nature portfolio

Peer Review File

Structural Visualization of HECT-type E3 ligase Ufd4 Accepting and Transferring Ubiquitin to Form K29/K48-branched Polyubiquitination

Corresponding Author: Professor Man Pan

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)
Branched ubiquitin chains have emerged as important ubiquitin codes regulating broad biological pathways such as proteasomal degradation. However, the molecular basis how the E3 ligases generate branched ubiquitin chains remains largely elusive. In this manuscript, Wu et al. reports the mechanism of ubiquitin chain branching by revealing the cryo-EM structure of the HECT ubiquitin ligase Ufd4 in complex with K48-linked diUb. Previous studies have reported that yeast Ufd4, as well as the human ortholog TRIP12, specifically assemble K29-linkages and facilitates K29/K48-branched Ub chains. In the current manuscript, Wu et al. demonstrate that Ufd4 adds K29-linked branches off the K48-linkages in vitro. Subsequently, the authors conducted a cryo-EM analysis to elucidate the structure of Ufd4 in complex with K48-linked diUb. The structure revealed that the ARM region and the HECT C-lobe are key structural elements that recruit K48-linked diUb and orient the K29 residue of the proximal Ub to the active cysteine of Ufd4. The authors thus beautifully show how Ufd4 exclusively generates the K29-linked branch off the K48-linkages.

Overall, this is a very interesting study, revealing how the HECT E3 generates branched specific ubiquitin linkages. What is surprising is that Ufd4 directly recognizes K48-linked diUb and orients the K29 of the proximal Ub moiety, thus specifically generating the K29/K48 branched linkage. This is an important finding, and I highly recommend publication of this study in Nature Communications. However, the manuscript should be improved before acceptance.

1. Lines 51-58 in introduction. References are missing for the description of K29 linkage specificity (and branched chain formation) of Ufd4 and TRIP12.

2. As my understanding, Ufd4 and TRIP12 can elongate K29-linked chains, i.e., they may be able to recognize the distal end of the K29-linked chains for further modifications. I was wondering whether Ufd4 can efficiently add a K29-linkage on the distal moiety of the K29-linked chains, or Ufd4 predominantly adds mono-ubiquitins on the K29 residue of K48-linked chains?

3. Related to this, in Fig. 1e, Fig. 1f, and extended Fig. 8c, the generated K29/K48 branched chains are further modified by Ufd4/TRIP12, suggesting that the distal end of the K29 linkage is efficiently modified by Ufd4/TRIP12. The authors should mention about this.

4. The presented data regarding functional analyses (such as in vitro Ub assays) using the interaction surface mutants are important. I recommend some of the key data are moved to the main figures.

5. Ufd4 mutants such as R1468A and H317A are almost defective in generating the K29/K48 branched linkage from K48-diUb (Extended Fig. 5f, 5g, 6c, 6d), while ubiquitylation of model substrates is only partially affected (Extended Fig. 7a). The authors should explain this: perhaps, these mutations may not severely affect the elongation of K29-linkages per se? Moreover, it appears that TRIP12 can modify the substrate (Extended Fig. 7a) de novo, as judged from the mono-ubiquitylated bands.

Reviewer #2

(Remarks to the Author)
This manuscript interrogates the mechanism of branched ubiquitin chain formation by the large HECT E3 Ub ligase Ufd4 and its human homolog TRIP12 through cryo-EM and enzyme assays. Specifically, the group focuses on the conjugation of K29-linked Ub chains onto K48-linked Ub chains by Ufd4. This mechanism is involved in the degradation of N-end substrates in yeast, but many other forms of branched chains are used in many other cellular processes in humans as well. However, uncovering mechanistic insights into the formation of branched chains is a challenge in the field because of the multiple possible mechanisms and dynamic steps of Ub transfer. The group performs detailed biochemistry and structural analysis using chemically linked complexes to investigate the intermediates of Ub transfer from the E2 to the HECT of Ufd4 and the transfer of Ub from the HECT E3 to the K48-linked Ub. Additional data further support that the mechanism is largely conserved in TRIP12. Overall, the group performs several quality assays to match their structures, and I have relatively minor comments.

1. The authors often summarize their findings in the enzyme assays without an interpretation (lines 167-173) into the specific role of these surfaces because they haven't performed kinetic analyses with these mutants. The addition of these data would be helpful to understand the role of each surface in Ub chain formation. For example, can increasing the concentration of the substrate overcome some of the deficiencies made by these mutants that are supposed to bind K48-linked Ub, indicating a Km defect?

2. Certain residues of the Ufd4 homolog TRIP12 should be interrogated to a similar extent as the Ufd4 structure to validate the TRIP12 presented structure.

3. In demonstrating that the C lobe interacts with the donor Ub, it is important to know if these deficits in Ub chain elongation are due to poor Ufd4 loading with Ub rather than the second transfer step onto substrate.

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5. Can the authors speculate as to why the donor Ub is not visible in the TRIP12 structure when Ufd4 makes contacts with the donor? Are the surfaces diverged compared to the K48 Ub chain binding sites?

6. The discussion should be improved. Currently, it is very short and lacks meaningful insights by not comparing their work to other mechanisms of Ub chain elongating enzymes/branching or N-degron substrates. Similarly, they can speculate as to why the chain formation was improved with longer Ub chains?

