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K27-linked ubiquitylation promotes p97 substrate processing and is essential for cell proliferation

Robert Shearer, Dimitris Typas, Fabian Coscia, Sofie Schovsbo, Thomas Kruse, Andreas Mund, and Niels Mailand
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Corresponding author(s): Niels Mailand (niels.mailand@cpr.ku.dk) , Dimitris Typas (dimitrios.typas@cpr.ku.dk)

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Thank you again for submitting your manuscript, together with anonymous reviews from a previous journal, to The EMBO Journal. I have now received the input of three arbitrating referees of our own choice, who have looked at the original reports as well as your responses to them, and who had also been asked to comment more generally on the latest version. As you will see from the below-copied feedback, the arbitrating referees were divided in their opinions, with only referee 3 considering the study ready for publication at the present stage; referee 1 remained unsupportive for a number of reasons explained in their report, while referee 2 acknowledged the potential importance and interest of the work but listed several major concerns that would in their view need to be addressed to make the study more insightful and a more compelling candidate for The EMBO Journal.

I have now further discussed these reports with my colleagues, as well as with the arbitrators cross-commenting on each others reports. Unlike referee 1, we continue to see value in this work in principle, and do not consider all of his/her points as overriding issues precluding publication. Nevertheless, the remaining major reservations from two trusted experts prevent us from offering expedited publication with only minor revision, as in some other cases of arbitration on transferred manuscript. I would therefore understand if you in this light favored to seek rapid publication without major changes elsewhere. Still, should you be able to strengthen your conclusions and deepen the mechanistic insight with some additional experiments and more decisive data, as requested/suggested by arbitrating referee 2, we would remain happy to pursue this study further for The EMBO Journal. If you are interested in this opportunity, I would encourage you to get back to me with a tentative response to arbitrating referees 1 and 2, and a revision plan detailing how their comments (especially those of referee 2) could be addressed. We might then directly discuss further proceedings on this basis.

Thank you again for the opportunity to consider this work for The EMBO Journal. I am sorry that the previous review and revisions did not facilitate a more straightforward decision process in this case, but hope you find the arbitrator comments helpful and look forward to hearing from you.

REFEREE REPORTS

Referee #1:

This paper uses a ubiquitin replacement strategy originally developed by James Chen's lab to test for potential functions of K27-linked chains. They report defects in cell cycle progression and Ub-GFP degradation, potentially through dysregulation of p97/VCP. This paper had been seen by unknown reviewers who criticized both experimental issues, but also questioned the novelty of this work.

Previous reviews criticized that this paper does not provide much insight into the function of K27-linked chains, and I agree with this notion. The link to p97 was originally made upon observing p97-cofactors as proteins that are less ubiquitylated in K27R-cells. However, even the authors admit that the p97 co-factors are unlikely to be modified with K27-linked chains (i.e. their modification is indirectly impacted by the ubiquitin mutation; also, whether the modification has a function is unclear). How K27R-ubiquitin interferes with cell cycle progression is unclear, and there are no substrates reported in this paper. As a consequence, there is no direct evidence that a cellular protein is modified with K27-linked chains. The biology of K27-linked ubiquitylation remains weak.

The authors suggest that the model substrate Ub-GFP is modified with K27-linkages. However, I am confused about this finding, as earlier work by the Varshavsky and Li labs had indicated that Ub-GFP is modified with K29- and K48-linkages. The authors do not discuss this discrepancy to earlier work, and they do not show convincing data that Ub-GFP is actually modified with K27-linked chains, and not with a few K27-linkages buried within a largely K29- and K48-rich conjugate. Notably, their apparent

substrate trap (UCHL3-CS) seems to mostly associate with Ub-GFP, but not with a substrate modified with substantial K27-linked chains.

The authors responded to criticism by overexpressing a disease mutant p97, which they refer to as "hyperactive". They state that this hyperactive p97 overexpression did not rescue the phenotypes of K27R-ubiquitin expression. However, at least in animal models, these p97 disease mutants are loss of functions, likely caused by a defect in exchanging adaptor proteins (thus, although the mutant hydrolyses ATP more rapidly, it is impaired in dealing with many substrates that require adaptor exchange to gain access to p97). I therefore doubt that this experiment is helpful.

My biggest concern had not been raised by the previous reviewers, i.e. that K27 of ubiquitin is buried. Modification of this site thus requires rather strong conformational changes in ubiquitin, and the K27R mutation is likely destabilizing. The authors argue against this issue (in their discussion), but they never probe whether ubiquitin-K27R is less stable than wildtype ubiquitin (in vitro, through thermal unfolding) or whether ubiquitin-K27R is degraded more rapidly in cells than wildtype ubiquitin (by pulse chase in E1 inhibited cells). A recent paper in Cell Chem Biol (Kudriaeva et al., Cell Chem Biol. 28, 1192-1205 (2021)) actually shows that the K27R mutation is destabilizing. In this absence of a clear substrate modified with K27-linked chains, the potential destabilizing effect of this mutation raises the possibility that the phenotypes observed in this paper are secondary in nature.

Referee #2:

