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Recognition of the CCT5 di-Glu degron by CRL4DCAF12 is dependent on TRiC assembly

Carlos Pla-Prats, Simone Cavadini, Georg Kempf, and Nicolas Thomä
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Thank you for submitting your study on CRL4-DCAF12 degron recognition in monomeric CCT5 to The EMBO Journal. It has now been assessed by three expert referees, whose comments are copied below for your information. I am happy to say that all reviewers appreciate the interest and timeliness of the findings and the general quality of the work, and that we would therefore be interested in pursuing the work further for publication. As you will see, the reports do still bring up a number of specific concerns and queries for clarification, which I would invite you to address in a revised version of the manuscript.

Referee #1:

The manuscript by C. Pla-Prats et al. presents structural and functional characterization on the recognition of the CCT5 C-terminal di-Glu degron by CRL4DCAF12 E3 ligase. They determine the cryo-EM structures of the DDB1-DCAF12-CCT5 complex and the DDB1- DCAF12 complex, and their structural and biochemical analyses indicate the molecular determinants of CCT5 di-Glu degron recognition. They also show that DCAF12 binds and ubiquitinates monomeric CCT5, instead of TRiC complex. Their study suggests a role for the CRL4DCAF12 E3 ligase in overseeing the assembly of the key molecular folding machinery TRiC. The overall topic is very interesting, still, the cryo-EM map and model quality need to be validated, and the structural analysis need to be better presented. Here are my comments and questions the authors need to address.

1) The authors claim their DDB1-DCAF12-CCT5 map has been resolved to 2.8 Å resolution. From the model-map fitting illustrated in Fig. S4B, it is hard to see high-resolution structural features. Please show such high-resolution features including especially the side chain model- map fitting details. Also, for Fig. 3A, it is hard to tell the fitting quality in the DCAF12-CCT5 interaction interface. Please illustrate the model-map fitting for DCAF12 and the CCT5 C- terminal 5 residues in this interface, respectively.

2) For the cryo-EM analysis of the DDB1-DCAF12-CCT5 complex, please explain why there is no indication of presence of a more complete CCT5, even in the 2D class averages. If it is due to the dynamics of the bound CCT5, the authors are suggested to perform 3D variability analysis in cryoSPARC to capture the presence of CCT5.

3) For the negative staining EM (NS-EM) map determination of the DDB1-DCAF12-CCT5 complex, please add a supplementary figure to illustrate the data processing process, especially representative reference-free 2D class averages to illustrate the presence and orientation of the associated CCT5. Also, in the NS-EM map, does the position of CCT5 allows its C-terminus to fit in the orientation obtained through the corresponding cryo-EM map?

4) Please show the detailed cryo-EM data collection, processing, and model quality in Table S1.

Minor points:

1) L.118, please define "ATTO488".

2) L. 298-299, the authors describe that "CCT5 adopts a curved shape formed by equatorial (EQ), middle (MD) and apical (AP) domains .....". In TRiC structure, the three domains are usually defined as equatorial (E), intermediate (I), and apical (A) domains.

Referee #2:

Because signal transduction relies on both transient and stable protein interactions, all organisms have evolved quality control pathways that monitor proper complex formation. While the importance of such assembly quality control has been clearly established, the underlying molecular mechanisms are still very incompletely understood and more structural and biochemical work is needed. Here, the authors present strong structural data that implicates the CRL4-DDB1-DCAF12 E3 ligase, known to function in C-end rule mediated degradation, as an assembly QC enzyme that could detect a chaperonin subunit in its free, but not complexed, state.

The authors focused their elucidation of the CRL4-DDB1-DCAF12 complex onto the candidate substrate CCT5, a subunit of the metazoan TRiC chaperonin. CCT5 contains a C-terminal di-Glu degron that they find is directly recognized by DCAF12 with decent (although maybe not high, as suggested by the authors) mid-nanomolar affinity. Having reconstituted the complex between DDB1-DCAF12 and a CCT5 C-terminal peptide, the authors proceeded to solve its cryo-EM structure, thereby revealing the mechanistic basis of C-end rule recognition. DCAF12 possesses multiple positively charged surface patches that are all essential for binding and thus read out combinatorically the carboxy-terminus and the gamma-carboxy functions of the most critical C-terminal Glu-residue in the -2 position. They noted some flexibility in recognition of the Glu side chain in the -1 position, likely explaining some variability in the C-terminal residue among DCAF12 substrates. The authors then show by a combination of negative stain EM using full-length CCT5 and published structures of assembled TRiC that both the C-terminus in CCT5 as well as the surface of CCT5 oriented towards DCAF12 are shielded from E3 ligase recognition by assembly of the TRiC complex. Using fully assembled TRiC, they accordingly found that DCAF12 can bind and ubiquitylate free CCT5, but not the CCT5 protein that is part of TRiC. This finding suggests that CUL4-DDB1-DCAF12 is a quality control E3 ligase that detects free CCT5, potentially as a consequence of aberrant or abortive TRiC assembly.