7. Line 54-56 "Notably, a recent study observed that E3 ligase TRIP12, the human homologue of Ufd4, can promote small-molecule-induced degradation through K29/K48-branched ubiquitin chains." Is missing a reference. I believe the authors are referring to Kaiho-Soma et al.

8. For kinetics, the authors should round off to two significant digits.

9. Type of "door" instead of "donor" in line 255.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In the revised manuscript, the authors have adequately addressed my previous concerns. The manuscript is now suitable for acceptance.

Reviewer #2

(Remarks to the Author)
The authors were highly responsive to reviewer comments and have alleviated any concerns. I support the publication of this manuscript.

Reviewer #1 (Remarks to the Author):

Branched ubiquitin chains have emerged as important ubiquitin codes regulating broad biological pathways such as proteasomal degradation. However, the molecular basis how the E3 ligases generate branched ubiquitin chains remains largely elusive. In this manuscript, Wu et al. reports the mechanism of ubiquitin chain branching by revealing the cryo-EM structure of the HECT ubiquitin ligase Ufd4 in complex with K48-linked diUb. Previous studies have reported that yeast Ufd4, as well as the human ortholog TRIP12, specifically assemble K29-linkages and facilitates K29/K48-branched Ub chains. In the current manuscript, Wu et al. demonstrate that Ufd4 adds K29-linked branches off the K48-linkages in vitro. Subsequently, the authors conducted a cryo-EM analysis to elucidate the structure of Ufd4 in complex with K48-linked diUb. The structure revealed that the ARM region and the HECT C-lobe are key structural elements that recruit K48-linked diUb and orient the K29 residue of the proximal Ub to the active cysteine of Ufd4. The authors thus beautifully show how Ufd4 exclusively generates the K29-linked branch off the K48-linkages.

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Our response: We thank the reviewer for the positive comments.

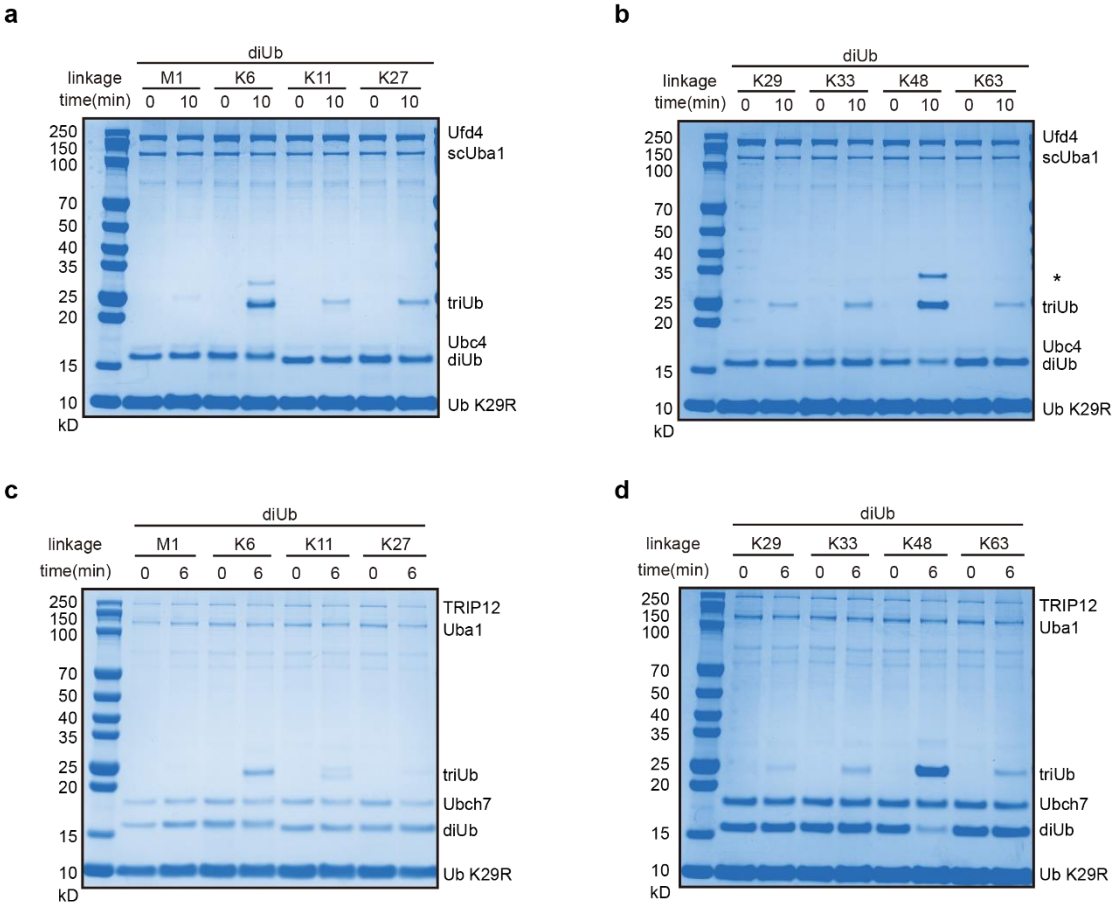
1. Lines 51-58 in introduction. References are missing for the description of K29 linkage specificity (and branched chain formation) of Ufd4 and TRIP12.