In this manuscript, Shearer et al. provide new insight into the occurrence and function of K27 Ub-Ub linkages. Although some functions for K27-polyUb in mammalian cells have been reported (e.g., roles in DNA damage repair, innate immunity, and regulation of PTEN), the mechanisms involved, other possible functions, and if K27-polyUb promotes proteasomal degradation are unknown. K27-polyUb is rare relative to other Ub-Ub linkage types and few tools have been available to study it. Here, Shearer et al. have described the refinement and validation of new experimental approaches to investigate K27-polyUb. They have shown that K27-polyUb is predominantly nuclear, is essential for cell viability and cell cycle progression, and that K27-polyUb is needed for p97-dependent proteasomal degradation of the artificial substrate Ub(G76V)-GFP. Inhibition of p97 recapitulates phenotypes when K27-polyUb formation is prevented by Ub replacement with Ub(K27R) or when its recognition is blocked by competition with the K27-specific binding protein UCH-L3(C95S).
The conclusion that K27-polyUb and p97 are epistatic is unexpected, important, and well-supported by multiple experiments. Overall, though, the paper is a bit of a mixed bag. The successful adoption and careful validation of the Ub-replacement strategy to eliminate K27 linkages are impressive, as is the use of the K27-specific binder UCHL3(C95S) described recently by van Tilburg et al.; the authors' use of UCHL3(C95S/D33A) as a negative control is particularly clever. Other very positive aspects of the work are the finding that K27-polyUb is predominantly nuclear, and the discovery of new phenotypes associated with K27-linkage depletion. However, the paper comes up short in the quality of the evidence used to decipher - or, at least, to constrain - models for how K27-polyUb is involved in p97-dependent pathways. Specific issues are elaborated below.
1. A general problem is that the label-free mass spec analyses are subject to sample-to-sample and instrumental variations; the need to normalize peak intensities to other peptides compounds opportunities for error. A more reliable approach would be to use isotopic labeling (e.g., TMT labeling).
(a) Ub-Ub linkages were not quantified directly; rather, changes in specific linkages were inferred by quantifying peptides corresponding to the unconjugated Ub. Thus, it was concluded from Fig 1H&I that the Ub(K27R) replacement had little effect on K48 or K63 linkages because the amounts of Ub not linked at those positions were relatively unchanged. However, particularly for relatively minor linkage types, the precision of those assays may not be sufficient to detect changes of potential significance. For example, K63-linked Ub already is a minor fraction of the total Ub (~38% of polyUb was reported by Dammer et al., which would be ~19% of total Ub), so even a 25% change in abundance of that linkage type would be difficult to discern. The analysis should have quantified the linkages directly by analyzing abundances of the Ub peptides containing GG on lysine sidechains, as was done in Fig. 3D. Moreover, quantitation of the other Ub-Ub linkages should be included. These analyses would be facilitated by peptide enrichment with anti-GG-K antibody, a common practice in Ub proteomics.
(b) In Fig. 5I, shouldn't the diGly peptide intensities be normalized to GFP peptide(s)? Doing so would substantially decrease the apparent increases in K27 associated with p97 and proteasome inhibition.
2. The key conclusion that the degradation substrate Ub(G76V)-GFP is itself modified by K27-polyUb hinges on the results in Fig. 5C&I. For the reason indicated above, the interpretation of the data in Fig. 5I is questionable. That leaves as supporting evidence the finding that Ub(G66V)-GFP together with a higher MW band are pulled-down with the K27-polyUb binding protein, UCHL3(C95S). Although it might not have been stated explicitly, the higher MW band presumably corresponds to K27-linked Ub-Ub(G76V)-GFP. An experiment should be done to test that: Does a K27R mutation in the substrate (i.e., Ub(K27R,G76V)-GFP) prevent its degradation and pull-down of UCHL3(C95S)?
Without additional data, the statement (p. 18) that turnover of ubiquitylated proteins "...is at least partially mediated by K27-linked ubiquitylation of p97 substrates" is not warranted.
3. In Fig. 2H, the data for Wee1i treatments are too sparse to be conclusive - even if WT and K27R-replacement cells do behave differently, the scatter and paucity of data points are too great to expect statistical significance.
4. The authors speculate (p. 19) that p97 might facilitate K27-ubiquitination by partially (and presumably, transiently) unfolding Ub to expose the K27 to E3 Ub ligases. That seems inconsistent with their observation that K27-polyUb is promoted by p97 inhibition (e.g., Fig. 5C).

5. (p. 14,15; Fig. 5; Fig. S5A,B) The authors have used catalytically-inactive versions of UCHL5 and other DUBs as negative controls for UCHL3(C95S). This really doesn't make much sense to me. Even the structurally closest relative, UCHL5, is very dissimilar to UCHL3 with respect to activity, substrate specificity, and localization (UCHL5 associates with 26S proteasomes). The experiments and results using inactive UCHL5 and other DUBs contribute little to the study and probably should be deleted from the paper.
6. How do the authors reconcile the absence of K27-polyUb in p97 cofactors (e.g., UFD1 and p47) with the mass spec results in Fig. 3E? Were the "stringent" anti-HA IP conditions not completely denaturing?
Some minor issues:
7. (p. 13, line 4) Perhaps change the word "likely" to "possibly" - I'm not convinced that proteasome-associated DUBs are responsible.
8. (p.17) The statement that "...Ub(K27R)-replaced cells show unchanged levels of most other Ub linkage topologies and ubiquitylation processes..." is incorrect; the effects on only a few linkage types were assessed.
9. (p. 27) "StageTipping" isn't a word.
10. (p. 34, Fig. 1G legend) "Lys-C digestion" is written here but wouldn't yield the peptide indicated; trypsin probably was meant.
11. For Fig. 3E, it would be helpful to explain the criteria used to select the protein hits highlighted in the figure.

Referee #3:

This is a well-executed study -specifically this revised version- and it makes a strong case for the importance of K27-linked ubiquitin (Ub) chains in human cell cycle progression. It also tightly links K27 Ub chain formation to p97/VCP action on Ub conjugates using both epistasis analyses of K27-Ub replacement with p97 inhibitor treatment as well as between overexpressed UCHL3-C95S, a K27-chain-specific binder, and p97 inhibition. The latter provide strong corroborative data for the Ub replacement experiments.

In the revision, the interpretative focus has shifted to substrate modification by K27 chains, rather than p97 cofactor modification, and this is underscored with direct mass spectrometric identification of K27 linkages on a p97-proteasome substrate and correlative data linking K27 chains with p97 processing and not proteasome action per se. The authors did an excellent job responding to the reviewer comments, including some detailed experimental suggestions from original Reviewer #2. These changes have greatly improved the manuscript.

There are now many questions to answer regarding how exactly Ub-K27R causes G2 cell cycle arrest and how K27 chains on substrates and/or p97 cofactors work in K27 chain-mediated physiological pathways such as replication. This paper makes these interesting questions to ask, and they should be addressable in future studies. I believe the data in the current paper are excellent and the experiments well controlled, and support publication in the EMBO Journal.