The biochemistry and structural biology reported in this paper are beautiful. The experiments have been designed and interpreted clearly and the findings are very important - they provide insight into C-end rule specificity and point towards a biological function of this pathway. I have, in fact, no criticism with respect to the data of this paper and would not ask the authors for any revision with respect to their experiments.

While the authors focus their discussion on a potential role of DCAF12 as an assembly QC enzyme, it stands to reason whether this is the key role of the E3 ligase in cells. They provide arguments that also for other targets, C-terminal complex formation might modulate recognition by DCAF12. However, it is not known whether the substrates investigated here are essential DCAF12 targets in cells. It could very well be that a crucial target that drove DCAF12 evolution uses a different mechanism (for example, unleashing a C-terminus that might be bound in cis as a response to a signaling event important for cell function or homeostasis). As distinguishing between such possibilities or identifying such a target will be a study in itself that requires very different technologies, I would not ask them to perform such experiments. However, I propose that the authors should include a discussion of potential alternative functions of DCAF12 in their paper.

Referee #3:

In the manuscript 'Recognition of the CCT5 di-Glu degron by CRL4DCAF12 is incompatible with TRiC 3 assembly', Carlos et al. detail in the recognition mechanism of di-Glu-containing substrates by DCAF12 by presenting the cryo-EM structure of the DDB1-DCAF12-CCT5 complex. They found that DCAF12 serves as a canonical WD40 DCAF substrate receptor and uses a positively charged pocket at the center of the β-propeller to bind the C-terminus of CCT5. Specifically, di-Glu motif of CCT5 displays a decisive role for the interaction. While subsequent results verified that DCAF12 only binds and ubiquitinates monomeric CCT5, and CCT5 in assembled TRiC complex is not competent for binding to DCAF12. Thus they concluded that a structurally intact TRiC complex therefore protects CCT5 from recognition by the CRL4DCAF12 E3 ligase.

Overall, most of the work is well supported by the data. This study represents an important contribution to our understanding of how CRL4DCAF12 targets di-Glu substrates. Despite these considerable strengths, there are several key areas for improvement of the manuscript.

Major comments:
1. The authors indicated that DCAF12 His144, Arg203 and Lys254 formed a positively charged patch for the stabilization of gamma carboxyl group of C-terminal Glu, this interaction seems just electrostatic attraction without forming of any direct hydrogen bond or salt bridge interactions. As the structural similarity of Asp or Gln with Glu, we are curious about whether the -1 position can be substituted by these two residues. Additionally, the authors mentioned that DCAF12 also binds Glu-Thr or Glu-Leu degron. Please titrate the mutant peptides of -1 position substituted by Asp, Gln, Thr and Leu against TbDDB1-DCAF12488, which is of importance for the analysis of specific selectivity of C-terminal Glu for DCAF12 binding.
2. In the competition experiments of different lengths of CCT5 degron peptides, the authors tested the truncation of degron peptide from 20 to 2 amino acids. They found that truncating the degron peptide to 5 residues brought a sharp decline of the binding affinity (~80-fold) comparing with the 10 residues peptide. They should narrow the gap of sequence length between 5-10 residues, such as addition of 6 or 8 residue peptide, to further confirm the minimum length of CCT5 peptide for DCAF12 binding.
3. Line 163, the authors mentioned that 'Among the residues preceding the C-terminal glutamates, the largest effects were seen for Glu538 (position -4; IC50 = 571 {plus minus} 103 nM), Pro536 (position -6; IC50 = 417 {plus minus} 53 nM) and Arg534 (position -8; IC50 = 395 {plus minus} 68 nM) (Fig 1D).'. All of these binding affinities are comparable with that of wild type CCT510 (IC50 = 390 {plus minus} 115 nM), thus this statement of 'largest effects' is not accurate and should be rewritten. However, we found that -3 or -5 substitution of CCT510 peptide by Ala showed a ~3-fold increasing of the binding, indicating some extent preference of these two positions. Please analyse this case and give a rational interpretation.
4. Line 263, the authors mentioned that 'At the base of the pocket, Arg344 further contributes to substrate binding through interactions with the CCT5 peptide backbone (Fig 3A and C). '. What kind of interactions here refer to?
5. The authors demonstrated that CRL4DCAF12 can effectively ubiquitinate monomeric CCT5, and it showed no ubiquitination activity towards TRiC. While whether this ubiquitination would lead to degradation of CCT5? Please verify this with cellular experiments, such as GPS assays or other similar experiments.
6. The authors uses time-resolved fluorescence energy transfer assay (TR-FRET) to monitor binding of a CCT5 C-terminal peptide to DDB1-DCAF12. The data indicated that the Kd for the 488CCT520 peptide was 215{plus minus}135 nM. The error value exceeds to half of the base value, and we think it is not exact enough, please redetermine this measurement.