Our response: We thank the reviewer for pointing this out. These references have now been added.

2. As my understanding, Ufd4 and TRIP12 can elongate K29-linked chains, i.e., they may be able to recognize the distal end of the K29-linked chains for further modifications. I was wondering whether Ufd4 can efficiently add a K29-linkage on the distal moiety of the K29-linked chains, or Ufd4 predominantly adds mono-ubiquitins

on the K29 residue of K48-linked chains?

Our response:



Rev. Figure 1 Ufd4/TRIP12 mediates ubiquitination using different linkage diubiquitins as substrates.

a, Ubiquitination catalyzed by Ufd4 on M1/K6/K11/K27-linked diubiquitins (diUbs). **b**, Ubiquitination catalyzed by Ufd4 on K29/K33/K48/K63-linked diUbs. The asterisk may indicate a product resulting from Ufd4-mediated ubiquitination, where Ufd4 first generates the K29/K48-branched triUb on the K48-linked diUb substrate and subsequently ubiquitinates the distal ubiquitin of K48-linked diUb. **c**, Ubiquitination catalyzed by TRIP12 on M1/K6/K11/K27-linked diUbs. **d**, Ubiquitination catalyzed by TRIP12 on K29/K33/K48/K63-linked diUbs. These diUbs were prepared in our previous study through chemical protein synthesis or enzymatic methods, as described in our submitted manuscript.

To answer the reviewer's question, we performed biochemical assays using K29-linked or K48-linked diubiquitin (diUb) as substrates for Ufd4-mediated ubiquitination, and employed the Ub K29R mutant as the ubiquitin source to prevent free K29-linked chains generated by Ufd4. We observed that K48-linked diUb were efficiently ubiquitinated, while K29-linked diUb exhibited relatively low ubiquitination efficiency

(**Rev. Figure 1b**). This indicates that Ufd4 preferentially recognizes K48-linked ubiquitin chains as substrates. We further tested Ufd4' s preference for other types of ubiquitin linkages, and found that K48-linked diUb were the most efficiently ubiquitinated substrate (**Rev. Figure 1a/b**). We also performed the same experiments on TRIP12 and also observed its preference for K48-linked diUb (**Rev. Figure 1c/d**). Thus, Ufd4/TRIP12 preferentially adds ubiquitin to the K48-linked ubiquitin chain.

3. Related to this, in Fig. 1e, Fig. 1f, and extended Fig. 8c, the generated K29/K48 branched chains are further modified by Ufd4/TRIP12, suggesting that the distal end of the K29 linkage is efficiently modified by Ufd4/TRIP12. The authors should mention about this.

Our response: We thank the reviewer's suggestion. Indeed, the generated K29/K48 branched chains can be further extended by Ufd4/TRIP12, and we have added this clarification in the manuscript to provide readers with a better understanding.

In main text: "*Meanwhile, the Ufd4-mediated polyubiquitination activity on K48-linked ubiquitin chain substrates was significantly reduced when Ub-K29R mutant was used, supporting K29-linked ubiquitination on K48-linked ubiquitin chain substrates*".

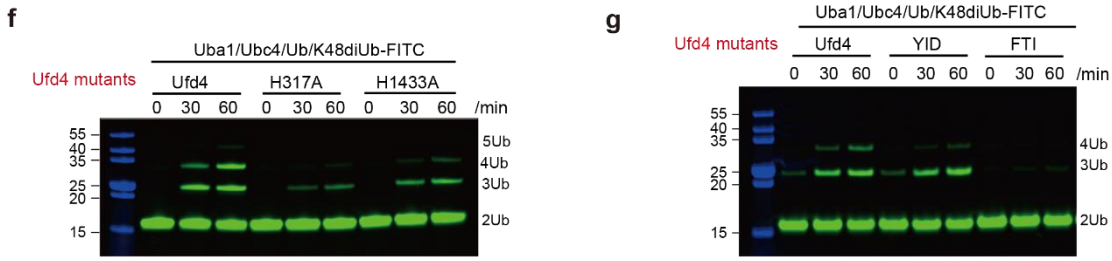
In discussion: "*Additionally, our ubiquitination assays revealed that Ufd4 possesses the capacity to extend K29-linked polyubiquitination on K48-linked ubiquitin chain substrates. However, the structural data presented in this study only describe the mechanism by which Ufd4 initiates the formation of K29/48-branched ubiquitin chains. The structural mechanisms involved in the elongation of these branched chains remain an open question.*"

4. The presented data regarding functional analyses (such as in vitro Ub assays) using the interaction surface mutants are important. I recommend some of the key data are moved to the main figures.

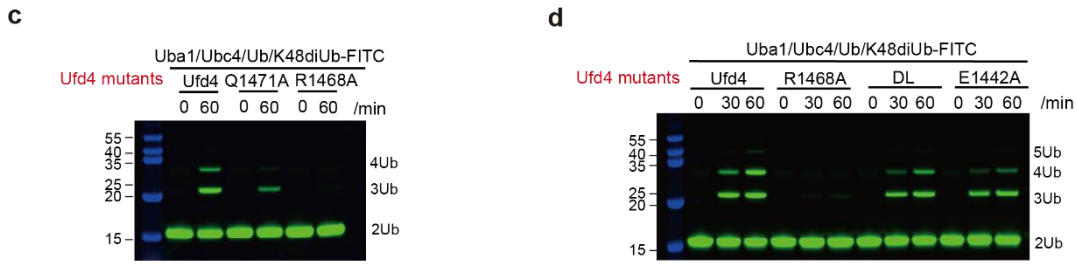
Our response: We thank the reviewer's suggestion. As per your recommendation, we have moved these key data to the main figures.