Referee #1:

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In our opinion, the notion that Ub(G76V)-GFP is modified with K29- and K48-linked Ub chains does not exclude that this substrate could also be modified by other Ub linkage types, as there are many examples of proteins being modified with a range of different Ub linkages. Indeed, our unbiased mass spectrometry (MS) analysis of Ub(G76V)-GFP immunoprecipitated under stringent buffer conditions to prevent the co-purification of other proteins confirmed that the Ub(G76V)-GFP model substrate is modified wit both K48- and K29-linked ubiquitylation as shown in previous work (which we refer to in the manuscript), but also demonstrated that K27-linkages are formed on this substrate (Figure 4F in the revised manuscript). It is clear from this analysis that K29- and K48-linkages on Ub(G76V)-GFP are more abundant than K27-linkages (which we point out in the text), and it is indeed possible that Ub(G76V)-GFP is not modified by extensive K27-linked chains. However, this does not rule out that short K27-linked chains on a substrate could be functionally important. In the revised manuscript, we included new data strengthening the notion that Ub(G76V)-GFP is directly modified by K27-linked ubiquitylation (new Figure 4G). Consistent with our MS data, we found that the ubiquitylated forms of Ub(G76V)-GFP were recognized by the partially specific Ub-K27 antibody (new Figure 4G; compare lanes 1 and 2). Importantly, a K27R mutation within Ub(G76V)-GFP (Ub(K27R,G76V)-GFP), which prevents ubiquitylation at K27 within this model substrate but not overall K27-linkage formation, selectively reduced the reactivity of the Ub-K27 antibody with the band corresponding to Ub(G76V)-GFP monoubiquitylated at K27 but not slower-migrating ubiquitylated forms of Ub(G76V)-GFP (new Figure 4G; compare lanes 2 and 3). By contrast, expressing Ub(G76V)-GFP in Ub(K27R)-replaced cells, which allows Ub(G76V)-GFP ubiquitylation at

K27 but not K27-linked extensions of Ub modifications on Ub(G76V)-GFP, strongly reduced all but the Ub(G76V)-GFP monoubiquitylation band recognized by the Ub-K27 antibody (new Figure 4G; compare lanes 2 and 4). This band was stronger than the corresponding band in Ub(WT)-replaced cells, consistent with the impaired processing of ubiquitylated forms of Ub(G76V)-GFP in Ub(K27R)-replaced cells and the inability of these cells to extend this initial K27-linkage on Ub(G76V)-GFP into longer K27-linked chains. When Ub(K27R,G76V)-GFP was expressed in Ub(K27R)-replaced cells, thereby preventing all K27-linkage formation on this substrate, little reactivity with the Ub-K27 antibody was observed (new Figure 4G; lane 5). Together with our MS data, these findings demonstrate that the Ub(G76V)-GFP model p97 substrate is targeted for K27-linked ubiquitylation impacting both the K27 residue in Ub(G76V) as well as Ub moieties attached to other lysines in Ub(G76V)-GFP. The modification of Ub(G76V)-GFP with K27-linkages is consistent with the co-purification of UCHL3 C95S but not K27-linkage binding-deficient UCHL3 C95S/D33A in Ub(G76V)-GFP IPs, and the lack of UCHL3 C95S binding to the variant Ub(G76V)-GFP-20AA substrate argues that this K27-linkage-specific binder does not associate with unmodified Ub(G76V) (Figure 5C,I).

The authors responded to criticism by overexpressing a disease mutant p97, which they refer to as "hyperactive". They state that this hyperactive p97 overexpression did not rescue the phenotypes of K27R-ubiquitin expression. However, at least in animal models, these p97 disease mutants are loss of functions, likely caused by a defect in exchanging adaptor proteins (thus, although the mutant hydrolyses ATP more rapidly, it is impaired in dealing with many substrates that require adaptor exchange to gain access to p97). I therefore doubt that this experiment is helpful.

The reviewer is correct in pointing out that the phenotypes seen in animal models of disease-associated p97 mutants, which have elevated ATPase and unfoldase activity *in vitro* (Blythe *et al*, 2019), are complex in nature and not solely due to excessive p97 ATPase activity. Our data show that the disease-associated p97 A232E (MSP) mutant accelerates the turnover of the Ub(G76V)-GFP model substrate in cells as would be expected from a hyper-active p97 protein (Figure S5G in the original manuscript). However, we cannot rule out that overexpression of p97 A232E may adversely impact other p97-driven cellular processes, and we concur that it may thus be difficult to draw unambiguous conclusions from experiments involving the p97 MSP mutant. We have therefore removed the data obtained with this mutant (Figure S5G-I in the original submission) from the revised manuscript.

My biggest concern had not been raised by the previous reviewers, i.e. that K27 of ubiquitin is buried. Modification of this site thus requires rather strong conformational changes in ubiquitin, and the K27R mutation is likely destabilizing. The authors argue against this issue (in their discussion), but they never probe whether ubiquitin-K27R is less stable than wildtype ubiquitin (in vitro, through thermal unfolding) or whether ubiquitin-K27R is degraded more rapidly in cells than wildtype ubiquitin (by pulse chase in E1 inhibited cells). A recent paper in Cell Chem Biol (Kudriaeva et al., Cell Chem Biol. 28, 1192-1205 (2021)) actually shows that the K27R mutation is destabilizing. In this absence of a clear substrate modified with K27-linked chains, the potential destabilizing effect of this mutation raises the possibility that the phenotypes observed in this paper are secondary in nature.

This is a valid concern in principle. However, we would like to point out that prompted by the study mentioned by the referee, we did in fact address this issue by comparing the stability of ectopic Ub(WT) and Ub(K27R) proteins in our Ub replacement cell lines using the protein synthesis inhibitor cycloheximide (CHX) (Figure S1J in the original manuscript). This data showed that total Ub abundance is reduced to a similar extent in Ub(WT)- and Ub(K27R)-replaced cells upon CHX treatment, suggesting that at least in our Ub replacement cells the stability of the ectopic Ub(WT) and Ub(K27R) proteins is not markedly different. Notably, in line with this notion, we show that the levels of most Ub linkage topologies and cellular ubiquitylation processes remain virtually unaffected by Ub(K27R) replacement (Figure 1I; new Figure EV2C; Figure 3E; Dataset EV1), which would not be expected if the Ub(K27R) protein was destabilized to an extent that impairs its general functionality and ability to support cellular ubiquitylation processes. It is also worth pointing out that cells expressing a sub-endogenous level of Ub(WT) display a full rescue of the proliferation defect arising from depletion of endogenous Ub, unlike the effect of Ub(K27R) expressed at a comparably higher level (Figure EV2F,G). This further indicates that the phenotypes seen in cells expressing the Ub(K27R) are unlikely to reflect decreased stability of the Ub(K27R) mutant relative to Ub(WT). Finally, the use of an orthogonal, Ub mutant-independent approach for blocking recognition of K27 linkages in cells via UCHL3-C95S overexpression, which we show phenocopies the impaired p97 substrate processing seen in Ub(K27R)-replaced cells (Figure 5; Figure EV5), further strengthens the notion that this and other phenotypes of Ub(K27R) replacement cells are not simply a secondary consequence of reduced stability of the Ub(K27R) protein. Notwithstanding these considerations, we carried out additional experiments to further probe the stability of the Ub(WT) and Ub(K27R) proteins in our Ub replacement cells. In time course experiments, we observed no visible difference in the decline of Ub(WT) and Ub(K27R) conjugates upon CHX treatment (new Figure EV1K, replacing Figure S1J in the original manuscript). In addition, we followed the reviewer's suggestion to test whether unconjugated Ub(K27R) is degraded more rapidly than Ub(WT) in E1-inhibited Ub-replaced cells. Under these conditions, we also observed no noticeable destabilization of Ub(K27R) (see Figure R1 below). It should be noted that combined CHX and E1i treatment induced extensive and rapid cell death in our hands, precluding us from assessing time points beyond 3 hours of treatment. Combined, these data show that in our Ub replacement cells the stably expressed Ub(WT) and Ub(K27R) proteins show no pronounced difference in stability.