Minor comments:
Some description is redundant and repetitive, such as '50 nM DDB1-DCAF12, 2 nM Tb-SA, 400 nM 488CCT520 (TbDDB1-DCAF12488) complex', please simplify related description or transfer the specific description into the Method section.

PLA-PRATS C, CAVADINI S, KEMPF G, THOMÄ NH
"Recognition of the CCT5 di-Glu degron by $CRL4^{DCAF12}$ is dependent on TRiC assembly"

RESPONSE TO THE REVIEWERS

REVIEWER #1: The manuscript by C. Pla-Prats et al. presents structural and functional characterization on the recognition of the CCT5 C-terminal di-Glu degron by CRL4DCAF12 E3 ligase. They determine the cryo-EM structures of the DDB1-DCAF12-CCT5 complex and the DDB1- DCAF12 complex, and their structural and biochemical analyses indicate the molecular determinants of CCT5 di-Glu degron recognition. They also show that DCAF12 binds and ubiquitinates monomeric CCT5, instead of TRiC complex. Their study suggests a role for the CRL4DCAF12 E3 ligase in overseeing the assembly of the key molecular folding machinery TRiC. The overall topic is very interesting, still, the cryo-EM map and model quality need to be validated, and the structural analysis need to be better presented. Here are my comments and questions the authors need to address.

Author comment: We thank the reviewer for their positive comments on our manuscript and helpful feedback, and have addressed their questions below.

Major comments:
1) The authors claim their DDB1-DCAF12-CCT5 map has been resolved to 2.8 Å resolution. From the model-map fitting illustrated in Fig. S4B, it is hard to see high-resolution structural features. Please show such high-resolution features including especially the side chain model- map fitting details. Also, for Fig. 3A, it is hard to tell the fitting quality in the DCAF12-CCT5 interaction interface. Please illustrate the model-map fitting for DCAF12 and the CCT5 C- terminal 5 residues in this interface, respectively.

Author response: We thank the reviewer for pointing this out. We have adapted our structural figures (Fig 2B, Fig EV2B, Fig EV4B, Fig EV5B) to better reflect the resolution of the map, and we have shown the density in Fig 3A as surface to facilitate analysis by the reader. We have also made a new figure to illustrate the DCAF12 model-map fitting and the CCT5 C-terminus interface which can be found in the Appendix (Appendix Fig S6).

2) For the cryo-EM analysis of the DDB1-DCAF12-CCT5 complex, please explain why there is no indication of presence of a more complete CCT5, even in the 2D class averages. If it is due to the dynamics of the bound CCT5, the authors are suggested to perform 3D variability analysis in cryoSPARC to capture the presence of CCT5.

Author response: We thank the reviewer for their suggestion. 3D classification were performed at several steps along the processing workflow for the 2.8 and 3.0 Å cryo-EM maps, but they did not reveal the presence of CCT5. Substantial efforts were directed at improving the completeness of the map, and over a dozen samples were analyzed by cryo-EM. The particles were homogeneous, and overpicking of particles did not reveal a subpopulation of particles with visible CCT5 regardless of whether the dataset was refined through 2D classification prior to 3D classification. We only once saw a 2D class of free DDB1 as evidence of broken particles. We believe that CCT5 is recognized almost exclusively through its C-terminus (Fig 1C; was Fig 5A at first submission) and retains significant flexibility when bound to DCAF12. We note that cross-linking the sample prior to EM analysis also did not show additional signal for CCT5. Signal for CCT5 was not observed regardless of whether 3D variability analysis was performed with RELION or cryoSPARC.