5. Ufd4 mutants such as R1468A and H317A are almost defective in generating the K29/K48 branched linkage from K48-diUb (Extended Fig. 5f, 5g, 6c, 6d), while ubiquitylation of model substrates is only partially affected (Extended Fig. 7a). The authors should explain this: perhaps, these mutations may not severely affect the elongation of K29-linkages per se?

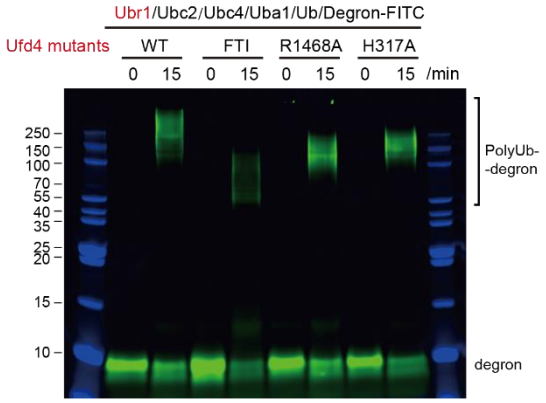
Extended Fig 5



Extended Fig 6



Extended Fig 7a



Our response: We thank the reviewer for the insightful comment. We would like to clarify that the data presented in Extended Fig. 5f, 5g, 6c, and 6d, and in Extended Fig. 7a, correspond to two different ubiquitination experiment setups, which explain the varying ubiquitination intensities and chain lengths observed for the Ufd4 mutants. In Extended Fig. 5f, 5g, 6c, and 6d, **only Ufd4 acts as the E3 ligase for the K48-diUb substrate**. The R1468A and H317A mutants of Ufd4 significantly impair ubiquitination, resulting in almost no detectable modification of the substrate. This result corroborates

our structural observation.

In contrast, in Extended Fig. 7a, **the ubiquitination is mediated by both Ubr1 and Ufd4 as E3 ligases**. The R1468A and H317A mutants of Ufd4 weaken its contribution to substrate ubiquitination, while Ubr1 is still able to ubiquitinate the substrate. Therefore, in Extended Fig. 7a, the overall ubiquitination is only partially reduced, unlike in Extended Fig. 5f, where it nearly disappears.

Despite the explanations provided in the submitted manuscript, we would like to offer additional context regarding the experimental setup for Extended Fig. 7a. Previous studies have shown that both E3 ligases, Ubr1 and Ufd4, contribute to the ubiquitination of substrates (such as type 1 degron and SCC1) via K29- and K48-linked polyubiquitin chains (*Proc. Natl. Acad. Sci. USA, 2009, 106(7): 2142-2147 and Nat. Cell Biol., 2010, 12(12): 1177-1185*). It has been established that Ubr1 can directly mediate K48-linked polyubiquitination, while Ufd4 does not directly mediate ubiquitination but can elongate the polyubiquitin chain and enhance substrate degradation (*Nat. Cell Biol., 2010, 12(12): 1177-1185)*. In this study, we show that Ufd4 preferentially recognizes K48-linked ubiquitin chains and extends them through branched ubiquitination, which deepens our understanding of Ufd4's role in the Ubr1/Ufd4 dual E3 ligase-mediated ubiquitination process. Therefore, in Extended Fig. 7a, we tested the effect of Ufd4 mutants on the overall ubiquitination in the Ubr1/Ufd4 dual E3 ligase system. The results show that the Ufd4 mutants reduce the elongation of the ubiquitin chains on the substrate, indicating that Ufd4-mediated branched ubiquitination plays a crucial role in this dual E3 ligase system.

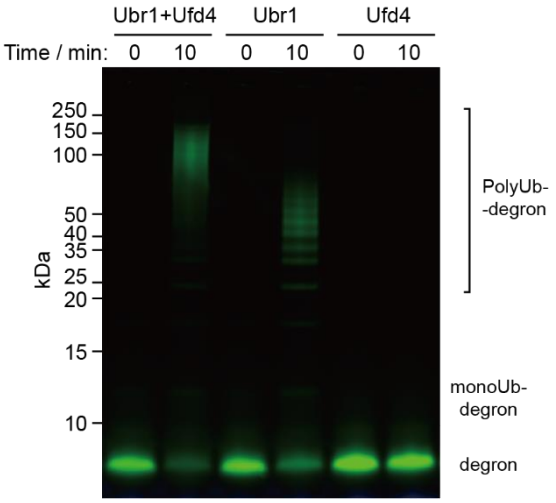
Additionally, the reviewer raised an interesting hypothesis that the R1468A and H317A mutations may not affect Ufd4's ability to elongate K29/K48-branched ubiquitin chains. While this is an intriguing possibility, addressing this biochemical question requires the preparation of structurally precise K29/K48-branched ubiquitin chains, which could serve as substrates for further tests. Unfortunately, the preparation of such samples remains a challenging and unresolved task, which has hindered our ability to investigate this further. On the other hand, the structural data presented in this study only represent the mechanism by which Ufd4 initiates the formation of K29/48-branched ubiquitin chains. In fact, Ufd4 is capable of further elongating these K29/48-branched chains, and understanding the structural mechanism of the elongation of these branched chains could provide valuable insights into the roles of R1468 and H317A during this process. These investigations are part of our future research plans.

