaction limits the conformational space of the donor Ub,enriching the "closed" E2~Ub conformation, the only known active conformation of UBE2S~Ub."

In my mind, this may also represent a mechanism towards keeping E2 activity at bay in the absence of E3 activator. This would prevent the wasteful discharge of E2~UB thioesters to acceptors other than E3-bound substrate. Indeed, CDC34 is greatly activated in the presence of CRLs, albeit through a distinct mechanism.

We liked this hypothesis and incorporated these thoughts in the discussion, lines 555-559.

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Roberto Sitia
Journal Submitted to: EMBO Journal
Manuscript Number: 108518R

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	NA
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	NA

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Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Alexa Fluor® 647 anti-mouse IgM Antibody RMM-1. Monoclonal Anti-ERp4436C9. Polyclonal Anti-Halo (Cat.G9281). Goat anti Mouse Anti-lambda-chain HRP Sputhern Biotech (Cat.1060-05)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All the cells that are used in the paper are available in the Sitia's laboratory and they are tested for myc every 2 months

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
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11. Identify the committee(s) approving the study protocol.	NA
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
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