3) For the negative staining EM (NS-EM) map determination of the DDB1-DCAF12-CCT5 complex, please add a supplementary figure to illustrate the data processing process, especially representative reference-free 2D class averages to illustrate the presence and orientation of the associated CCT5. Also, in the NS-EM map, does the position of CCT5 allows its C-terminus to fit in the orientation obtained through the corresponding cryo-EM map?

Author response: A figure with the data processing workflow for the negative-stain DDB1-DCAF12-CCT5 structure has been added to the manuscript. The figure (Appendix Figure S4) is made to be comparable to Figures EV4 and EV5 that detail the cryo-EM processing workflows. Regarding the CCT5 C-terminal tail, binding of the CCT5 equatorial domain largely covers the pocket, but leaves an opening around DCAF12 β-propeller blade 6, at the entrance of the pocket. The mode of binding observed in the negative-stain structure allows the CCT5 C-terminus to fit in the pocket. Although this was briefly mentioned in the text, we have expanded our description of the binding mode and made a figure to illustrate it that can be found in the Appendix (Appendix Fig S5).

4) Please show the detailed cryo-EM data collection, processing, and model quality in Table S1.

Author response: A table is incorporated into the manuscript (Table EV1; Cryo-EM data collection, refinement and validation statistics) that shows the detailed cryo-EM data collection, processing and validation statistics for both 2.8 and 3.0 Å cryo-EM structures. Table EV2 similarly presents the equivalent parameters for the negative-stain structure.

Minor points:
1) L.118, please define "ATTO488".

Author response: We are not aware of an alternative name for the fluorescent dye ATTO488. It appears to belong to a series of fluorescent labels named after the parent company (ATTO-TEC GmbH) and wavelength in nm of the maximum fluorescent emission. We have reworded our introduction to the label to be clearer to readers (lines 120-124). It now reads: "The resulting $_{Tb}$DDB1-DCAF12 complex was mixed with a peptide corresponding to the 20 C-terminal amino acids of CCT5 (^{488}CCT5$_{20}$; CCT5 amino acids 522-541) conjugated to the fluorescent label ATTO488, which contains the di-Glu motif and acts as a fluorescence acceptor".

2) L. 298-299, the authors describe that "CCT5 adopts a curved shape formed by equatorial (EQ), middle (MD) and apical (AP) domains .....". In TRiC structure, the three domains are usually defined as equatorial (E), intermediate (I), and apical (A) domains.

Author response: We thank the reviewer for pointing this out. We have corrected the name of the domain in the manuscript (line 306) and in the related figures (Fig 2A, Fig 4A, Appendix Fig S5).

REVIEWER #2: Because signal transduction relies on both transient and stable protein interactions, all organisms have evolved quality control pathways that monitor proper complex formation. While the importance of such assembly quality control has been clearly established, the underlying molecular mechanisms are still very incompletely understood and more structural and biochemical work is needed. Here, the authors present strong structural data that implicates the CRL4-DDB1-DCAF12 E3 ligase, known to function in C-end rule mediated degradation, as an assembly QC enzyme that could detect a chaperonin subunit in its free, but not complexed, state.

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Author response: We thank the reviewer for their very positive comments on our manuscript and helpful feedback. As per their suggestion, we have added a discussion on the potential alternative functions of DCAF12 in the manuscript (Discussion; lines 434-441). It now reads: "It is conceivable, however, that the evolution of DCAF12 might have been driven by a substrate whose degradation is independent of assembly into a complex. Recognition might follow the allosteric release of a C-terminal tail in response to a post-translational modification. DCAF12 might also act on specific splicing isoforms or products of caspase cleavage, and have ubiquitin-independent functions. In drosophila, it has recently been shown that the pro-apoptotic functions of DCAF12 are partially underlain by its non-degradative inhibition of inhibitor of apoptosis proteins (IAPs), which do not contain di-Glu degrons".

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Major comments:
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Author response: We have performed the suggested experiment and incorporated the results into a new figure panel (Figure EV1F; described in lines 164-166). We found that the affinity between Glu541Leu and Glu541Ala mutants was similar, and higher than the affinity of the polar mutants (Glu541Gln, Glu541Thr). We also found that a Glu541Asp mutant had the lowest affinity of all the peptides, below that of polar and hydrophobic substitutions and additional Glu540Asp and Glu541Lys mutant peptides that we designed. This suggests a more complex recognition code than previously anticipated.