6. Moreover, it appears that TRIP12 can modify the substrate (Extended Fig. 7a) de novo, as judged from the mono-ubiquitylated bands.

Our response: We thank the reviewer's comment. However, we did not find any data related to TRIP12 in Extended Fig. 7a, as this figure specifically focuses on Ufd4. It seems the reviewer may have been referring to Ufd4, which aligns with the content presented in Extended Fig. 7a.

As mentioned in our fifth response, we utilized the Ubr1/Ufd4 dual E3 ligase system to catalyze ubiquitination. In this system, previous studies have shown that Ubr1 directly mediates K48-linked polyubiquitination of substrates (such as type 1 degron and SCC1), while Ufd4 does not independently catalyze ubiquitination but instead extends pre-existing polyubiquitin chains (*Nat. Cell Biol., 2010, 12(12): 1177-1185*). Therefore, in Extended Fig. 7a, the mono-ubiquitylated bands are most likely generated by Ubr1.

To further validate this, we conducted ubiquitination assays using type 1 degron as a substrate, the same substrate used in Extended Fig. 7a. Our results showed that Ufd4 alone is unable to ubiquitinate the substrate, and no faint mono-ubiquitylated bands were observed. In contrast, Ubr1 efficiently catalyzes substrate ubiquitination, producing weak mono-ubiquitylated bands similar to those seen in Extended Fig. 7a (**Rev. Figure 2**). These findings strongly suggest that Ufd4 does not catalyze de novo ubiquitination of the substrate type 1 degron.



Rev. Figure 2 Ubiquitination mediated by the Ufd4 and Ubr1 dual E3 ligases.

Reviewer #2 (Remarks to the Author):

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Our response: We thank the reviewer for the positive comments.

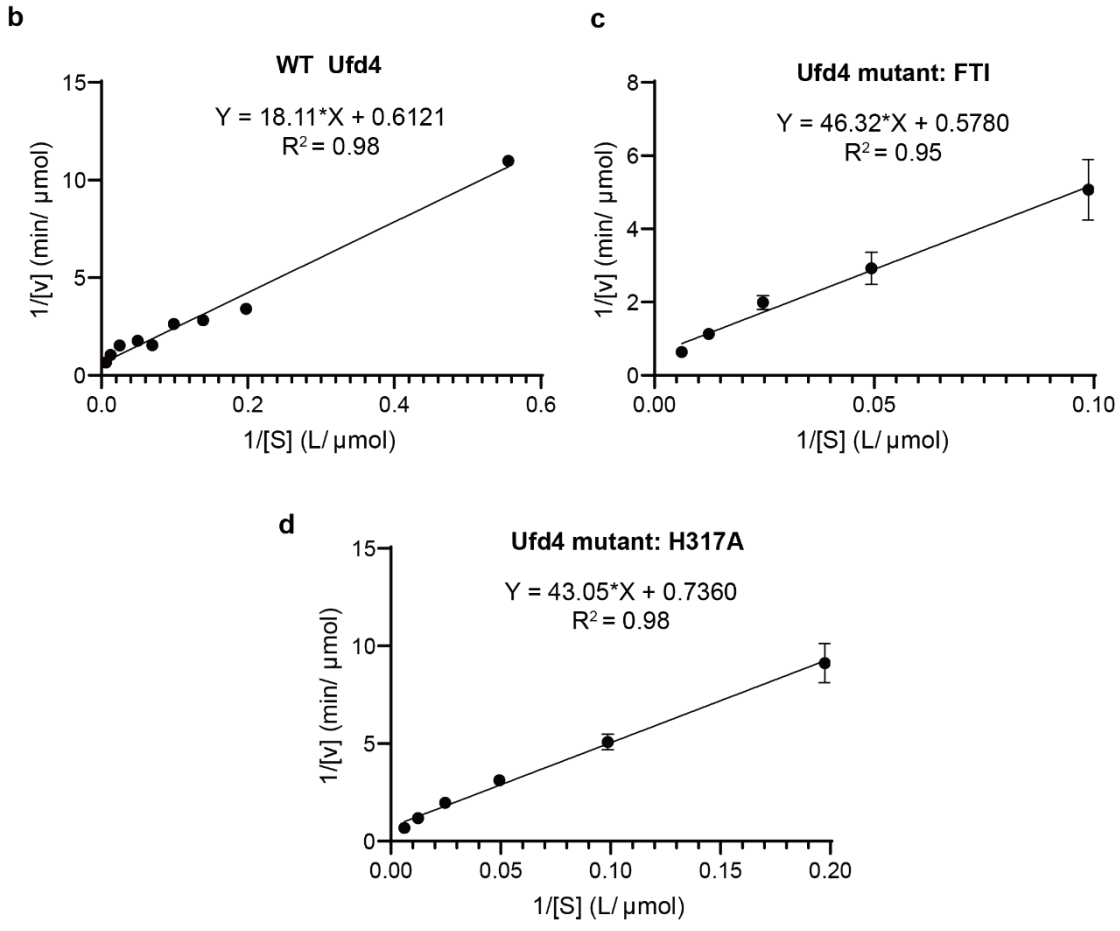
1. The authors often summarize their findings in the enzyme assays without an interpretation (lines 167-173) into the specific role of these surfaces because they haven't performed kinetic analyses with these mutants. The addition of these data would be helpful to understand the role of each surface in Ub chain formation. For example, can increasing the concentration of the substrate overcome some of the deficiencies made by these mutants that are supposed to bind K48-linked Ub, indicating a Km defect?