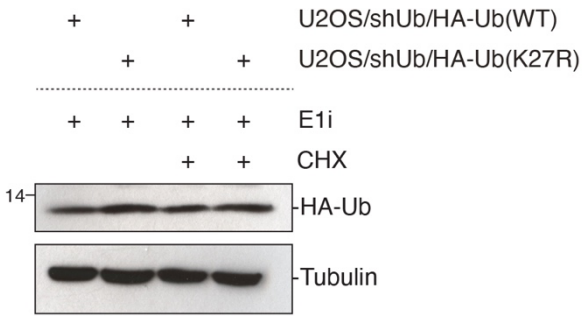

Figure R1.
Analysis of Ub(WT) and Ub(K27R) stability in E1-inhibited Ub replacement cells
U2OS/shUb/HA-Ub(WT) and U2OS/shUb/HA-Ub(K27R) cells grown in the presence of DOX for 72 h to induce Ub replacement were incubated with Ub E1 inhibitor (E1i) and, where indicated, CHX for 3 h and processed for immunoblotting with HA and Tubulin antibodies. Data are representative of three independent experiments with similar outcome.

Referee #2:

In this manuscript, Shearer et al. provide new insight into the occurrence and function of K27 Ub-Ub linkages. Although some functions for K27-polyUb in mammalian cells have been reported (e.g., roles in DNA damage repair, innate immunity, and regulation of PTEN), the mechanisms involved, other possible functions, and if K27-polyUb promotes proteasomal degradation are unknown. K27-polyUb is rare relative to other Ub-Ub linkage types and few tools have been available to study it. Here, Shearer et al. have described the refinement and validation of new experimental approaches to investigate K27-polyUb. They have shown that K27-polyUb is predominantly nuclear, is essential for cell viability and cell cycle progression, and that K27-polyUb is needed for p97-dependent proteasomal degradation of the artificial substrate Ub(G76V)-GFP. Inhibition of p97 recapitulates phenotypes when K27-polyUb formation is prevented by Ub replacement with Ub(K27R) or when its recognition is blocked by competition with the K27-specific binding protein UCH-L3(C95S).
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1. A general problem is that the label-free mass spec analyses are subject to sample-to-sample and instrumental variations; the need to normalize peak intensities to other peptides compounds opportunities for error. A more reliable approach would be to use isotopic labeling (e.g., TMT labeling).

We acknowledge that isotopic labeling approaches such as TMT labeling have been widely used in the Ub field in combination with di-Gly enrichment strategies (e.g. (Udeshi *et al*, 2020)). However, label-free approaches are now being increasingly employed, and their suitability for in-depth profiling of the Ub system has been clearly demonstrated in a number of recent papers (e.g. (Hansen *et al*, 2021; Steger *et al*, 2021)). In our opinion, one distinct advantage of the label-free approach is that it is better suited than TMT labeling for covering a large dynamic range, which is highly relevant in the context of this study considering the extensive differences in abundance of individual Ub linkages and Ub-modified proteins. Our rationale was to quantify changes in Ub linkages and the ubiquitylation state of individual proteins within the same experiment, for which we would argue using label-free analysis is appropriate. Indeed, we observed very low sample-to-sample variation between individual replicates; to better illustrate this, we have added correlation plots for the label-free mass spectrometry experiments showing strong reproducibility between individual replicates (average Pearson correlation of 0.96) (new Figure EV3D). It should also be mentioned that we verified several aspects of the mass spectrometry data, incl. the unaltered levels of K48- and K63-linked Ub conjugates and loss of ubiquitylation of factors including XPC, RAD23, p47 and UFD1 upon Ub(K27R) replacement (Figure EV1I,J; Figure 3F,G), further supporting the validity of our proteomic approach.

(a) Ub-Ub linkages were not quantified directly; rather, changes in specific linkages were inferred by quantifying peptides corresponding to the unconjugated Ub. Thus, it was concluded from Fig 1H&I that the Ub(K27R) replacement had little effect on K48 or K63 linkages because the amounts of Ub not linked at those positions were relatively unchanged. However, particularly for relatively minor linkage types, the precision of those assays may not be sufficient to detect changes of potential significance. For example, K63-linked Ub already is a minor fraction of the total Ub (~38% of polyUb was reported by Dammer et al., which would be ~19% of total Ub), so even a 25% change in abundance of that linkage type would be difficult to discern. The analysis should have quantified the linkages directly by analyzing abundances of the Ub peptides containing GG on lysine sidechains, as was done in Fig. 3D. Moreover, quantitation of the other Ub-Ub linkages should be included. These analyses would be facilitated by peptide enrichment with anti-GG-K antibody, a common practice in Ub proteomics.