2. In the competition experiments of different lengths of CCT5 degron peptides, the authors tested the truncation of degron peptide from 20 to 2 amino acids. They found that truncating the degron peptide to 5 residues brought a sharp decline of the binding affinity (~80-fold) comparing with the 10 residues peptide. They should narrow the gap of sequence length between 5-10 residues, such as addition of 6 or 8 residue peptide, to further confirm the minimum length of CCT5 peptide for DCAF12 binding.

Author response: We have performed the suggested experiment and incorporated the results into the manuscript (Figure 1D; described in lines 144-151). We found the affinities of the $CCT5_6$ and $CCT5_8$ peptides were very close to that of the $CCT5_5$ and $CCT5_{10}$ peptides respectively, indicating that a big increase in binding affinity occurs between 7 and 8 residues. This is now described in the text as follows: "We observed maximal binding when the C-terminal CCT5 peptides were eight residues or longer (Fig 1D). Truncating the degron peptide to six residues or less impaired binding, such that the $^{488}CCT5_{20}$ probe was not fully outcompeted at a concentration of 12.5 μM. Only traces of binding were observed for a CCT5 di-peptide at 12.5 μM, our highest tested experimental concentration (Fig 1D). The sequence features of di-Glu degrons were initially identified in peptides of at least ten residues in length. Our measurements thus show that a sequence context of seven to eight residues is sufficient for di-Glu degron binding".

3. Line 163, the authors mentioned that 'Among the residues preceding the C-terminal glutamates, the largest effects were seen for Glu538 (position -4; IC50 = 571 {plus minus} 103 nM), Pro536 (position -6; IC50 = 417 {plus minus} 53 nM) and Arg534 (position -8; IC50 = 395 {plus minus} 68 nM) (Fig 1D).'. All of these binding affinities are comparable with that of wild type CCT510 (IC50 = 390 {plus minus} 115 nM), thus this statement of 'largest effects' is not accurate and should be rewritten. However, we found that -3 or -5 substitution of CCT510 peptide by Ala showed a ~3-fold increasing of the binding, indicating some extent preference of these two positions. Please analyse this case and give a rational interpretation.

Author response: We thank the reviewer for pointing this out. While we see a clear and reproducible trend in the rank-order of these peptides, it is absolutely correct that the differences are small and that the errors are overlapping. We have therefore acknowledged the magnitude of these differences and the increase in binding affinity after alanine mutations in the -3 and -5 positions particularly, re-writing the paragraph as follows (lines 166-180): "Mutations in the amino acids preceding the C-terminal glutamates did not exhibit equally pronounced effects {{referring to mutations in the C-terminal glutamates}} when mutated to alanine, and displayed different behaviors (Fig 1E). Peptides mutated in degron positions -4 (Glu538Ala; IC_{50} = 571 ± 103 nM), -6 (Pro536Ala; IC_{50} = 417 ± 53 nM) and -8 (Arg534Ala; IC_{50} = 395 ± 68 nM) displayed similar affinities than the wild type sequence (WT; IC_{50} = 363 ± 78 nM), while mutations in positions -3 (Ser539Ala; IC_{50} = 125 ± 15 nM), -5 (Gly537Ala; IC_{50} = 100 ± 11 nM), -7 (Lys535Ala; IC_{50} = 208 ± 26 nM), -9 (Ile539Ala; IC_{50} = 209 ± 25 nM) and -10 (Glu532Ala; IC_{50} = 222 ± 25 nM) gave rise to slightly better binding when mutated to alanine (Fig 1E). Taken together, our measurements confirm that degron binding is driven by the C-terminal glutamates and highlight the importance of the -2 degron position for binding. We find that DCAF12 shows only moderate preference for individual degron residues preceding the C-terminal glutamates, in line with degradation reporters in cells that show little effect for mutations N-terminal of the di-Glu motif. However, the increased binding of alanine mutants of degron positions -3, -5, -7, -9 and -10 suggest that the CCT5 C-terminus is not the optimal di-Glu degron sequence bound by $CRL4^{DCAF12}$ ". In our structure, the -3 and -5 positions are close to the ceiling, which is formed by hydrophobic residues that drive interactions with the DCAF12 Loop (Fig EV3F). Assuming that the peptide chain trajectory remains unchanged, we speculate that alanine residues in the -3 and -5 positions interact favorably with ceiling amino acids Leu440, Pro441 and Phe411.