Our response: We thank the reviewer's valuable suggestion. To further clarify the specific roles of these residues in ubiquitination, we performed the enzyme kinetic analysis on the two mutants (FTI and H317A) that exhibited the most pronounced defects in ubiquitination activity (**Rev. Figure 3)**. Given that these two mutants showed the most significant reduction in activity, we prioritized them for kinetic analysis to gain the most relevant functional insights. Our results showed that, compared to WT Ufd4, both FTI and H317A mutations had minimal impact on the catalytic rate constant (k_{cat}), but led to a significant increase in the Michaelis constant (K_m). This suggests that these residues play a crucial role in substrate binding, rather than in catalysis, which is

consistent with their location at the enzyme-substrate interface in the complex. The increase in Km indicates that higher substrate concentrations can compensate for the binding defects, and at full binding saturation, the activity should be indistinguishable from that of the wild type. These results reinforce the structural observation that these residues are primarily involved in substrate recognition rather than catalysis. Additionally, these data have been incorporated into the main text.

a

Enzyme	k_{cat} (min^{-1})	k_{cat} Fold Change	K_m (μM)	K_m Fold Change
WT Ufd4	3.3	1.00	30	1.00
FTI mutant	3.5	1.06	80	2.71
H317A mutant	2.7	0.82	59	1.98



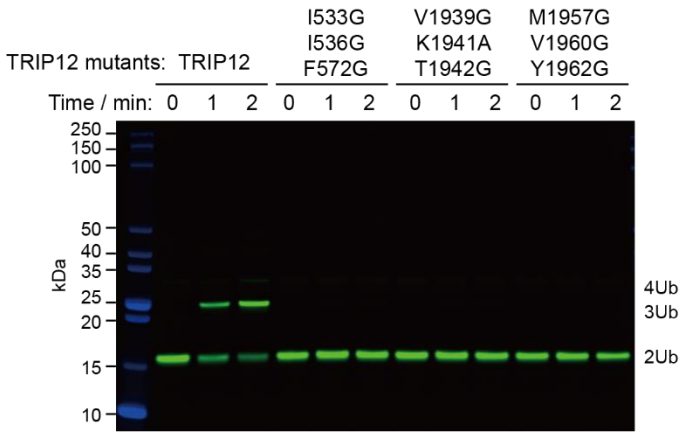
Rev. Figure 3 Enzymatic Kinetic Analysis of Ufd4 and Its Mutants

a, A summary table of kinetic parameters for Ufd4 and its mutants. **b-d**, Double reciprocal Lineweaver – Burk plots of wild-type (WT) Ufd4 (b), Ufd4 FTI mutant (c), and Ufd4 H317A mutant (d).

2. Certain residues of the Ufd4 homolog TRIP12 should be interrogated to a similar extent as the Ufd4 structure to validate the TRIP12 presented structure.

Our response: We thank the reviewer's insightful comment. In our structural analysis of TRIP12 binding to K48-linked diUb, we identified key residues involved in interactions with both the distal and proximal ubiquitins, located in the UICH1/UICH2 regions and the C-lobe. Specifically, residues I533, I536, and F572 in the UICH1 and UICH2 regions interact with hydrophobic patches of the distal Ub, while residues in the C-lobe, including V1939, K1941, T1942, M1957, V1960, and Y1962, engage with the proximal Ub. The C-lobe residues V1939, K1941, and T1942 contribute to recognizing the proximal ubiquitin, interacting with R54 and E18/P19/S20 of the proximal Ub. Additionally, M1957, V1960, and Y1962 in the C-lobe form hydrophobic interactions with N25 of the proximal Ub.