We apologize if the intended purpose and our interpretation of the data originally shown in Figure 1H,I (now Figure 1H; Figure EV1G in the revised manuscript) was not clear. We would like to emphasize that we do not conclude based on these data (showing total intensity of Ub peptides spanning the K48 and K63 residues) that K48-Ub and K63-Ub linkages remain relatively unchanged upon Ub(K27R) replacement. We only use these data to substantiate that the total abundance of Ub in cells is not significantly changed upon the DOX-induced replacement of endogenous Ub with Ub(K27R) in the Ub(K27R) replacement cells. This complements the data in Figure 1G showing a gradual exchange of the WT peptide spanning the K27 residue in Ub with the corresponding mutant K27R peptide upon DOX treatment of Ub(K27R) replacement cells, thus providing an important validation of this experimental system. The accompanying text in the manuscript reads as follows (page 7): *"Importantly, successful DOX-induced replacement of endogenous Ub with Ub(K27R) in U2OS/shUb/HA-Ub(K27R) cells was verified by mass spectrometry (MS), showing a progressive exchange of the tryptic peptide spanning Ub-K27 with the corresponding mutant K27R peptide, while the levels of other Ub-derived peptides remained essentially unchanged (Figure 1G,H; Figure EV1G)".* We think the confusion may have been caused by the way we labeled the Ub peptides in these panels, which has now been changed to indicate the residues spanned by these peptides while not giving the impression that they correspond to diGly-modified peptides. As pointed out by the reviewer, direct quantification of Ub linkage abundance (as determined by the abundance of di-Gly modifications on individual Ub lysine residues) was originally provided in Figure 3D. Considering the reviewer's point, we decided to move this data into Fig. 1 (now Figure 1I in the revised manuscript). As requested by the referee, we also included data on the abundance of di-Gly modifications on K33 in Figure 1I, showing that Ub(K27R) replacement has no significant impact on the level of K33-linked Ub conjugates. The Ub(K27R) substitution presented technical challenges to the quantification of di-Gly modifications on the neighboring K29 residue that is present within the peptide containing the K27R mutation. To quantify K29-linkage abundance, we therefore instead took advantage of a recently published specific affinity reagent for K29-linked Ub chains, sAB-K29 (Yu *et al*, 2021). This not only verified the specificity of this reagent for detecting K29-linkages in cells by showing a near-complete loss of signal in Ub(K29R)-replaced cells, but also demonstrated that the abundance of K29-linked Ub chains remains largely unaffected by (Ub(K27R) replacement (new Figure EV2C). In addition to quantifying diGly modifications on individual Ub lysines, we used antibodies specific to K48- and K63-linked Ub conjugates as an independent approach to validate our mass spectrometry data that the abundance of these linkages remains largely unchanged upon Ub(K27R) replacement (Figure EV1I,J).

Collectively, these data show that except for the expected strong decline in K27-linked ubiquitylation, Ub(K27R) replacement has little impact on the abundance of other Ub linkage types.

(b) In Fig. 5I, shouldn't the diGly peptide intensities be normalized to GFP peptide(s)? Doing so would substantially decrease the apparent increases in K27 associated with p97 and proteasome inhibition.

We agree and have normalized the diGly peptide intensities to GFP peptides as suggested by the reviewer. From this it can be seen that K27-linked ubiquitylation of Ub(G76V)-GFP is increased moderately (approx. two-fold) upon inhibition of p97 but not the proteasome, an effect that follows a similar trend as K29-linkages (Figure 4F in the revised manuscript).

2. The key conclusion that the degradation substrate Ub(G76V)-GFP is itself modified by K27-polyUb hinges on the results in Fig. 5C&I. For the reason indicated above, the interpretation of the data in Fig. 5I is questionable. That leaves as supporting evidence the finding that Ub(G66V)-GFP together with a higher MW band are pulled-down with the K27-polyUb binding protein, UCHL3(C95S). Although it might not have been stated explicitly, the higher MW band presumably corresponds to K27-linked Ub-Ub(G76V)-GFP. An experiment should be done to test that: Does a K27R mutation in the substrate (i.e., Ub(K27R,G76V)-GFP) prevent its degradation and pull-down of UCHL3(C95S)? Without additional data, the statement (p. 18) that turnover of ubiquitylated proteins "...is at least partially mediated by K27-linked ubiquitylation of p97 substrates" is not warranted.

We believe the mass spectrometry experiment originally shown in Figure 5I (now Figure 4F in the revised manuscript) provides direct evidence that K27-linkages are formed on the Ub(G76V)-GFP model substrate, but we agree that this finding would be strengthened by additional experimental evidence. We therefore followed the reviewer's excellent suggestion to test the impact of a K27R mutation within Ub(G76V)-GFP (i.e. Ub(K27R,G76V)-GFP), taking advantage of the partially specific K27-Ub antibody (Figure 3A), whose recognition of K27-linkage formation on Ub(G76V)-GFP we were able to control for through the use of the Ub(K27R,G76V)-GFP mutant and our Ub(K27R) replacement cells. As expected from our mass spectrometry data, we found that ubiquitylated forms of Ub(G76V)-GFP were recognized by the Ub-K27 antibody (new Figure 4G; compare lanes 1 and 2). Importantly, a K27R mutation within Ub(G76V)-GFP (Ub(K27R,G76V)-GFP), which prevents ubiquitylation at K27 within this model substrate but not overall K27-linkage formation, selectively reduced the reactivity of the K27-Ub antibody with the band corresponding to Ub(G76V)-GFP monoubiquitylated at K27 but not slower-migrating ubiquitylated forms of Ub(G76V)-GFP (new Figure 4G; compare lanes 2 and 3). By contrast, expressing Ub(G76V)-GFP in Ub(K27R)-replaced cells, which allows Ub(G76V)-GFP ubiquitylation at K27 but not K27-linked extensions of Ub modifications on Ub(G76V)-GFP, strongly reduced all but the Ub(G76V)-GFP monoubiquitylation band recognized by the Ub-K27 antibody (new Figure 4G; compare lanes 2 and 4). This band was stronger than in Ub(WT)-replaced cells, consistent with the impaired processing of ubiquitylated forms of Ub(G76V)-GFP in Ub(K27R)-replaced cells and the inability of these cells to extend this initial K27-linkage on Ub(G76V)-GFP into longer K27-linked chains. When Ub(K27R,G76V)-GFP was expressed

in Ub(K27R)-replaced cells, thereby preventing all K27-linkage formation on this substrate, little reactivity with the Ub-K27 antibody was observed (new Figure 4G; lane 5). Together with our MS data, these findings firmly demonstrate that the Ub(G76V)-GFP model p97 substrate is targeted for K27-linked ubiquitylation impacting both the K27 residue in Ub(G76V) as well as Ub moieties attached to other lysines in Ub(G76V)-GFP. We note that even in Ub(K27R)-replaced cells, the Ub(K27R,G76V)-GFP protein underwent monoubiquitylation (new Figure 4G), in agreement with the notion that other Ub lysines within Ub(G76V)-GFP are also targeted for ubiquitylation. Extending these observations, we analyzed the impact of a K27R mutation in the Ub(G76V)-GFP reporter on its expression level. If K27-linkage signals were involved in facilitating p97-mediated Ub(G76V)-GFP turnover, then a K27R mutation within Ub(G76V)-GFP (Ub(K27R/G76V)-GFP) would be expected to partially stabilize Ub(G76V)-GFP, albeit not to a full extent as K27-linkages are still formed on Ub modifications targeting other lysines in Ub(G76V)-GFP (new Figure 4G). This is precisely what we observed, as in Ub(WT)-replaced cells Ub(K27R/G76V)-GFP was expressed at a moderately higher level than Ub(G76V)-GFP but was further stabilized in Ub(K27R)-replaced cells (new Figure 4G; new Figure EV4D). These observations strengthen the notion that K27-linked ubiquitylation of the Ub(G76V)-GFP model substrate facilitates its proteasomal turnover.