4. Line 263, the authors mentioned that 'At the base of the pocket, Arg344 further contributes to substrate binding through interactions with the CCT5 peptide backbone (Fig 3A and C). '. What kind of interactions here refer to?

Author response: We thank the reviewer for pointing out the lack of clarity in our writing. DCAF12 Arg344 interacts through electrostatic interactions with the backbone carbonyl between CCT5 Glu540 and Glu541. This interaction persists during the (minimal) rotation of the CCT5 peptide backbone that accompanies the flexibility of the Glu541 side chain, and is therefore expected to persist with amino acid substitutions of the -1 degron position.

5. The authors demonstrated that CRL4DCAF12 can effectively ubiquitinate monomeric CCT5, and it showed no ubiquitination activity towards TRiC. While whether this ubiquitination would lead to degradation of CCT5? Please verify this with cellular experiments, such as GPS assays or other similar experiments.

Author response: We thank the reviewer for their suggestion, which is well taken. The study by Koren I. *et al*. that is referenced throughout our manuscript identified degrons recognized by DCAF12 by carrying out GPS reporter assays in cells. They first identified proteasomally degraded proteins *in vivo*, and then used targeted E3 ligase disruption to assign degradation activity for each reporter construct to a specific E3 ligase, reaching the conclusion that DCAF12 downregulated protein constructs that ended in a di-Glu motif. They showed DCAF12-mediated degradation for reporters containing a CCT5 C-terminal peptide and the full length

CCT5 protein. The *in vivo* ubiquitin-dependent degradation of CCT5 by DCAF12 has therefore already been demonstrated. Building on that study, we now provide a molecular rationale for the CRL4^{DCAF12} ubiquitination activity, and propose a biological role for the CRL4^{DCAF12} ligase in AQC. A study by Elliot K.L. *et al.* (https://doi.org/10.1091/mbc.E15-01-0048) indicates that recombinantly fusing GFP to the N-terminus of CCT5 prevents TRiC assembly and renders CCT5 monomeric *in vivo*. We believe, in fact, that the degradation of the overexpressed GFP-CCT5 reporter constructs reported by Koren I. *et al* reflects the degradation of monomeric CCT5. Engineering a GFP-CCT5 construct that maintains TRiC assembly and CRL4^{DCAF12} binding and ubiquitination is non-trivial. We believe that, additionally, the difficulty of establishing the right controls would yield GPS reporter experiments inconclusive. We thus consider this experiment outside the scope of this work.

6. The authors uses time-resolved fluorescence energy transfer assay (TR-FRET) to monitor binding of a CCT5 C-terminal peptide to DDB1-DCAF12. The data indicated that the Kd for the 488CCT520 peptide was 215{plus minus}135 nM. The error value exceeds to half of the base value, and we think it is not exact enough, please redetermine this measurement.

Author response: We thank the reviewer for their observation. We have repeated the measurements, obtaining a value for the affinity of 245 {plus minus} 52 nM. The new data has been incorporated into the results section (line 129 and Fig 1B, as well as the associated K_i value in the main text (line 134)).

Minor comments:
Some description is redundant and repetitive, such as '50 nM DDB1-DCAF12, 2 nM Tb-SA, 400 nM 488CCT520 (TbDDB1-DCAF12488) complex', please simplify related description or transfer the specific description into the Method section.

Author response: We thank the reviewer for their suggestions to improve the clarity of our manuscript. We have kept the qualitative descriptions of the $_{Tb}$DDB1-DCAF12 and $_{Tb}$DDB1-DCAF12^{ATTO488} complexes in the main text, which we believe help understand the described experiments, but have restricted the detailed concentration information to the methods section (lines 526-541).

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that in light of the positive re-reviews copied below, we have now accepted it for publication in The EMBO Journal.

Referee #1:

The authors have addressed my comments and questions in a satisfactory way. I would suggest publication of the manuscript.

Referee #3:

The authors largely addressed our comments on their manuscript. I agree with the publication of the revised manuscript on EMBO Journal.

EMBO Press Author Checklist

Corresponding Author Name: Nicolas H. Thomä
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2022-112253

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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Not Applicable	

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Not Applicable	
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

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Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgments

Design

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If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Materials and Methods
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure legends

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Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
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Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
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Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

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State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Yes	Throughout the manuscript
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data Availability
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Yes	Throughout the manuscript