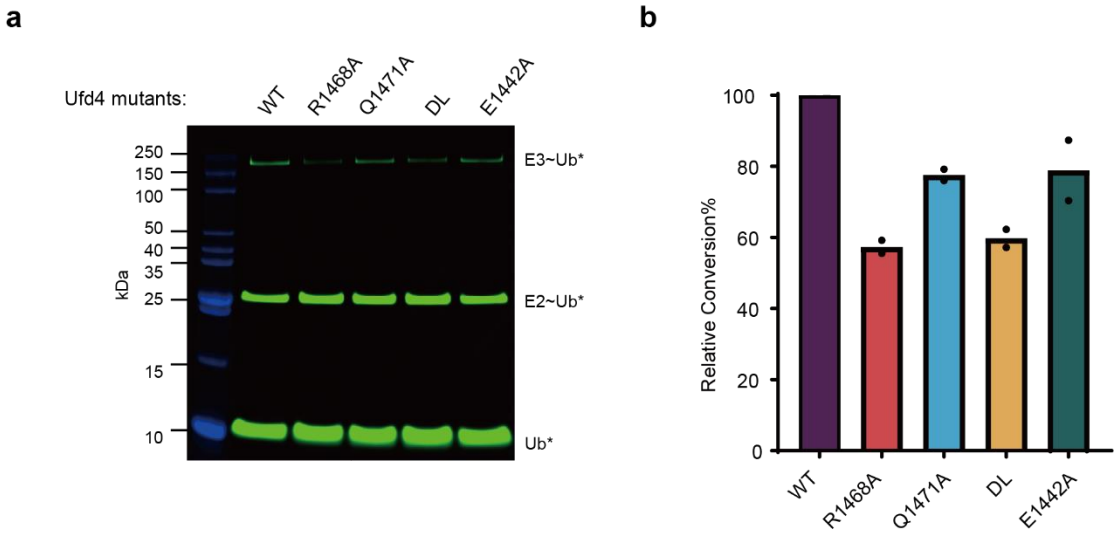
To investigate the functional relevance of these interactions, TRIP12 mutants, including I533G/I536G/F572G, V1939G/K1941A/T1942G, and M1957G/V1960G/Y1962G, were prepared and subjected to ubiquitination assays. Biochemical assays showed that these mutations significantly reduced substrate ubiquitination (**Rev. Figure 4**), highlighting the importance of these interactions in TRIP12's ability to catalyze K29/K48-branched ubiquitination. These results support the structural data and emphasize the role of these conserved residues in the ubiquitination. These data have also been incorporated into the main text.



Rev. Figure 4 A comparison of ubiquitination activity between TRIP12 and its mutants.

3. In demonstrating that the C lobe interacts with the donor Ub, it is important to know if these deficits in Ub chain elongation are due to poor Ufd4 loading with Ub rather than the second transfer step onto substrate.

Our response: We thank the reviewer's insightful suggestion. In the structure of the Ufd4-triUbK29/K48 complex, we identified key residues (D1436, L1438, E1442, R1468, Q1471) in the C-lobe of Ufd4 that interact with the donor Ub and found that mutations in these residues impair substrate ubiquitination. To further investigate whether these interactions affect Ub loading onto Ufd4, we performed E3 ligase Ufd4~Ub thioester formation assays based on previous reports. Our results show that wild-type Ufd4 efficiently loads Ub, while Ufd4 mutants display varying degrees of defects in donor Ub loading (**Rev. Figure 5**). This supports our observation that these interactions play a crucial role in Ufd4's Ub loading and subsequent substrate ubiquitination. These findings have also been integrated into the main text.



Rev. Figure 5 In vitro E3~Ub thioester formation assays.

a, In vitro E3~Ub thioester formation assays using wild-type (WT) Ufd4 and mutants R1468A, Q1471A, D1436A/L1438A (DL), and E1442A. N-terminally fluorescently labeled ubiquitin was used, and the reactions were detected via fluorescence. **b**, The columns of relative E3~Ub thioester formation (%) in (a) are shown, with each data point represented individually.

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Our response: We thank the reviewer's valuable suggestion. In accordance with your recommendation, we have moved these key data to the main figures for better clarity and accessibility.

5. Can the authors speculate as to why the donor Ub is not visible in the TRIP12 structure when Ufd4 makes contacts with the donor? Are the surfaces diverged compared to the K48 Ub chain binding sites?

Our response: We thank the reviewer's insightful question. Sequence alignment reveals significant differences between the residues in Ufd4 that interact with the donor Ub and their corresponding residues in TRIP12 (**Rev. Figure 6**). Among the five identified interaction residues of Ufd4, four are not consistent: Q1471 (Q in Ufd4, I in TRIP12), E1442 (E in Ufd4, D in TRIP12), L1438 (L in Ufd4, E in TRIP12), and D1436 (D in Ufd4, S in TRIP12). These variations in critical residues may weaken the interaction between the C-lobe and the donor Ub, which could increase the flexibility of the donor Ub in the TRIP12 structure, presumably explaining why the donor Ub is not visible in the TRIP12 structure.

Rev. Figure 6 Sequence alignment of Ufd4 and TRIP12, focused on the the C-lobe residues involved in interactions with the donor Ub.

The key residues in Ufd4 that interact with the donor Ub are marked with black circles. The residues that are consistent in TRIP12 are indicated with hollow black circles, while the residues that differ are marked with solid black circles.

6. The discussion should be improved. Currently, it is very short and lacks meaningful insights by not comparing their work to other mechanisms of Ub chain elongating enzymes/branching or N-degron substrates. Similarly, they can speculate as to why the chain formation was improved with longer Ub chains?

Our response: We appreciate reviewer's suggestion to improve the discussion section. We have revised it as below.