3. In Fig. 2H, the data for Wee1i treatments are too sparse to be conclusive - even if WT and K27R-replacement cells do behave differently, the scatter and paucity of data points are too great to expect statistical significance.

Prompted by the reviewer's comment, we performed 4 additional repeats of this experiment (Figure 2H) to solidify the data. Despite the variation between experiments, it should be emphasized that all 8 individual experiments showed an identical trend that the Ub(WT)- and Ub(K27R)-replaced cells behave differently. To more appropriately demonstrate this, we now employ a paired t-test instead of Mann-Whitney test for the statistical analysis of these data.

4. The authors speculate (p. 19) that p97 might facilitate K27-ubiquitination by partially (and presumably, transiently) unfolding Ub to expose the K27 to E3 Ub ligases. That seems inconsistent with their observation that K27-polyUb is promoted by p97 inhibition (e.g., Fig. 5C).

Based on structural studies on substrate unfolding by the *S. cerevisiae* Cdc48-Ufd1-Npl4 complex showing that the K27 residue in Ub becomes exposed in the context of an unfolded Ub-modified substrate exiting the Cdc48 central channel (Twomey *et al*, 2019), it remains an interesting possibility that p97-driven unfolding of ubiquitylated substrates could facilitate K27-linked ubiquitylation under at least some conditions. Consistent with this idea, proteomic studies have shown that the total abundance of cellular K27 linkages is decreased by p97 inhibition but accumulate upon proteasomal inhibition (Heidelberger *et al*, 2018). This could be explained if K27 linkages are actively generated downstream of p97-mediated unfolding of ubiquitylated substrates and facilitate their subsequent proteasomal processing. In principle, we think it could be of relevance to the field to mention this hypothesis in the discussion. However, we acknowledge that our data are not consistent with such a mechanism impacting the Ub(G76V)-GFP substrate that we analyzed, and we have therefore removed the statements pertaining to this speculative point from the discussion.

5. (p. 14,15; Fig. 5; Fig. S5A,B) The authors have used catalytically-inactive versions of UCHL5 and other DUBs as negative controls for UCHL3(C95S). This really doesn't make much sense to me. Even the structurally closest relative, UCHL5, is very dissimilar to UCHL3 with respect to activity, substrate specificity, and localization (UCHL5 associates with 26S proteasomes). The experiments and results using inactive UCHL5 and other DUBs contribute little to the study and probably should be deleted from the paper.

The reviewer is correct in pointing out the limited similarity between UCHL3 and UCHL5. The purpose of including data for other DUBs was to show that the ability of UCHL3-C95S to stabilize the Ub(G76V)-GFP p97 model substrate is not shared by a range of other DUBs that are not known to interact with K27-linkages (i.e. this effect is not simply produced by overexpression of any catalytically inactive DUB). That said, we acknowledge and respect the reviewer's perspective, and we therefore removed the data showing the inability of a panel of overexpressed catalytically inactive DUBs other than UCHL3 to stabilize the Ub(G76V)-GFP model substrate (Figure S5A in the original manuscript). As a comparison for the effects of overexpressed UCHL3 proteins, we do think it is useful to show data for catalytically inactive UCHL5, which we simply refer to in the text as the DUB that is structurally most closely related to UCHL3. However, if deemed necessary, we can remove the data on UCHL5 as well.

6. How do the authors reconcile the absence of K27-polyUb in p97 cofactors (e.g., UFD1 and p47) with the mass spec results in Fig. 3E? Were the "stringent" anti-HA IP conditions not completely denaturing?

In line with the reviewer's point, we emphasize in the manuscript (page 17-18) that our mass spectrometry-based analysis of ubiquitylation changes induced by Ub(K27R) replacement (Figure 3D,E) does not allow us to conclude whether regulated proteins are directly modified by K27-linkages or if their ubiquitylation status is indirectly regulated by K27-linked ubiquitylation, the latter of which seems to be true for p97 cofactors seeing that they show no detectable interaction with the K27 binder UCHL3 C95S (Figure EV5B). We do not currently understand why nuclear p97 cofactors show reduced ubiquitylation in Ub(K27R)-replaced cells despite they do not seem to be direct targets of K27-linked ubiquitylation. Because abrogating K27-linked ubiquitylation selectively reduces ubiquitylation of nuclear p97 cofactors and only appears to impact p97 functionality in the nucleus, one possibility is that p97 cofactors simply become ubiquitylated due to their proximity to Ub signaling enzymes acting in the context of the p97 machinery, and the reduced ubiquitylation of nuclear p97 cofactors may at least to some extent be a consequence of impaired processing of p97 substrates in the nucleus when K27 linkages cannot be generated.

Some minor issues:
7. (p. 13, line 4) Perhaps change the word "likely" to "possibly" - I'm not convinced that proteasome-associated DUBs are responsible.

We modified the text as suggested.

8. (p.17) The statement that "...Ub(K27R)-replaced cells show unchanged levels of most

other Ub linkage topologies and ubiquitylation processes..." is incorrect; the effects on only a few linkage types were assessed.

We respectfully disagree with the reviewer that we only assessed the effects on only a few linkage types. In the original version of Figure 3D (now Figure 1I in the revised manuscript), we provided data on the impact of abrogating K27-linked ubiquitylation on the ubiquitylation status (di-Gly modification) of 5 of the 7 lysine acceptor sites in Ub (K6, K11, K27, K48 and K63). As described above, we have now added the corresponding data for K33-linkages to Figure 1I, showing no significant change in K33-linkage abundance upon Ub(K27R) replacement. Moreover, using a recently reported specific K29-linkage binder, sAB-K29 (Yu *et al.*, 2021) we show that Ub(K27R) replacement also has little if any impact on the abundance of K29-linkages (new Figure EV2C). Collectively, therefore, we provide evidence that apart from the expected impact on K27-linkages, the ubiquitylation of all other lysine acceptor sites in Ub remains largely unchanged upon abrogation of K27-linked ubiquitylation.

9. (p. 27) "StageTipping" isn't a word.

We have modified the text accordingly.

10. (p. 34, Fig. 1G legend) "Lys-C digestion" is written here but wouldn't yield the peptide indicated; trypsin probably was meant.

Trypsin was indeed used in this experiment – we apologize for the error and thank the reviewer for pointing this out. The legend for Figure 1G has been corrected accordingly.

11. For Fig. 3E, it would be helpful to explain the criteria used to select the protein hits highlighted in the figure.

We appreciate the suggestion. We originally highlighted proteins showing the most prominently reduced ubiquitylation state upon Ub(K27R) replacement but did not use strictly defined criteria for this selection. As labeling all significantly regulated proteins in the volcano plot (Figure 3E) is impractical, we now instead highlight only hits that have a known link to the p97 system, and we point this out accordingly in the figure legend. The identity of the other regulated proteins can be seen in the full dataset provided in Dataset EV1, which we also now state clearly in the figure legend.

Referee #3:

This is a well-executed study -specifically this revised version- and it makes a strong case for the importance of K27-linked ubiquitin (Ub) chains in human cell cycle progression. It also tightly links K27 Ub chain formation to p97/VCP action on Ub conjugates using both epistasis analyses of K27-Ub replacement with p97 inhibitor treatment as well as between overexpressed UCHL3-C95S, a K27-chain-specific binder, and p97 inhibition. The latter provide strong corroborative data for the Ub replacement

experiments.

In the revision, the interpretative focus has shifted to substrate modification by K27 chains, rather than p97 cofactor modification, and this is underscored with direct mass spectrometric identification of K27 linkages on a p97-proteasome substrate and correlative data linking K27 chains with p97 processing and not proteasome action per se. The authors did an excellent job responding to the reviewer comments, including some detailed experimental suggestions from original Reviewer #2. These changes have greatly improved the manuscript.

There are now many questions to answer regarding how exactly Ub-K27R causes G2 cell cycle arrest and how K27 chains on substrates and/or p97 cofactors work in K27 chain-mediated physiological pathways such as replication. This paper makes these interesting questions to ask, and they should be addressable in future studies. I believe the data in the current paper are excellent and the experiments well controlled, and support publication in the EMBO Journal.

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Yu Y, Zheng Q, Erramilli SK, Pan M, Park S, Xie Y, Li J, Fei J, Kossiakoff AA, Liu L *et al* (2021) K29-linked ubiquitin signaling regulates proteotoxic stress response and cell cycle. *Nat Chem Biol* 17: 896-905

Thank you again for sumitting your revised manuscript, "K27-linked ubiquitylation promotes p97 substrate processing and is essential for cell proliferation", and please excuse the delay in its re-evaluation. I have in the meantime heard back from referee 2, whose comments are copied below. I am happy to say that the referee is overall satisfied with the revisions, so that there should be no further principle objections towards publication. Still, you will see that the reviewer retains a few concerns revolving around Figure 4, which I would herewith invite you to address/clarify in a final round of minor revision. Once we will have received these modified final files, we should be ready to proceed with formal acceptance and publication of the study.

REFEREE REPORTS

Referee #2:

This newest revision from Shearer et al. has satisfactorily addressed most problems cited by the critiques of the earlier manuscript. However, I remain concerned about the authors' data and conclusions from the experiments that used Ub(G76V)-GFP as a model substrate.

1. In Fig. 4F, the data reporting relative peptide amounts as determined by mass spectrometry are results of four "technical replicates" from each condition. The authors base conclusions on apparent differences seen in comparisons of mean values, but the high degree of scatter (e.g., for the diGly-K27 peptide with p97 inhibition) makes these comparisons unreliable. Notably, no statistical tests of significance were reported. Also, what exactly is meant by "technical replicates" used for this experiment? Four repeats of injections of the same sample into the mass spectrometer, especially with the variability seen in the data, would not constitute a rigorous analysis. Fortunately, the authors' conclusion that Ub(G76V)-GFP ubiquitination products include K27 linkages is further supported by the results in Fig. 4G; nonetheless, unless Fig. 4F can be revised to show more convincing data, it should be removed from the paper.
2. The authors have shown that K27 Ub-Ub linkages are important to facilitate p97-dependent turnover of some proteins. Whether K27-polyUb is involved by modifying the substrate directly or if it has an indirect effect (e.g., by modifying p97 or its cofactors) is an important question. The authors conclude from experiments with Ub(G76V)-GFP that direct K27 ubiquitination of the substrate is involved, and that (p. 11) "...the extent of K27-linked ubiquitylation on Ub(G76V)-GFP model substrates inversely correlated with their stability in cells." The data supporting this conclusion (Fi. 4C,G; Fig. EV4D) are not very convincing. In Fig. 4C, the authors need to show that the increase in nuclear GFP upon replacement of Ub with Ub(K27R) is statistically-significant. Moreover, although Fig. 4G shows an apparent decrease of Ub(G76V)-GFP upon Ub replacement by Ub(K27R), the results as presented are only qualitative; even if the band intensities are quantified, it needs to be established that they are in a linear range.
3. Finally, a minor item needs correction. In the Abstract, the word "Consistently" is improperly used.

Point-by-point reply to referee comments

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In this experiment, the four technical replicates correspond to independent samples prepared from four individual plates and are not simply four injections of the same sample into the mass spectrometer. We have clarified this in the figure legend for Fig. 4F (page 42 in the manuscript). Because of the variability of the individual data points for the diGly-K27 peptide with p97 inhibition, the trend indicating increased levels of K27-linkages on Ub(G76V)-GFP upon p97i treatment did not reach statistical significance. We point this out in the text as follows: "*In line with this, our MS analysis showed a trend that both K27- and K29-linked Ub modifications accumulated on Ub(G76V)-GFP upon inhibition of p97, although in the case of K27-linkages the increase was modest and varied between individual samples (Figure 4F).*" (page 15-16).

2. The authors have shown that K27 Ub-Ub linkages are important to facilitate p97-dependent turnover of some proteins. Whether K27-polyUb is involved by modifying the substrate directly or if it has an indirect effect (e.g., by modifying p97 or its cofactors) is an important question. The authors conclude from experiments with Ub(G76V)-GFP that direct K27 ubiquitination of the substrate is involved, and that (p. 11) "...the extent of K27-linked ubiquitylation on Ub(G76V)-GFP model substrates inversely correlated with their stability in cells." The data supporting this conclusion (Fi. 4C,G; Fig. EV4D) are not very convincing. In Fig. 4C, the authors need to show that the increase in nuclear GFP upon replacement of Ub with Ub(K27R) is statistically-significant. Moreover, although Fig. 4G shows an apparent decrease of Ub(G76V)-GFP upon Ub replacement by Ub(K27R), the results as presented are only qualitative; even if the band intensities are quantified, it needs to be established that they are in a linear range.