"*Prior to proteasomal degradation, substrate proteins with destabilizing residues at the N-terminus (termed as N-degrons) are often recognized by UBR family E3 ligases and ubiquitinated in a K48 linkage-specific manner. This ubiquitination process on N-degron substrates can be further augmented by another E3 ligase Ufd4 (a K29-linkage-specific HECT-type E3 ligase), leading to accelerated substrate degradation. The mechanism of a representative yeast Ubr1-catalyzed process, including the initiation of*

N-degron ubiquitination and the K48 linkage-specific elongation step, was recently elucidated by cryo-electron microscopy (cryo-EM) studies in our group. Nonetheless, the mechanism through which Ufd4 introduces K29-linked Ub chains on substrates to increase the processivity of polyubiquitination remains unknown. Here, our biochemical and mass spectrometry experiments clarified key aspects of the molecular mechanism of Ufd4, where Ufd4 preferentially catalyzes branched ubiquitination on K48-linked ubiquitin chains, thereby elongating the ubiquitin chain. To gain structural insight into this process, we designed a mechanism-based chemical trapping strategy to study the Ufd4-specific ubiquitination processes, specifically the transfer of Ub from the E2 enzyme to the E3 ligase, and subsequently from the E3 ligase to the substrate. This strategy combined with cryo‐electron microscopy (cryo‐EM) provides valuable mechanistic insights into how Ufd4 mediate the K29/K48-branched ubiquitination and enhances our understanding of the catalytic mechanism underlying HECT E3 ligases. Meanwhile, our findings contribute to a deeper understanding of linkage-specific polyubiquitin chain assembly by providing mechanistic details specific to K29-linked ubiquitination, thereby complementing previous structural studies on M1-, K48-, and K63-linked ubiquitin chains. Interestingly, the human homologue of Ufd4, TRIP12, also preferentially assembles K29/K48-branched Ub chains through a conserved structural mechanism, suggesting an evolutionarily conserved function across species.

A variety of structural elements responsible for acceptor ubiquitin recognition have been identified within the catalytic domains of E2 and E3 enzymes, playing a critical role in assembling ubiquitin chain. For instance, a flexible loop in the E2 enzyme UBE2R2 is reshaped by the CRL RING domain to precisely position the acceptor ubiquitin within the catalytic site. Likewise, a zinc finger motif embedded in the RING2 domain of the RBR-type E3 ligase RNF216 engages the acceptor ubiquitin, thereby ensuring the selective generation of K63-linked ubiquitin chains. Despite these insights, the contribution of non-catalytic regions in E2 and E3 enzymes to acceptor ubiquitin recognition remains poorly understood. A recent structural study on the HECT-type E3 ligase UBR5, which specifically assembles K48-linked ubiquitin chains, revealed that its N-terminal UBA domain binds an acceptor ubiquitin, emphasizing the crucial role of non-catalytic domains in dictating ubiquitin chain assembly. In this study, we found that the non-catalytic N-terminal ARM domain of Ufd4/TRIP12 cooperates with the HECT catalytic domain to precisely position the acceptor ubiquitin, aligning its Lys29 toward the Ufd4/TRIP12 active site. Although the N-terminal ARM domain may play a secondary role in this process, its involvement further underscores the emerging concept that non-catalytic regions in ubiquitin ligases contribute to ubiquitin chain assemble. Moving forward, it will be essential to investigate ubiquitin chain assembly at the level of full-length ubiquitination enzymes rather than focusing solely on their catalytic domains. Such studies will provide deeper insights into the role of non-catalytic domains in acceptor ubiquitin recognition.

Currently, structural studies on homotypic ubiquitin chain assembly primarily focus on the formation of diUb chains, where only the acceptor ubiquitin unit needs to be recognized for chain elongation. In contrast, a structural model for the generation of longer homotypic ubiquitin chains has been established mostly through the structural

study of the UBE2K/RNF38 RING domain, which catalyzes the formation of K48-linked triUb from K48-linked diUb. In this model, similarly, only the acceptor (distal) ubiquitin is recognized, while the proximal ubiquitin remains unbound. This raises the intriguing question of whether branched ubiquitin chain formation follows a similar recognition pattern. Specifically, in the case of branched ubiquitination on a diUb substrate, does the recognition of the proximal ubiquitin alone suffice to initiate the branching process? Here, we found that for Ufd4/TRIP12, when K48-linked diUb serves as the substrate, both the proximal and distal ubiquitins are recognized simultaneously. This suggests that, at least in the case of Ufd4/TRIP12, unlike homotypic chain elongation, which relies solely on the recognition of the distal ubiquitin, the formation of branched ubiquitin chains requires the recognition of multiple ubiquitins. Building on this, we developed a working model for branched ubiquitin chain assembly, highlighting the key distinctions between homotypic and branched ubiquitin chain formation. This observation raises the question of whether the recognition of multiple ubiquitins for the position of the acceptor ubiquitin is a common feature among E3 ligases catalyzing branched ubiquitination, or if it is unique to Ufd4/TRIP12? We anticipate that future structural studies on other branched ubiquitin ligases will provide further insights."

7. Line 54-56 "Notably, a recent study observed that E3 ligase TRIP12, the human homologue of Ufd4, can promote small-molecule-induced degradation through K29/K48-branched ubiquitin chains." Is missing a reference. I believe the authors are referring to Kaiho-Soma et al.

Our response: We thank the reviewer for pointing this out. We indeed intended to reference the study by Kaiho-Soma et al., and it has now been added.

8. For kinetics, the authors should round off to two significant digits.

Our response: We thank the reviewer's suggestion. We have rounded the kinetics data to two significant digits as recommended.

9. Type of "door" instead of "donor" in line 255.

Our response: We thank the reviewer for pointing out the typo. We have corrected it.