We believe it is not appropriate to perform statistical analysis of the representative experiments showing quantitative image-based cytometry (QIBC) analysis of large numbers

(typically thousands) of individual cells. These experiments are similar in nature to conventional flow cytometry analyses, for which it is also common practice to display results as representative experiments. Most papers reporting QIBC data that we are aware of (see Ercilla et al., Cell Reports 30:2416-2429 (2020) and Toledo et al., Cell 155:1088-1103 (2013) for examples) are also showing representative experiments and consequently not performing statistical analysis of the data (where even tiny differences would tend to show statistical significance given the very large number of individual data points). A large majority of the QIBC experiments in our manuscript are representative of at least 3 independent experiments with similar outcomes. Below, we provide examples of independent replicates of the experiments in Fig. 4C and Fig. EV4D showing similar trends (Fig. R1A,B). It should also be mentioned that the immunoblotting data on Ub(G76V)-GFP expression shown in Fig. 4D effectively represent an independent confirmation of the effects seen by QIBC in Fig. 4C via an orthogonal approach. In a similar vein, the immunoblotting data on Ub(G76V)-GFP expression in Fig. 4G are consistent with the effects seen by QIBC in Fig. EV4D.

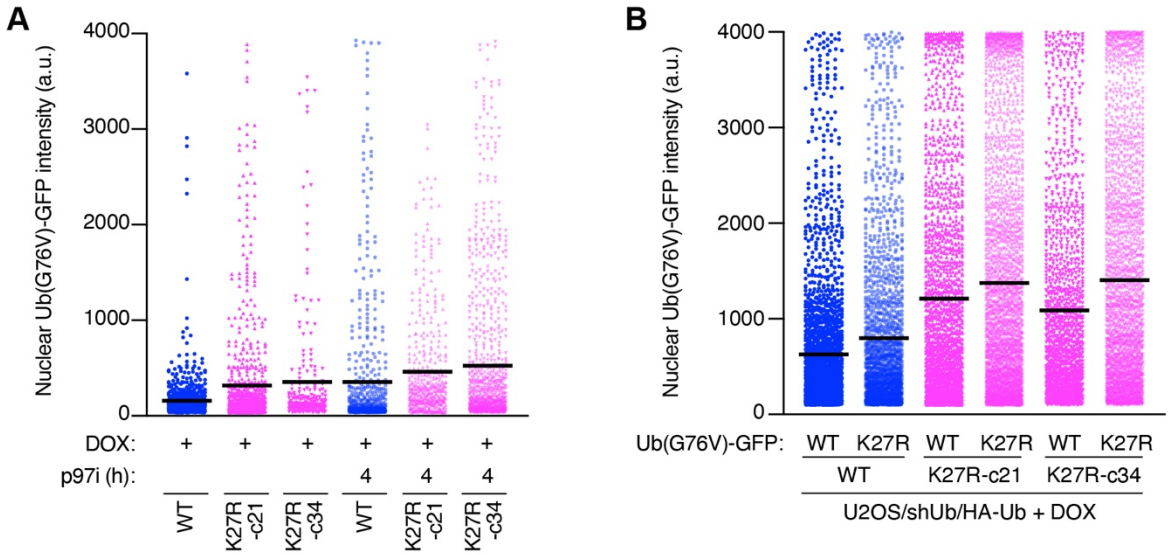

Figure R1.
Independent replicates of the QIBC experiments in Fig. 4C and Fig. 4EVD
A. Independent replicate of experiment in Fig. 4C. U2OS/shUb/HA-Ub replacement cell lines stably expressing shUb-resistant Ub(G76V)-GFP reporter were treated with DOX and p97i as indicated, and nuclear Ub(G76V)-GFP signal intensity was analyzed by QIBC (black bars, mean; 150-750 GFP-positive cells analyzed per condition). **B**. Independent replicate of experiment in Fig. EV4D. DOX-treated U2OS/shUb/HA-Ub(WT) and U2OS/shUb/HA-Ub(K27R) cell lines were transfected with the indicated Ub(G76V)-GFP expression constructs for 24 h, and nuclear GFP signal was analyzed by QIBC (black bars, mean; >1000 GFP-positive cells were analyzed per condition).

Our understanding of the referee's comment about the experiment in Fig. 4G is that the decrease in K27 ubiquitylation of Ub(G76V)-GFP seen upon Ub(K27R) replacement and/or a K27R mutation within the Ub(G76V)-GFP substrate is not fully convincing as the GFP immunoblot of the GFP IP is saturated, raising the possibility that the reduced amount of K27-linked ubiquitylation of Ub(G76V)-GFP in lanes 4 and 5 could be simply due to a lower amount of immunoprecipitated Ub(G76V)-GFP being loaded in these lanes. To rule out this

possibility, we have now added to Fig. 4G a Ponceau S stain we did of the membrane containing these IPs, which clearly shows that more Ub(G76V)-GFP is in fact present in lanes 4 and 5, consistent with the abundance of Ub(G76V)-GFP being increased when its modification by K27-linked ubiquitylation is impaired by Ub(K27R) replacement and/or K27R mutation of Ub(G76V)-GFP.

3. Finally, a minor item needs correction. In the Abstract, the word "Consistently" is improperly used.

We changed “Consistently” to “Moreover” in the abstract (page 2).

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.

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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 replicates.
- 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 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 χ^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

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New materials and reagents need to be available; do any restrictions apply?	Yes	Data Availability section (page 32)

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 (page 27-28)

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.	Yes	Materials and Methods (page 23-25)

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.	Yes	Materials and Methods (page 22-23)
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.	Yes	Materials and Methods (page 22-23)

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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	

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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?	Not Applicable	

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

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Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Materials and Methods (page 32)
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	Materials and Methods (page 32)
Include a statement about **blinding** even if no blinding was done.	Yes	Materials and Methods (page 32)
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.	Yes	Materials and Methods (page 32)
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?	Not Applicable	

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 (page 37-50)
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure legends (page 37-50)

Ethics

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 approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

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.

Adherence to community standards	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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 section (page 31)
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**.	Not Applicable	